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Award Number: DAMD17-99-1-9452

TITLE: Involvement of Human Estrogen Related Receptor Alpha 1 (hERR 1) in Breast Cancer and Hormonally Insensitive Disease

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REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

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

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Carole B. Christian _____

3/1/01 _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (1 Jul 99 - 1 Jul 00)

4. TITLE AND SUBTITLE
Involvement of Human Estrogen Related Receptor Alpha 1 (hERR 1) in Breast Cancer and Hormonally Insensitive Disease

5. FUNDING NUMBERS
DAMD17-99-1-9452

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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, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Aug 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

A plausible candidate for a novel breast cancer prognosticator and target for therapy is the estrogen-related receptor α (ERRα), an orphan nuclear receptor that shares significant sequence identity with estrogen receptors (ERs) but does not bind estrogens. The goals of these studies are to asses ERRα 's utility as a novel breast cancer prognosticator and to elucidate ERRα's role in modulating estrogen-responsive gene transcription. The data obtained to date indicate that:(i) ERRα is itself an estrogen-responsive gene (ii) ERRα expression levels are significantly lower in a small panel of breast carcinomas relative to normal mammary tissues, (iii) the ERRα /ERα ratio significantly correlates with S-phase fraction and DNA ploidy within the tumors, (iv) ERRα represses estrogen-stimulated transcription in MCF-7 cells, (v) ERRα may modulate expression of numerous estrogen-responsive genes whose promoters contain identified ERRα -binding sites, and (vi) ERRα -dependent transcription can be activated through increased c-*erb*B2 signaling, a gene implicated in tamoxifen resistance. These findings may provide the basis for future studies to determine whether ERRα status may indicate sensitivity to hormonal therapies, progression to tamoxifen resistance, and disease outcome.

14. SUBJECT TERMS
Breast Cancer Prognosticator, ERR alpha, ER alpha, Gene Transcription, c-erbB2/HER-2, Tamoxifen Resistance

15. NUMBER OF PAGES
40

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	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Approximately 50 % of breast cancer patients with estrogen receptor α (ERα) -positive tumors respond to antiestrogen therapy (1). Since expression of progesterone receptor (PgR) is dependent on ERα activity, further selection of patients for ERα-positive and PgR-positive tumors enhances the breast cancer hormonal therapy response rate to nearly 80 % (2). Estrogen-related receptor α (ERRα), an orphan nuclear receptor that shares significant sequence identity with ERα and ERβ but does not bind estrogens (3), has been shown by my research group (4, 5) and others (6-9) to bind and activate transcription through estrogen response elements (EREs) and extended steroid receptor half-sites previously identified as steroidogenic factor 1 (SF-1) response elements (SFREs). Recently, ERα has been shown to activate transcription through SFREs as well as EREs (9). My research group [Fig. 5; O'Reilly, Ariazi, and Mertz, in preparation; (4)] and Yang *et al.*(10) have also demonstrated that ERRα can interact via direct protein-protein contacts with ERα. Hence, there are several mechanisms whereby ERRα may modulate estrogen responsiveness through modulating or substituting for ERα activities, making it a plausible candidate for a novel breast cancer prognosticator and target for therapy.
The goals of this Post-doctoral Fellowship are: (i) to ascertain alterations which regulate ERRα activities and if these alterations impact the etiology of some breast cancers; (ii) to determine if ERRα impacts the phenotype of hormone-responsive and non-responsive breast tumor cells; and (iii) to elucidate the possible mechanism(s) by which ERRα modulates the transcriptional regulation of estrogen-responsive genes implicated in breast cancer.

BODY

Specific Aim I - To test whether alterations in expression, RNA splicing, phosphorylation status, subcellular localization, or mutations in ERRα significantly correlate with the development of some breast cancers. (A) Assays will be developed with human mammary carcinoma MCF-7 cell derivatives in Aim II to characterize ERRα RNA and protein abundance, possible splicing variants, possible phosphorylation isoforms, subcellular localization, and possible mutations. (B) These assays will be used to characterize clinical ERα-positive primary breast carcinomas for ERRα. (C) Additionally, ERα-positive/tamoxifen-resistant breast tumors will be examined for ERRα RNA expression, splicing variants, and possible mutations.

Task 1. To test whether alterations in ERRα significantly correlate with the development of clinical ERα-positive primary breast carcinomas and ERα-positive/tamoxifen-resistant breast tumors (months 1-36)

Patients with breast tumors that express ERα have an improved prognosis relative to patients with ERα-negative breast tumors (1, 2, 11). Given that ERα and ERRα share multiple biochemical activities including binding to many of the same transcriptional response elements to modulate estrogen responsiveness, I hypothesize that typing breast tumors for ERRα levels could further improve ERα's prognostic value.

Task 1A. Develop assays with breast cancer cell lines to look for alterations in ERRα (months 1-12).

Development of real-time quantitative polymerase chain reaction (PCR) assays.

To begin to test whether ERRs are involved in breast carcinogenesis through modulation of estrogen responsiveness, I initially determined whether ERRs themselves are estrogen-responsive genes. I also investigated whether breast carcinomas exhibit differences in expression of ERRs relative to normal mammary epithelial cells and whether expression levels of these genes among the tumors correlate with ER and PgR status and clinical properties indicative of tumor aggressiveness. To measure expression of ERRα, ERRβ, ERRγ, ERα, ERβ, and PgR, real-time quantitative PCR assays were developed. In these assays, continuous measurement of fluoresence due to specific complex formation of Sybr Green I with double-stranded PCR products versus single-stranded DNA facilitates accurate and sensitive quantitation of initial mRNA molar amounts (12-14). My assays were developed using an ABI 7700 sequence detection system (Applied Biosystems). Because PCR efficiency decreases with increasing number of cycles due to limiting polymerase, nucleotides, and primers, the critical parameter recorded in the real-time PCR assay is the threshold cycle (C_t), *i.e.*, the cycle (measured to a fractional value of the 1 minute extension phase of the PCR) when PCR products are initially detected over background fluorescence. Background fluorescence levels are determined from the signal intensity of no-template control reactions. Spectral compensation of signal intensity differences among sample wells is accomplished by the inclusion of ROX, a fluorescent reporter dye, in the enzyme reaction buffer (Perkin Elmer). In my real-time PCR assays, each transcript's initial copy number in an unknown sample was calculated based on regression analysis of its corresponding C_t standard curve, which was generated by serial dilutions covering 6 logs of known starting amounts of the target template in every experiment. PCR primers were optimized for high efficiency, *i.e.*, product size smaller than 125 bp and no spurious bands. All RNA samples were pre-treated with DNase to eliminate contaminating cellular DNA. To minimize non-specific product amplification, the real-time PCR assays employed AmpliTaq Gold (Perkin Elmer), a chemically modified form of Taq polymerase that is completely inactive at room temperature and requires an initial 10-minute heat activation step at 95° C; thus, the use of this enzyme results in an automatic "hot start". Specific PCR products were verified by analysis of DNA melting curves (15). To control for variability in RNA integrity and cDNA synthesis efficiency, assays were normalized to the estradiol (E_2)-independent 36B4 gene, previously identified as the acidic ribosomal phosphoprotein P0 gene (16).

Effects of estrogens and antiestrogens on expression of ERRs.

Because the effects of estrogens and antiestrogens on ERα and PgR mRNA expression in MCF-7 cells are well documented, I used this model system to develop and verify my real-time quantitative PCR assays (Fig. 1). I found, as expected, that E_2 repressed ERα expression while it induced PgR

expression (17-20). Also expected, the partial antiestrogen 4-hydroxy tamoxifen (4OHT) did not significantly affect ERα expression (20), though it increased PgR expression to a lesser degree than E_2, likely because 4OHT does not block ERα's AF-1 transactivation domain (21, 22). The complete antiestrogen ICI-182780 treatment increased ERα mRNA expression and did not change PgR expression. Taken together, these data demonstrate that the real-time quantitative PCR assays are working properly.

Interestingly, I found that E_2 significantly induced, while 4OHT probably repressed ERRα mRNA expression. Induction of ERRα mRNA levels by the synthetic estrogen diethylstilbestrol (DES) has been independently confirmed in murine endometrium *in vivo* (23). Thus, ERRα is responsive to estrogens. Given that ERs and ERRs bind both EREs and SFREs [see Figs. 3, 9 and 11 below; (4, 8, 9)], my finding that the ERRα/ERα ratio changed 45-fold with E_2 treatment relative to the control conditions is especially important. This dramatic change in the ERRα/ERα ratio could lead to significant changes in response element occupancy which could, in turn, modulate estrogen responsiveness. In support of this hypothesis is my finding in transient transfection assays that an increase in the ERRα/ERα ratio in MCF-7:WS8 cells leads to antagonism of E_2-stimulated transcription (see Fig. 6B below). Thus, the increased ERRα/ERα molar ratio that occurs in E_2-treated cells may lead to antagonism of endogenous estrogen-responsive gene transcription, thereby acting as a negative feedback on regulation of E_2 signaling. I also found that the ERRα and ERα mRNAs were present at comparable levels in MCF-7:WS8 cells, while ERβ and ERRβ mRNA levels were very low (data not shown). The finding of very low ERβ levels is also consistent with an independent report (24), further validating the real-time PCR assays.

Screening for ERRα mRNA splice-variants and mutations in cell lines.

Many variant ERα mRNAs arising from alternative splicing (25-28) and mutations (29-33) have been identified in breast carcinomas and postulated to be involved in the development of the disease. To determine whether some breast carcinomas contain ERRα1 mRNA splice-variants as evidenced by changes in the size of ERRα cDNA fragments, a series of twelve overlapping reverse transcriptase (RT)-PCR primers were designed spanning ERRα1's 5' untranslated region (UTR) and its complete open reading frame (ORF). These primer sets span exon-intron junctions to prevent the amplification of potential contaminating genomic DNA. The RT-PCR primers were used in various combinations to amplify ERRα1's 5' UTR and ORF in six overlapping ~350-bp cDNA fragments and to amplify its entire ORF in a single 1325-bp fragment. Initially, I tested my RT-PCR assays with established mammary cell lines. I found that ERRα1 mRNA is expressed and its structure is normal in ERα-positive MCF-7:WS8, MCF-7:5C and T47D cells, and ERα-negative MDA-MB-468 and MDA-MB-231 cells (data not shown). However, ERRα1 mRNA abundance appeared lower in MDA-MB-231 cells compared to the other cell lines. I also examined these cell lines for ERRα2 mRNA using a primer that anneals to its unique 5' region. An ERRα2-specific cDNA fragment of correct size was amplified in 3 of the 4 mammary cell lines; interestingly, the MDA-MB-231 cells were devoid of ERRα2 mRNA (data not shown). As a control for cDNA integrity, I confirmed the presence of 36B4 mRNA in all of the cell lines. Thus, ERRα mRNA abundance is low in at least one ERα-negative mammary carcinoma cell line.

To identify mutations in ERRα and to discern the fine structure of alternatively spliced ERRα mRNA species, I have developed automated sequencing protocols using these same series of diagnostic ERRα mRNA RT-PCR primer sets. Automated DNA sequencing equipment is readily available as a core facility at the McArdle Laboratory. Initially, the sequencing protocols were developed using cDNAs derived from normal mammary gland and uterine tissues. I found a 3-bp insertion (CAG) between nucleotides 745 and 746 that introduces an additional Gln amino acid relative to the ERRα sequence deposited in Genbank by C.T. Teng's research group [Accession No. L38487; (10)]. The insertion of 3 bases in ERRα has been independently confirmed (34). Additionally, I have sequenced and confirmed the 3-base CAG insertion in ERRα from the same endometrial RL95-2 cells that C.T. Teng's group used to determine ERRα's sequence. Furthermore, I sequenced ERRα from MCF-7:WS8, MCF-7:5C, T47D, MDA-MB-231, MDA-MB-468, and HeLa cells, always finding the same 3-base insertion. Accordingly, it is likely the 3-base CAG insertion exists in the wild-type ERRα sequence.

Development of ligand affinity-purified polyclonal anti-ERRα antibodies.

I needed ERRα-specific antisera to be able to examine breast tissues for ERRα protein expression by immunohistochemistry (IHC) as proposed in above and to identify definitively and to characterize ERRα-binding sites as proposed in **Revised Aim IIIA**. Because the lab group's previous α-GST-ERRα1$_{17-329}$ antibody interfered with ERRα's ability to bind DNA in EMSAs [see Fig. 3; (4)], I raised new anti-ERRα antibodies. ERRα amino acid sequences were evaluated for predicted antigenicity, hydropathy, and surface exposure using the Protean program of the DNASTAR bioinformatics package (Madison, WI). Basic local alignment search tool (BLAST) analyses (35) allowed further selection of candidate immunogenic peptides; queries showing an exact sequence match with only ERRα and not other family members were chosen. The amino acid sequences were each synthesized in the form of multiple antigen peptides (MAPs) or eight peptide copies attached to a non-immunogenic polylysine core and used to immunize New Zealand white male rabbits. Polyclonal antibodies were ligand-affinity purified from the collected rabbit serum using sepharose columns coupled to each immunizing-ERRα MAP and given designations corresponding to the respective location of the immunizing peptide: anti-ERRα1$_{18-32}$, anti-ERRα1$_{49-66}$, and anti-ERRα2$_{31-45}$, the last of which is specific to the unique N-terminal region in ERRα2. The anti-ERRα1$_{49-66}$ antibodies were found to be suitable for Western blotting applications (Fig. 2) and immunoshifting ERRα-DNA complexes in EMSAs (Fig. 3).

Studies on ERRα phosphorylation and development of monoclonal anti-ERRα phosphospecific antibodies are discussed in ***Task 2C****.*

Task 1B. Examine primary ERα-positive breast carcinomas (months 2-24).

Expression of ERRs in breast tissues and correlations with clinicopathological factors.

ERα levels are usually low in normal mammary epithelium and high in breast tumor cells (36-41). This alteration in ERα expression is likely an important etiologic event in breast carcinogenesis [reviewed in (42)]. Thus, I examined clinical breast tumors and normal mammary epithelial cells (MECs) for expression of nuclear receptor mRNAs to confirm that my assays detect this change in ERα expression and to test whether expression of ERRs is also altered between these tissues. The breast carcinomas were obtained from the National Breast Tissue Resource (SPORE) at The Baylor College of Medicine through Dr. Gary Clark in a blinded fashion regarding clinical breast tumor properties, including ER and PgR status as measured by biochemical ligand-binding assays (ER-LB and PgR-LB, respectively), and cellular S-phase fraction and DNA ploidy as determined by flow cytometry analysis. For my preliminary experiments, I used as a control normal MECs derived from mammoplastic reduction surgeries obtained from the University of Wisconsin-Madison through Dr. Colin Jefcoate. Because it is technically very difficult to isolate high quality RNA from such a fatty tissue, bulk mammary gland tissue was enzymatically dispersed with collagenase and subjected to differential centrifugation to enrich and collect MECs. Dr. Jefcoate's lab supplied us with MECs after expansion in culture for a single passage under defined culture conditions that supported the outgrowth of both luminal and basal MECs (43, 44).

Using the real-time quantitative PCR assays, I have determined to date expression levels of ERα, PgR, ERβ, ERRα, and ERRβ in a small panel of 8 clinical random primary breast carcinomas and normal MECs derived from 3 individual mammoplastic reductions (Fig. 4). I evaluated whether a given gene's mRNA expression levels were significantly different in normal MECs as a group compared to the breast carcinomas as a group using the Wilcoxon non-parametric two-sided rank sum test (45). I found ERα levels were significantly higher ($p=0.01$), while ERβ levels were significantly lower ($p=0.05$) in breast carcinomas compared to normal MECs. My findings are consistent with previous reports that ERα mRNA levels are significantly higher (36) and ERβ levels are significantly lower in breast carcinomas than normal mammary glands (37, 46, 47), though ERβ is sometimes co-expressed with ERα in breast tumors (48) albeit at much lower levels than ERα (49). Thus, my real-time PCR assays again show expected results.

I also found that ERRα ($p=0.02$) and ERRβ ($p=0.01$) were significantly lower in breast carcinomas relative to normal MECs by the Wilcoxon rank sum test. These significant differences in expression of ERRα and ERRβ suggest that these receptors may also be involved in the development of breast cancer. Of potential importance, the ERRα/ERα ratio was quite significantly lower in breast

carcinomas relative to MECs [22-fold (p=0.01) comparing means; 48-fold comparing medians]. This large difference in the relative ratio of these two receptors probably has important implications in estrogen responsiveness since, as discussed above, it is likely that the ERRα/ERα ratio largely determines which receptor preferentially occupies EREs and SFREs. Thus, many sites usually occupied by ERRα in normal mammary epithelium may be primarily occupied by ERα in breast carcinomas, thereby resulting in differential expression of these estrogen-responsive genes. I will test further whether ERRα and the ERRα/ERα ratio are significantly lower in breast carcinomas than MECs. Even though the MECs used here retain expression of differentiation markers (43), it is possible that their gene expression profiles do not fully reflect the gene expression profiles of MECs *in vivo* due to their having been expanded through a single cell-culture passage. Hence, I will use in my future studies normal MECs enymatically dispersed from bulk mammary gland tissues, but not expanded in culture.

Next, I assessed for the carcinomas whether correlations existed between mRNA levels of each nuclear receptor and any previously determined clinicopathological factors. I were unblinded to the breast tumors' clinical biomarkers following the completion of my assays. The non-parametric Spearman's Rank Correlation Coefficient, designated ρ_s (Spearman's rho) (45), was used as a measure of relationships between breast carcinoma properties and mRNA levels of each gene. Absolute values of ρ_s near 1 or 0 indicate a strong or weak correlation, respectively, while the sign indicates a positive or negative relationship. Because the DNA ploidy data were in the form of dichotomous observations, tumors containing aneuploid or diploid nuclei were assigned ranks of 1 and 2, respectively. Raw data were used for ranking all other parameters and mRNA levels. Spearman's rhos for pairwise correlations of breast carcinoma properties are summarized in Table 1.

While the findings presented here are quite preliminary due to the small sample size, the data indicate some intriguing trends. ERα mRNA abundance as determined by real-time quantitative PCR showed a strong and significant correlation ($\rho_s = 0.83$, $P = 0.01$) with ER protein levels as determined by the ligand-binding assay (ER-LB), which classified 7 of the 8 tumors as ER-positive. Similarly, Chevillard and colleagues (50) found that a greater number of breast tumors were ERα-positive by semi-quantitative RT-PCR (86 of 105 samples) than by standardized enzymoimmunoassays (EIA; 79 of 105 samples). These discrepancies may be due to alterations in ERα that abrogate its ability to bind ligand; such mutations in ERα's LBD have been documented to occur in breast tumors [reviewed in (33)]. These discrepancies may also be explained by the heterogeneous nature of tumor cell subpopulations and tissue sampling error. Nonetheless, the strong correlation between ERα mRNA levels and ER-LB provides further support that the real-time quantitative PCR assays are working as expected.

ERRα mRNA levels did not correlate with expression of other genes or clinical properties. The ERα mRNA and ER-LB levels also did not correlate with other clinical biomarkers. Nevertheless, the ERRα/ERα ratio correlated with ER-LB ($\rho_s = -0.76$, $P < 0.05$), ERα expression ($\rho_s = 0.88$, $P < 0.05$), and PgR expression ($\rho_s = -0.81$, $P < 0.05$). The potential clinical importance of the ERRα/ERα ratio is illustrated by its significant correlations with DNA ploidy ($\rho_s = -0.85$, $P < 0.05$) and S-phase fraction ($\rho_s = 0.71$, $P < 0.10$). These preliminary findings provide actual support to the hypothesis of the prognostic importance of the ERRα/ERα ratio.

ERRβ mRNA levels correlated with ERβ mRNA levels ($\rho_s = 0.81$, $P < 0.05$), suggesting a possible functional relationship between these genes as well. It has been reported that ERβ-positive tumors are associated with clinical indicators of breast cancer (47, 48). Likewise, it is possible that ERRβ levels may also have clinical significance. I plan to follow up on this finding by continuing to analyze tumor samples for ERRβ mRNA levels along with ERRα in the experiments proposed in **Aim IB**. However, biochemical and functional analysis of ERRβ is beyond the scope of this application and is the focus of a separate, small "concept" grant supported by the Department of Defense (BC995935).

Screening for ERRα mRNA splice-variants and mutations in breast tumors.

The diagnostic RT-PCR assays for characterization of ERRα's mRNA structure as described in **Task 1A** were used to examine 9 random clinical breast carcinomas. The RT-PCR assays indicated that ERRα1 was expressed and its mRNA was properly spliced in all of the tumors (data not shown). The ERRα2-specific primers also enabled amplification of a PCR product of correct size in all 9 tumors, though a second round of PCR amplification was required to detect ERRα2 in 2 of the 9 tumors [Fig. 5

(round 1 of PCR); data not shown (round 2 of PCR)]. Interestingly, in one breast carcinoma (number 9), an additional variant ~90 bp larger than the expected ERRα2 product was detected. DNA sequence analysis of the larger PCR product indicated that it was an mRNA splice-variant with a precise insertion of part of ERRα1's intron 1 sequence at the exon-1/2 splice junction. Therefore, at least one ERRα splice variant likely exists in breast tumor cells.

Task 1C. Examine wild-type ERα/tamoxifen-resistant breast tumors (months 25-36).

No progress on the examination of wild-type ERα/tamoxifen-resistant breast tumors has as yet been accomplished.

Specific Aim II - To test whether the genetic ablation of ERRα affects cellular growth and death characteristics following estrogen and antiestrogen treatment of matched MCF-7 cell clones which are either sensitive (i.e., MCF-7:WS8 cells) or insensitive (i.e., MCF-7:5C cells) to these drugs. (A) Because the ligand for ERRα is not yet identified, somatic cell knockouts of the ERRα gene in MCF-7:WS8 and MCF-7:5C cells will be generated. (B) The resulting ERRα knockout cells and their parental ERRα wild-type cells will be characterized for cellular growth characteristics and apoptosis after treatment with estrogen and antiestrogens, as well as examined in standard cell transformation assays.

Task 2. To knockout the ERRα gene in the matched hormone responsive and non-responsive cell lines, MCF-7:WS8 and MCF-7:5C cells, respectively, and characterize possible alterations in cellular growth and apoptosis after treatment of these cells with estrogens and antiestrogens (months 1-24)

Task 2A. Determination of ERRα copy number by karyotype coupled with FISH analysis (months 1-6).

Task 2B. Generation of ERRα somatic cell knockouts using MCF-7:WS8 and MCF-7:5C cells (months 7-18).

Task 2C. Characterization of ERRα genetic ablation on cellular growth and apoptosis of MCF-7:WS8 and MCF-7:5C cells following treatment with estrogen and antiestrogens (months 19-24).

In my original fellowship application, I hypothesized that ERRα may play a role in the development of TAM-resistant breast cancer. As a model system of TAM resistance, I proposed to characterize the matched hormonally sensitive MCF-7:WS8 and insensitive MCF-7:5C cells for potential alterations in ERRα-dependent transcription. These cell sublines were generated by long-term growth selection of MCF-7 parental cells cultured under estrogenized and estrogen-free conditions, respectively (51). Pilot transient-transfection and ERE-driven luciferase reporter gene assays were developed to initially address ERRα's transactivation potential in the MCF-7:WS8 and MCF-7:5C cells. These studies showed that ERRα interfered with basal and E_2-stimulated transcription in the WS8 cells (Fig. 6B, discussed in detail in **Revised Task 3B**). In contrast, the hormonally insensitive 5C cells did not exhibit significant E_2-stimulation of ERE-mediated reporter gene activity as expected. If alterations in ERRα's transcriptional potential were involved in the TAM-resistant phenotype in 5C cells, one may predict that ERRα would activate ERE-mediated transcription in these cells rather than repress transcription as in the WS8 cells. However, the presence of an ERRα expression plasmid did not significantly affect the very low basal reporter activity in these 5C cells (data not shown). Moreover, as described above in **Task 1A**, 5C cells do not express ERRα splice-variant mRNAs or exhibit mutations in ERRα cDNA coding sequence. These preliminary data suggest that factors other than ERRα are involved in the development of TAM-resistance in this particular model system. However, given that ERRα likely modulates estrogen-responsive transcription, ERRα may still play a role in the development of a significant number of TAM-resistant breast cancers, though not in all cases. Hence, a better-defined model system of TAM-resistant breast cancer other than matched WS8 and 5C cells may be better suited for exploring ERRα's potential role in TAM-resistant disease progression.

Overexpression of epidermal growth factor receptor (EGFR) (52) and c-*erb*B2 [also termed HER-2 and *neu*; (53, 54)] has been implicated in the failure of antiestrogen therapy in some breast

cancer patients. Several reports describe the reproducibility of TAM-stimulated growth of MCF-7 cells as solid tumors in athymic nude mice (55-58). These tumors overexpressed EGFR relative to normal MCF-7 cells (59). Elevated MAPK activity was demonstrated in T5-PRF cells, another mammary carcinoma cell line growth-selected for estrogen independence from parental estrogen-responsive T5 human breast cancer cells (60). Stable introduction of c-*erb*B2 into MCF-7 cells conferred TAM resistance (61, 62). Both EGFR and c-*erb*B2 signal through the Ras/MAPK pathway and MAPK signaling mediates activation of unliganded ERα through phosphorylation events (62-66). Because c-*erb*B2 can induce activation of unliganded ERα, I tested whether increased c-*erb*B2 signaling could also modulate ERRα's transcriptional potential using transient-transfection and reporter gene assays (Fig. 7). These assays employed a matched set of plasmids that encode rat versions of oncogenic c-*erb*B2 (c-*erb*B2$_{Onc}$, pJRneu) and dominant-negative H*ras* (H*ras*$_{DN}$, pJRH*ras*$_{DN}$) under the control of a retroviral promoter [(67-69); obtained from Michael N. Gould's laboratory]. MCF-7:WS8 cells were co-transfected with the indicated combinations of plasmids and incubated in the presence or absence of the antiestrogen ICI-182780 to distinguish between effects of endogenous ERα and ERRα. I found that an increased level of ERRα1 by itself had no significant effect on SFRE-driven transcription (Fig. 7, 2 vs. 1), but did activate transcription in the presence of c-*erb*B2$_{Onc}$ (Fig. 7, 4 vs. 2), with this activation being attenuated when H*ras*$_{DN}$ was present as well (Fig. 7, 7 vs. 4). These data indicate that increased MAPK signaling may activate ERRα-dependent transcription. Next, I constructed an ERRα1 truncation mutant (ERRα1$_{1-376}$) that lacks a conserved coactivator binding site termed a nuclear receptor (NR) box in ERRα's C-terminal putative AF2 domain (70). NR boxes, comprised of the consensus sequence LXXLL [where L is leucine and X is any amino acid], mediate protein-protein interactions between NRs and coactivators, leading to recruitment of histone acetylation complexes and the basal transcription machinery (71-73). A similar ERRα expression plasmid has been previously shown to be incapable of activating transcription (8, 9). ERRα1$_{1-376}$ failed to activate transcription in the presence of c-*erb*B2$_{Onc}$ (Fig. 7, 10 vs. 4 and 8). Moreover, the presence of ERRα1$_{1-376}$ inhibited full-length ERRα's (ERRα1$_{1-423}$) activation potential with c-*erb*B2$_{Onc}$ (Fig. 7, 11 and 12 vs. 4). Therefore, I tentatively conclude that (i) ERRα1$_{1-376}$ can act as a dominant-negative mutant, and (ii) c-*erb*B2 signaling through the MAPK pathway targets ERRα to modulate its activation potential.

Upon original consideration of my fellowship application, the Review Panel suggested that rather than generating ERRα knockout cells, I should consider an antisense approach because of the likelihood of multiple endogenous ERRα alleles. However, the mechanisms whereby an antisense RNA interferes with translation are largely undefined and often do not satisfactorily block protein expression of a given target. I interpret the Panel's suggestion to mean that I should consider generating the equivalent of ERRα knockout cells using an approach whereby the caveat of potential multiple ERRα alleles are circumvented. Towards this end, I will employ the dominant-negative ERRα1$_{1-376}$ variant. In light of the recent data described directly above and the Panel's Comments, rather than generating ERRα gene knockout sublines using matched parental WS8 and 5C cells as originally proposed, I would like to revise the original **Specific Aim II** as follows:

Revised Specific Aim II - To test whether c-*erb*B2 induced activation of ERRα-dependent transcription may contribute to the TAM-resistant phenotype by generating and characterizing MCF-7 sublines stably-transfected with plasmids inducibly expressing wild-type or dominant-negative ERRα variants in the presence and absence of activated c-*erb*B2.

Revised Task II. Establishment MCF-7 cell sublines that inducibly express ERRα variants in the presence and absence of activated c-*erb*B2, thereby facilitating examination of ERRα's potential role in a defined TAM-resistant cell line model system (months 15-36).

Revised Task 2A. Establishment of MCF-7 cell sublines inducibly expressing ERRα variants in the presence and absence of activated c-erbB2 (months 15-24).

Matched stably transfected MCF-7 cell lines will be established that inducibly express wild-type or dominant-negative ERRα1 variants with or without activated c-*erb*B2 under the control of a Tetracycline (Tet) –regulated bi-directional promoter and are referred to herein as *erb*B2^{+}/ERRα1$_{WT}$, *erb*B2^{+}/ERRα1$_{DN}$, *erb*B2^{-}/ERRα1$_{WT}$, and *erb*B2^{-}/ERRα1$_{DN}$ cells, respectively. These stably-transfected cell lines will express the transgenes in an inducible manner using a variant of the Tet repressor that

binds DNA when occupied by Tet to repress transcription (Tet-off repressor), but does not bind DNA when unoccupied by Tet (74). An MCF-7 cell line that stably expresses the Tet-off repressor (MCF-7/Tet-off) is available from Clontech (Palo Alto, CA). This MCF-7/Tet-off cell line has been shown to induce a Tet-regulated luciferase reporter gene 75-fold when Tet is removed from the culture medium. I will employ the bi-directional Tet-regulated expression plasmid pBI [Clontech;(75)] that allows two genes of interest to be regulated coordinately in a Tet-responsive manner through seven Tet-repressor DNA-binding sites flanked on both sides by minimal cytomegalovirus (CMV) promoters. Activated c-*erb*B2 in combination with ERRα1$_{WT}$ or ERRα1$_{1-376}$ (dominant-negative variant, ERRα1$_{DN}$) will be subcloned into pBI to generate pBI-*erb*B2/ERRα$_{WT}$, pBI-*erb*B2/ERRα$_{DN}$, pBI-ERRα$_{WT}$, and pBI-ERRα$_{DN}$. I will stably transfect MCF-7/Tet-off cells with these bi-directional Tet-regulated expression plasmids to generate sublines in which ERRα is predicted to activate (*erb*B2$^+$ sublines) transcription (Fig. 7) or repress (*erb*B2$^-$ sublines) transcription (Fig. 6B). The pBI-based vectors will be co-transfected with a hygromycin-resistance expression plasmid to facilitate selection of the stably transfected sublines. I will screen the *erb*B2$^+$ sublines for significantly greater MAPK activity compared to the *erb*B2$^-$ sublines by immunoprecipitating the MAPK enzyme from these sublines and incubating it with the positive control substrate, PHAS-I, in the presence of [γ-^{32}P]ATP. Subsequently, these cell lines will be further screened for expression of equivalent amounts of exogenous ERRα variants by Western blotting methods employing the ligand affinity-purified anti-ERRα1$_{49-66}$ antibodies.

Using similar methods as those described for Fig. 6 and Fig 7, I will assay expression of pEREx3-luc and pSFREx3-luc reporter genes in *erb*B2$^-$/ERRα1$_{WT}$ and *erb*B2$^+$/ERRα1$_{WT}$ cells before and after withdrawal of Tet from the media to induce transgene expression. I predict that induction of ERRα1 expression should result in repression of ERE- and SFRE-regulated transcription in the *erb*B2$^-$/ERRα1$_{WT}$ subline, whereas induction of both c-*erb*B2 and ERRα1 expression should give rise to increased reporter gene activity in the *erb*B2$^+$/ERRα1$_{WT}$ subline. The ERRα dependence of the effects on transcription will be verified by performing similar reporter gene assays in the control *erb*B2$^-$/ERRα1$_{DN}$ and *erb*B2$^+$/ERRα1$_{DN}$ sublines. As an additional control for c-*erb*B2-induced modulation of ERRα-dependent transcription through the MAPK pathway, similar co-transfection reporter gene assays will also be performed with the inclusion of a dominant-negative H*ras* construct (pJRH*ras*$_{DN}$). These inducible MCF-7 sublines will be used in **Revised Task 3B** to examine the modulatory effects of ERRα on E_2-responsive transcription and to examine c-*erb*B2 induced activation of ERRα on transcriptional regulation of genes whose promoters contain putative ERRα-binding sites (see Table 2 and Fig. 9).

Revised Task 2B. Examination of ERRα's potential role in c-*erb*B2-mediated TAM-resistant MCF-7 sublines (months 25-36).

Since MCF-7 cells overexpressing c-*erb*B2 have been previously reported to acquire TAM resistance (61, 62), I will test by cell culture growth curve analysis multiple *erb*B2+/ERRα1$_{WT}$ sublines, cultured in the absence of Tet to induce c-*erb*B2 expression, for c-*erb*B2-dependent attenuation of 4OHT's growth suppressive effects. However, long-term induction of c-*erb*B2 expression may be required to attenuate sufficiently 4OHT's effects. If I observe this attenuation of 4OHT-mediated growth suppression or TAM resistance, I will likewise evaluate *erb*B2$^+$/ERRα1$_{DN}$ cells to test whether ERRα1$_{DN}$ blocks c-*erb*B2's ability to promote TAM resistance. If *erb*B2$^+$/ERRα1$_{DN}$ cells are not able to acquire TAM-resistance, this result would suggest ERRα, probably in an activated form, may be involved in the development of some TAM-resistant breast tumors. If ERRα1$_{DN}$ is implicated in interfering with c-*erb*B2-dependent acquisition of TAM resistance, this finding would suggest that activated ERRα may functionally bypass 4OHT's antagonism of ERα-dependent growth stimulation. In future studies, I would extend these findings by testing whether *erb*B2+/ERRα1$_{WT}$ cells grown as solid tumors in athymic mice frequently develop TAM resistance, whereas *erb*B2$^+$/ERRα1$_{DN}$ cells do not.

Revised Task 2C. Characterization of ERRα's phosphorylation status (months 25-36).

Because c-*erb*B2 signals through the MAPK pathway, I reasoned that activation of ERRα-dependent transcription in the presence of activated c-*erb*B2 (Fig. 7) may be mediated by changes in ERRα's phosphorylation state. To begin to address this issue, I tested whether activated MAPK can directly phosphorylate ERRα *in vitro*. As a source of activated MAPK, I used rat p42 ERK2 (Calbiochem) that had been previously phosphorylated *in vitro* by a constitutively active MEK1 (MAPK

kinase) mutant. GST-fusion proteins were expressed and purified from *E. coli*. Activated ERK2 and [γ-^{32}P]ATP were incubated with equimolar amounts of purified full-length ERRα1 (GST-ERR$\alpha 1_{1\text{-}423}$), truncated ERRα1 variants (GST-ERR$\alpha 1_{1\text{-}376}$, GST-ERR$\alpha 1_{1\text{-}173}$) and control substrates. PHAS-1 [phosphorylated heat- and acid-stable protein (Calbiochem)] and GST-βglobin served as positive and negative controls, respectively. Following the kinase reactions, the mixtures were resolved by denaturing PAGE and scanned with a PhosphorImager (Molecular Dynamics; Fig.8). As expected, ERK2 phosphorylated PHAS-I, but not GST-βglobin. I found all of the ERRα1 variants were phosphorylated. Although this kinase assay is not quantitative, the increased signal intensity of GST-ERR$\alpha 1_{1\text{-}423}$ versus GST-ERR$\alpha 1_{1\text{-}376}$ and GST-ERR$\alpha 1_{1\text{-}173}$ suggests ERRα1 may be phosphorylated within the region 377-423 in addition to 1-173. Thus, ERRα can serve as a MAPK substrate *in vitro*.

Since c-*erb*B2 can induce activation of ERRα-dependent transcription (Fig. 7) and MAPK can phosphorylate ERRα (Fig. 8), I propose that changes in ERRα's phosphorylation state may regulate its transcriptional activity. Since MAPK-dependent phosphorylation of ERα results in loss of corepressor binding (76) and SMRT can interact with ERRα (O'Reilly, Ariazi, and Mertz, in preparation), changes in ERRα's phosphorylation state may regulate its transactivation potential through differential recruitment of coregulators. I will determine ERRα's phosphorylation status in MCF-7 cell lines inducibly expressing *erb*B2$^+$/ERRα1 and *erb*B2$^-$/ERRα1 and map the phosphorylated residues. In future studies beyond the scope of this Fellowship Proposal, my lab group will likely determine whether changes in ERRα's phosphorylation state cause differential recruitment of coactivators and corepressors. To determine whether ERRα is differentially phosphorylated in *erb*B2$^+$/ERRα_{WT} cells compared to *erb*B2$^-$/ERRα_{WT} cells and to map the targeted residues, I will initially immunoprecipitate ERRα proteins from these sublines using the ligand affinity-purified anti-ERR$\alpha 1_{49\text{-}66}$ antibodies, followed by separation of ERRα isoforms in 2D polyacrylamide gels in which the gel's pI gradient dimension should allow resolution of differentially phosphorylated ERRα isoforms. The 2D gels will be transferred to membranes for Western blotting analysis with anti-ERR$\alpha 1_{49\text{-}66}$ antiserum to confirm the identity of ERRα. As additional controls, ERRα isoforms corresponding to various phosphorylation states in the 2D gels will be confirmed by co-migration of ERRα proteins treated with protein phosphatases and activated MAPK using methods similar to those used for Fig. 8. Later, ERRα phosphopeptides will be identified by radiolabeling cells with [γ-^{32}P]ATP, immunoprecipitating ERRα using the anti-ERR$\alpha 1_{49\text{-}66}$ antibodies, digesting the protein with trypsin, and resolving the peptides in a 2D polyacrylamide gel. Radiolabeled peptide spots will be recovered from the gel and sequenced using a triple quadrapole mass spectrophotometer available at the UW-Biotech Center. Thus, I will be able to identify the specific phosphorylation events that may regulate ERRα's transcriptional potential as an activator or repressor due to the presence or absence of activated c-*erb*B2.

Upon review of this Fellowship Proposal, Reviewer B commented that generation of phosphospecific anti-ERRα antibodies might be a difficult and time-consuming undertaking. Hence, generation of such phosphospecific antibodies will likely not be able to be accomplished during the time of this award. However, my lab group intends to accomplish this task following the current granting period. In future studies beyond this Fellowship Proposal, following identification of phosphorylated residues, my lab intends to introduce amino acid substitutions to test whether these specific phosphorylation events are important for determining ERRα's transcriptional potential by transient-transfection and reporter gene assays. If these phosphorylation events are important in determining ERRα's transactivation potential, we would be encouraged to investigate in future studies whether ERRα's phosphorylation status modulates the recruitment of corepressors and coactivators. If phosphorylation events modulate ERRα's activities, we would eventually develop a phosphospecific anti-ERRα antibody that could distinguish activated from unactivated ERRα. Such phosphospecific antibodies might enable diagnostic determination of ERRα's phosphorylation state in clinical tumors and examination for correlations between ERRα's phosphorylation state and prognosis, sensitivity to antiestrogen therapy, and progression to TAM resistance. This concept has already been employed successfully with a phosphospecific anti-c-*erb*B2 antibody (PN2A) where it was demonstrated by multivariate analyses that phosphorylated c-*erb*B2 provided significant prognostic value in node-positive primary breast carcinomas (77).

Specific Aim III - To test whether genetic ablation of ERRα modulates the activity of transcriptional regulatory elements in the hormonally sensitive and insensitive cell lines described in Aim II. (A) ERRα-mediated transcriptional regulation will be investigated with heterologous reporter genes containing various EREs from promoters known to affect breast cancer, e.g. progesterone receptor (PgR), pS2, HER-2/neu, et cetera, as well as documented AP-1 sites modulated by ERα. (B) These EREs and AP-1 sequences will also be examined by gel-mobility-shift assays (GMSAs) and immunoshift assays. DNA binding and protein-protein interactions of ERα and hERRα1, including possible specific isoforms, will be correlated with transcriptional activity.

Task 3. To test whether genetic ablation of ERRα modulates ERE- or AP-1-dependent transcription, and to correlate transcription activity with DNA binding and protein-protein interations of ERα and ERRα, including possible specific isoform(s) (months 25-36)

Task 3A. Characterization of ERE and AP-1 DNA-binding activity by GMSAs and immunoshift assays (months 25-36).

Task 3B. Evaluation of ERE- and AP-1-dependent transcription using heterologous reporter genes (months 25-36).

ERRα and ERα bind EREs and SFREs [Figs. 3, 9 and 11; (4, 9)], directly interact through protein-protein contacts [Fig. 10; O'Reilly, Ariazi, and Mertz, in preparation; (4)], and are themselves estrogen-regulated genes [Fig. 1; (23)]. Thus, ERRα could modulate estrogen responsiveness through several non-mutually exclusive mechanisms. ERRα and ERα may compete for binding to the same element through protein-DNA interactions (model I; Fig. 11), bind separate subelements within a composite element [model II; (10)], form true ERRα-ERα heterodimers capable of binding DNA (model III), form ERRα-ERα complexes not capable of binding DNA (model IV), and/or compete for binding co-regulators of ERα (model V). Moreover, estrogen-dependent changes in ERRα and ERα expression (e.g., Fig. 1) may indirectly affect response element occupancy. ERRα has been demonstrated to modulate estrogen-responsive transcription of lactoferrin (10) and osteopontin (9, 78, 79). Thus, it is highly likely ERRα modulates other estrogen-responsive genes, including ones involved in breast cancer. Identification of ERRα-regulated genes and elucidation of the mechanisms by which it regulates these genes could impact the management of breast cancer and lead to the development of ERRα as a new therapeutic target.

Upon the review of this Fellowship Proposal, both Reviewers A and B commented that there were too many proposed experiments. Hence, I would like to alter **Specific Aim III** to focus on the characterization of DNA sequences directly bound by ERRα such as EREs and SFREs rather than indirectly bound by the receptor through protein-protein interaction such as AP-1 sites. Hence, I would like to revise the originally proposed **Specific Aim III** as follows:

Revised Specific Aim III -To Begin to Elucidate Mechanisms By Which ERRα May Play Roles in Breast Carcinogenesis By Identifying Estrogen-responsive Breast Cancer Prognosticator Genes Which Are Transcriptionally Modulated Through ERRα and Determining the Effects of ERRα on Transcriptional Regulation of These Genes.

Revised Task 3A. Characterization of putative ERRα-binding sites by GMSAs and immunoshift assays (months 25-36).

Identification of ERRα-binding sites in promoters of genes implicated in breast cancer.

Knowing which genes are transcriptionally modulated by ERRα may help to clarify ERRα's potential role in breast cancer. To identify potential ERRα-responsive genes, the transcriptional regulatory regions of genes known to be implicated in breast cancer were examined for sequences potentially capable of being bound by ERRα by searching a eukaryotic promoter database [http://www.epd.isb-sib.ch/; (80)] and Genbank (http://www.ncbi.nlm.nih.gov/) (Table 2). The core ERRα-binding sequences used to query the databases were taken from studies conducted by the laboratories of J.M. Vanacker (8) and V. Giguère (7). ERRα binding to many of these potential sites was

confirmed with EMSAs employing lysates of COS cells transfected with an ERRα1 expression plasmid (Fig. 9). Although not quantitative, these preliminary EMSAs clearly indicate that ERRα binds these sites to varying degrees. Almost all of the genes tested whose promoters contain confirmed ERRα-binding sites are estrogen responsive and exhibit prognostic significance in breast cancer (Table 2). I propose to characterize the DNA-binding properties of ERRα to many of these binding sites and to test whether some of the corresponding genes are responsive to changes in ERRα expression in **Revised Aim IIB**.

Cross talk between ERα and ERRα

One model by which ERRα can affect estrogen responsiveness is via the formation of heterodimers with ERα, thereby affecting ERα's DNA binding specificity and/or affinity for EREs and SFREs. My lab group (4) and others (10) previously reported that ERRα and ERα directly interact. To map the region of ERRα that interacts with ERα, we used GST pull-down assays that employed GST-ERRα truncation variants and [^{35}S]-labeled ERα. We found that ERα interacts with a region overlapping ERRα's T/A box located in the D domain (amino acids 145-173, Fig. 10; O'Reilly, Ariazi, and Mertz, in preparation). An amino acid sequence alignment of the T/A boxes from ERα, ERRα, ERRβ, and ERRγ reveals 100 % conservation among the ERRs and 50 % conservation between ERα and the ERRs. Thus, ERRα binding to ERα is likely of physiological significance.

Models by which ERRα can affect estrogen responsiveness include (i) competing with ERα for binding to EREs and SFREs via direct protein-DNA interactions, and (ii) affecting ERα's ability to bind DNA via direct protein-protein interactions. Intriguingly, such interactions could provide an additional level of modulation of receptor-dependent transcriptional activity by sequestering either receptor away from DNA. My lab group have found that the addition of increasing amounts of ERRα both interferes with ERα binding to the vitERE and results in the appearance of ERRα-DNA complexes (Fig. 11). However, either of these non-mutually exclusive models can account for this finding. We will distinguish between these two hypotheses using ERRα mutants that interact with ERα but do not bind DNA in EMSAs. The results of these studies will also likely provide a biochemical basis for the potential prognostic significance of the ERRα/ERα ratio correlating with DNA ploidy and S-phase fraction (Table 1).

Revised Task 3B. Evaluation of ERRα-binding sites using heterologous reporter genes (months 25-36).

ERRα-mediated repression of E_2-induced transcription.

ERRα has been demonstrated to activate (8-10, 34, 79, 81, 82) or repress (4) transcription depending upon the specific promoter and cell line. Given ERRα and ERα directly interact and bind many of the same HREs, ERRα could either interfere or enhance estrogen-responsive transcription. To test directly the affects of ERRα, I have developed reporter gene assays. My vectors contain 3 tandem consensus EREs or SFREs cloned upstream of a basal thymidine kinase (TK) promoter driving luciferase expression. They are termed pEREx3-luc (obtained from V.C. Jordan's laboratory) and pSFREx3-luc (obtained from J.M. Vanacker's laboratory). I co-transfected pEREx3-luc with an ERRα1 expression vector into CV-1 cells, which naturally lack ERα, and MCF-7:WS8 cells, which contain ERα. As expected, ERRα1 was found to activate transcription through the EREs in an E_2-independent manner in CV-1 cells (see Fig. 6A). However, ERRα1 repressed both basal ERE-driven transcription and, importantly, E_2-induced transcription in MCF-7:WS8 cells (Fig. 6B).

*Charactization of ERRα's direct effects on transcription of the genes whose promoters contain validated ERRα-binding sites using reporter gene assays with inducible MCF-7 cell lines established in **Revised Specific Aim IIA**.*

In **Revised Specific Aim IIA**, I propose to establish stably transfected MCF-7 cell lines that inducibly express specific ERRα1 variants with or without c-erbB2$_{Onc}$ under the control of a Tetracycline (Tet) –regulated bi-directional promoter where ERRα should activate (*erb*B2$^+$/ERRα1) transcription as in Fig. 7 or repress (*erb*B2$^-$/ERRα1) transcription as in Fig. 6B. I will use the inducible MCF-7/*erb*B2$^-$ cell lines to characterize the effects of ERRα-mediated modulation with various ERα-ligands of some of the genes whose promoters listed in Table 2 contain validated ERRα-binding sites. Next, I will examine in MCF-7/*erb*B2$^+$ cells the effects of c-*erb*B2-induced activation of ERRα on transcriptional regulation of these estrogen-responsive genes. One of the many possible findings is that

the presence of activated c-*erb*B2 will result in a reversal of ERRα's transcriptional potential on a given promoter: in cases where ERRα was a repressor it becomes an activator, but in cases where it was an activator it becomes a repressor. A second possibility is that the presence of activated c-*erb*B2 will result in constitutive activation of ERRα, independent of promoter context. A third possibility is that c-*erb*B2-induced alterations in ERRα may not result in a change in ERRα's transcriptional potential for some of the promoters. This last possibility would suggest ERRα's transcriptional potential, in certain cases, can be largely determined by promoter context independent of c-*erb*B2-induced activation of the MAPK signaling pathway. Later, I will study the effects of ERα ligands on c-*erb*B2-induced activation of ERRα by reporter gene assays as outlined directly above. In *erb*B2$^-$/ERRα1$_{WT}$ cells, I expect ERRα to interfere with E_2-stimulated transcription. However, in E_2-treated *erb*B2$^+$/ERRα1$_{WT}$ cells, the presence of activated c-*erb*B2 leading to activation of ERRα may allow ERRα and ERα to cooperate either additively or synergistically. Treatment of these cells with ICI-182780, a complete antiestrogen, may block ERα's ability to cooperate with activated ERRα induced by c-*erb*B2. The possible outcomes of treating *erb*B2$^+$/ERRα1$_{WT}$ cells with the partial antiestrogen 4OHT are difficult to predict because c-*erb*B2-mediated stimulation of MAPK signaling may result in loss of corepressor binding with 4OHT-occupied ERα (76); therefore, in this case, ERRα and ERα may or may not cooperate. Similarly, I will employ the matched ERRα1$_{DN}$ cell lines to control for ERRα-dependent effects on transcription. Additional controls will include promoters containing site-specific mutations in their ERRα-binding sites. The data obtained from these reporter gene assays should indicate whether the identified binding sites are authentic ERRα response elements. Moreover, these transcription assays will provide a means to evaluate the functional activities of any potential ERRα variants identified in **Revised Specific Aim IIIA** above. It will also be interesting to see whether the effects observed differ among the various promoters tested.

In summary, I expect the data obtained from these experiments to validate the hypothesis that ERRα can affect the estrogen responsiveness of some genes via interactions with ERα and/or transcriptional response elements. They will likely also indicate that ERRα can directly affect expression of some genes whose altered expression correlates with breast cancer pathogenesis and disease progression.

KEY RESEARCH ACCOMPLISHMENTS

- ERRα is itself a modestly estrogen-responsive gene (Fig. 1).

- Ligand affinity-purified anti-ERRa1$_{49-66}$ antibodies have been generated and found suitable for Western blotting applications (Fig. 2) and EMSAs (Fig. 3).

- In a small sample size, ERRα mRNA is frequently expressed at significantly lower levels in random clinical primary breast carcinomas than in normal mammary tissues (Fig 4).

- Among a small number of clinical breast carcinomas, the relative ERRα/ERα mRNA ratio correlates with S-phase fraction and DNA ploidy. Thus, the ERRα/ERα ratio may serve as a biomarker of tumor aggressiveness (Table 1).

- At least one ERRα mRNA splice-variant exists in breast carcinomas (Fig. 5).

- ERRα can activate transcription in CV-1 and repress E2-dependent transcription in MCF-7 cells (Fig. 6).

- Increased c-*erb*B2 signaling through the MAPK pathway can induce activation of ERRα (Fig. 7).

- ERRα can be phosphorylated by activated MAPK *in vitro* (Fig. 8).

- Potential ERRα DNA-binding sites have been identified in numerous promoters of genes implicated in breast cancer (Table 2) and have initially been authenticated by EMSAs (Fig. 9).

- ERRα directly interacts with ERα through protein-protein contacts mapped to ERRα's T/A box adjacent to the receptor's core DNA-binding domain (Fig. 10).

- ERRα and ERα can compete for binding DNA (Fig. 11).

REPORTABLE OUTCOMES

Publications

O'Reilly, G. H., Ariazi, E. A., and Mertz, J. E. Human Estrogen-related Receptor α1: Identification and Mapping of Functional Domains (manuscript in preparation).

Patents

Mertz, J. E., Johnston, S. D., Kraus, R. J., and Ariazi, E. A. Human Estrogen-related Receptor α. U.S. Patent (pending). Application Number 09/488,730. Filing date of January 20, 2000.

Awarded Funding

"Estrogen-related Receptor Beta in Breast Cancer and Estrogen Responsiveness"
Principal Investigator: Janet E. Mertz, Ph.D.
Co-principal Investigator: Eric A. Ariazi, Ph.D.
Agency: US Army Medical Research and Materiel Command
Type: Concept Award (BC995035) Period: 9/1/00 to 8/31/01
The aims of this proposal are: (1) to test whether alterations in ERRbeta expression correlate with the development of breast cancer; and (2) to begin to identify ERRbeta-regulated genes and to ascertain whether ERRbeta levels modulate estrogen responsiveness in mammary carcinoma cells.

Applied for Funding

"Estrogen-related Receptor alpha In Breast Cancer"
Principal Investigator: Janet E. Mertz, Ph.D.
Co-principal Investigator: Eric A. Ariazi, Ph.D.
Agency: National Cancer Institute, NIH
Type: R01 Period: 2001 to 2006
The aims of this proposal are: (i) to determine the utility of ERRalpha as a novel breast cancer prognosticator and target for therapy; and (ii) to elucidate the mechanisms by which ERRalpha modulates the transcriptional regulation of genes involved in breast cancer.

CONCLUSIONS

Several assays and reagents have been developed to study ERRα in breast cancer including real-time quantitative PCR assays, ligand-affinity purified anti-ERRα antibodies, EMSAs to investigate ERRα-binding to DNA, ERRα transient-transfection and reporter gene assay, and GST pull-down assays. I have found that ERRα is itself modestly estrogen responsive. I also found that expression of ERRα mRNA is significantly lower in a small panel of random breast carcinomas than normal MECs and that the ERRα/ERα ratio correlates with S-phase fraction and DNA ploidy, indicating potential as a novel breast prognosticator. Additionally, I found an ERRα2 splice-variant mRNA in the breast tumors. To investigate ERRα-dependent transcriptional mechanisms, I have identified numerous potential ERRα-binding sites in the promoters of genes implicated in breast cancer and initially verified these sites by EMSAs. ERRα and ERα were demonstrated to compete for binding to a consensus ERE. However, the mechanism of this cross talk may occur through protein-DNA or protein-protein interactions since the receptors were also demonstrated to interact through a region containing ERRα's T/A box (amino acids 145-173). ERRα activates transcription in CV-1 and represses E_2-stimulated transcription in MCF-7 cells. Furthermore, increased c-*erb*B2 signaling though the MAPK pathway leads to activation of ERRα and ERRα can be phosphorylated by activated MAPK *in vitro*.

I had originally proposed establishing ERRα knockout sublines derived from matched hormonally-responsive and unresponsive MCF-7 cells. The Review Panel commented that I should try another approach such as antisense RNA methods to overcome the caveat of multple endogenous ERRα alleles. I have since characterized a new ERRα truncation mutant that lacks a C-terminal coactivator binding domain that functions as a dominant-negative variant in transient-transfection and reporter gene assays. It has also been reported that stable-transfection of c-erbB2 into MCF-7 cells results in a TAM-resistant phenotype, and I found that c-erbB2 induces activation of ERRα. To study ERRα's potential role in TAM-resistance, I therefore propose to establish MCF-7 sublines inducibly expressing wild-type and dominant-negative ERRα variants in the absence and presence of activated c-*erb*B2.

The above findings could provide the basis for future studies to determine whether ERRα status may indicate sensitivity to hormonal therapies, progression to tamoxifen resistance, and disease outcome.

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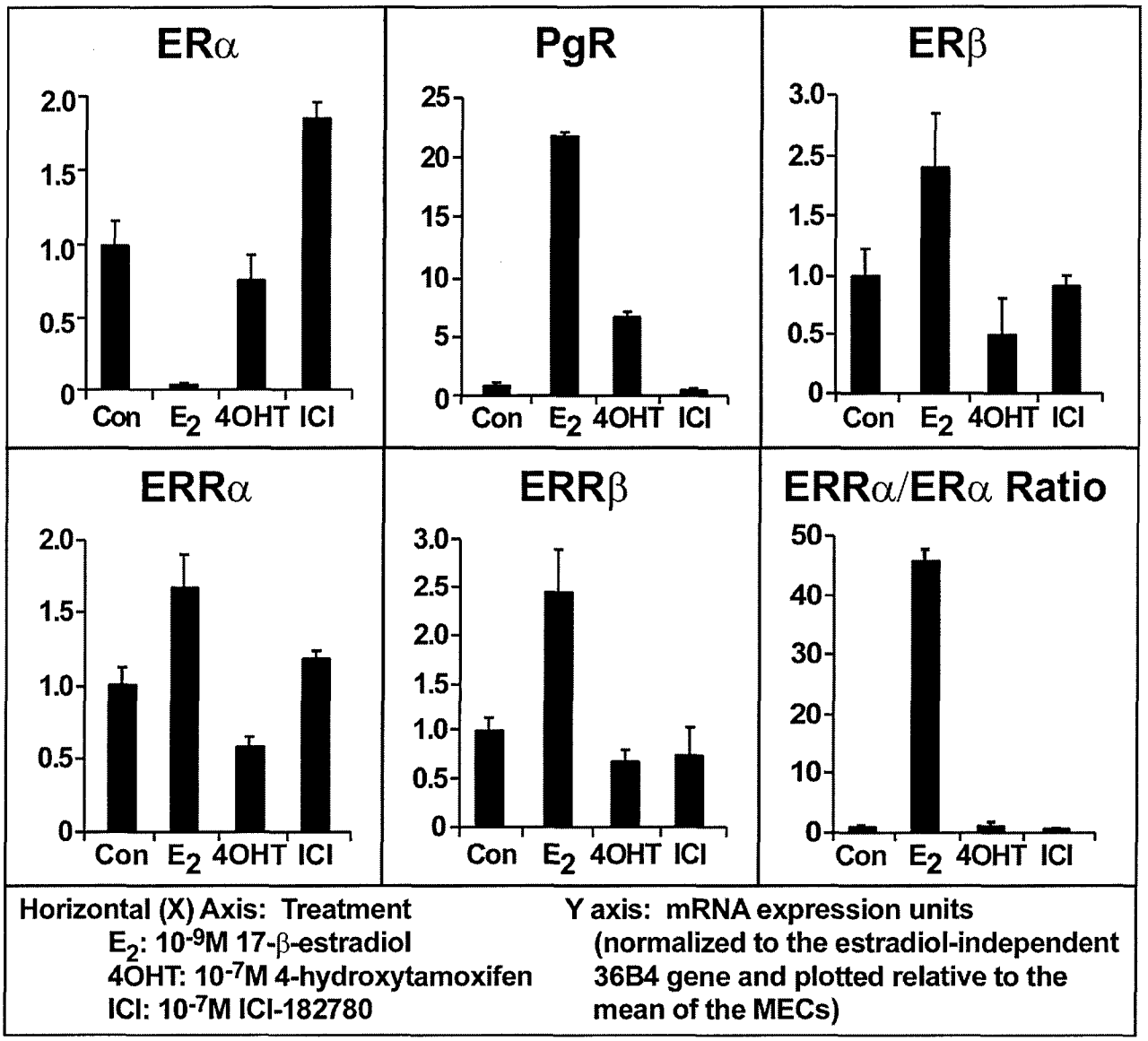


Fig. 1. Nuclear receptor levels in MCF-7:WS8 cells as determined by real-time quantitative PCR. Cells were seeded in estrogen-free medium [phenol red-free/dextran-coated charcoal-stripped fetal bovine serum (CS-FBS)] and treated with 10^{-8} M E_2, 10^{-6} M 4OHT, 10^{-6} M ICI-182780, or vehicle alone (control) for 48 h. Error bars represent the SDs calculated from triplicate measurements on the same day. This experiment has been independently repeated with similar results.

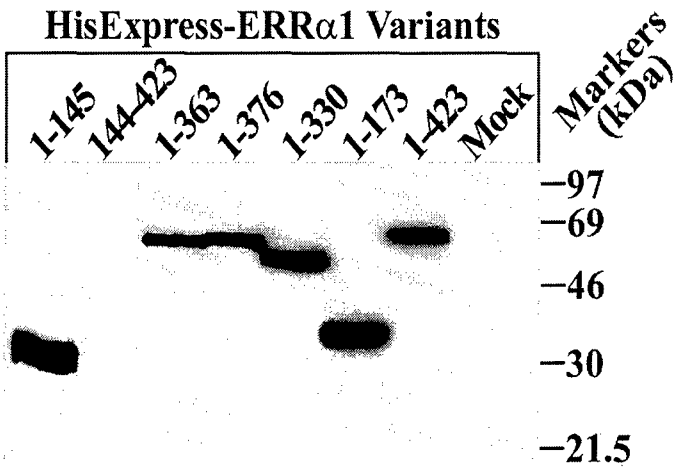


Fig. 2. Anti-ERRα1$_{49-66}$ antibody-probed Western blot of COS cells transfected with pExpERRα1 [N-terminal "Express" (Invitrogen) epitope-tagged ERRα1] corresponding to full-length (1-423) and truncated variants as indicated. The ERRα1$_{144-423}$ truncated variant does not contain the immunizing peptide sequence (ERRα1$_{49-66}$).

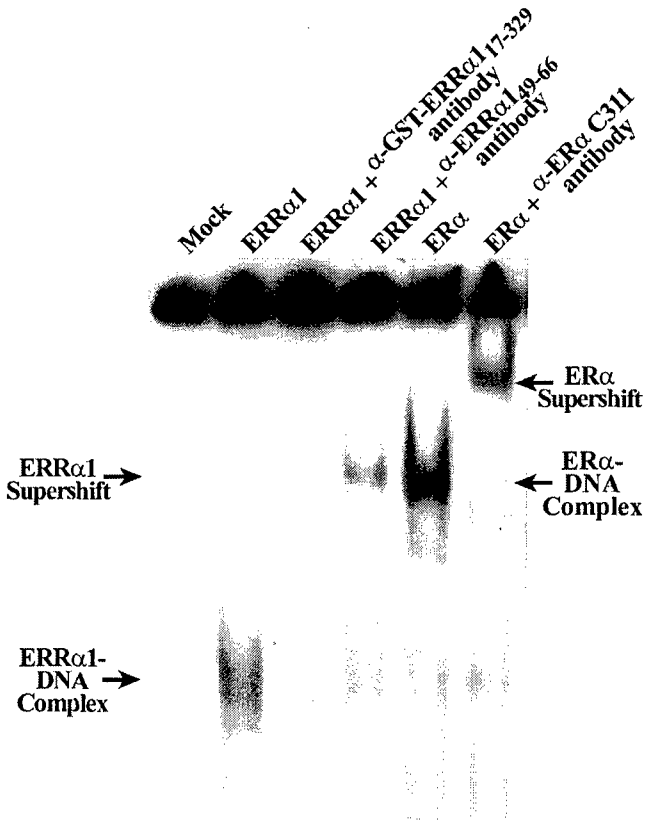


Fig. 3. EMSAs of ERRα and ERα binding to the vitERE probe DNA. Whole-cell extracts of COS cells transfected with pRSV-ERRα1 or mock-transfected were used as protein sources.

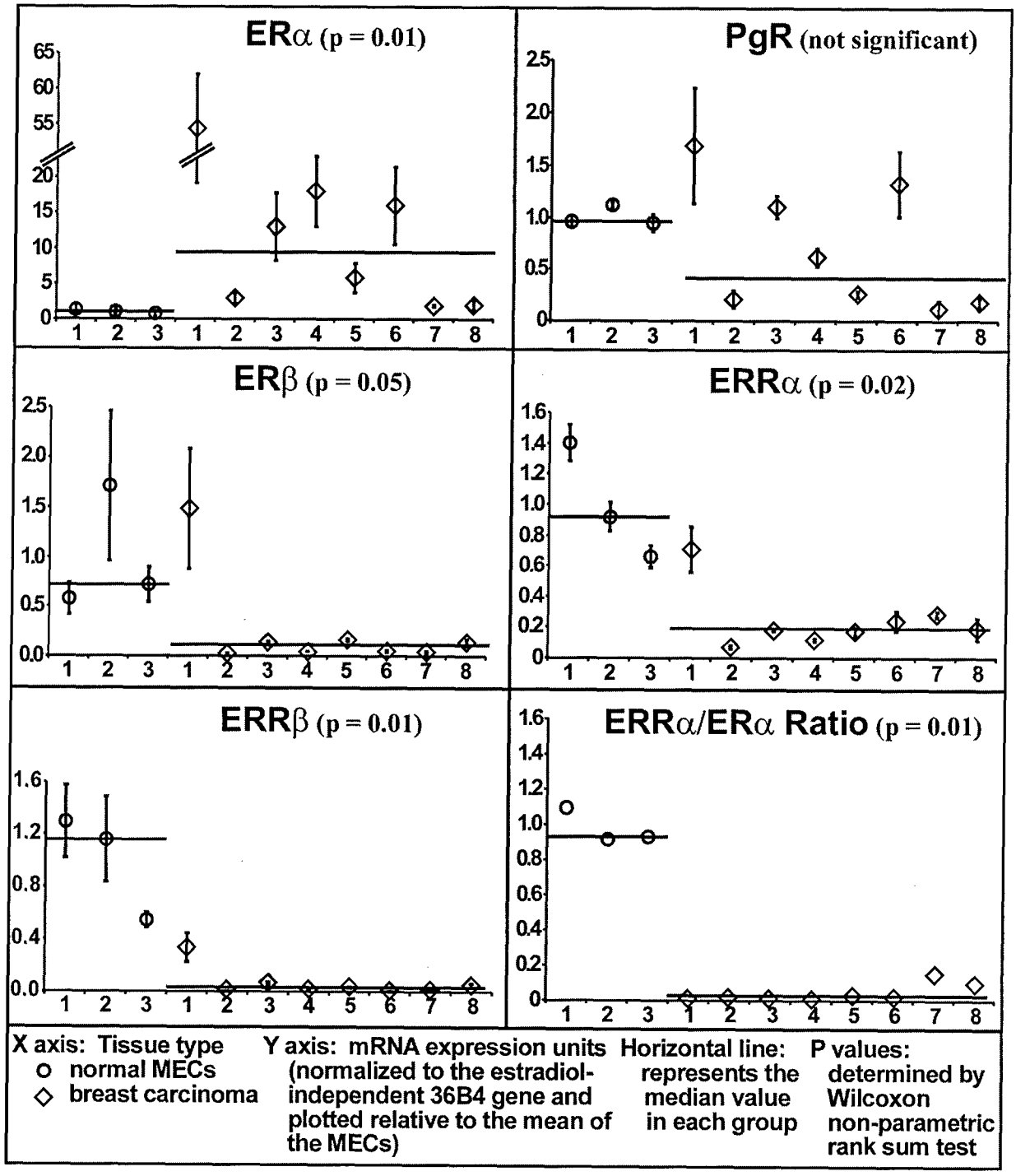


Fig. 4. Nuclear receptor mRNA levels in normal MECs and breast carcinomas as determined by real-time quantitative PCR. Error bars represent the SD of the mean from triplicate measurements on the same day.

	ER-LB	S-Phase	DNA Ploidy	ERα	ERβ	ERRα	ERRβ	ERRα/ERα
ER-LB	1.00‡	-0.50	0.39	**0.83‡**	0.48	0.17	0.52	**-0.76†**
S-Phase	-0.50	1.00‡	-0.58	-0.50	-0.07	0.57	-0.14	**0.71***
DNA ploidy	0.39	-0.58	1.00‡	-0.62	-0.17	-0.17	-0.17	**-0.85‡**
ERα	**0.83‡**	-0.50	0.62	1.00‡	0.52	0.19	0.33	**-0.88‡**
ERβ	0.48	-0.07	-0.17	0.52	1.00‡	0.43	**0.81†**	-0.17
ERRα	0.17	0.57	-0.17	0.19	0.43	1.00‡	0.52	0.14
ERRβ	0.52	-0.14	-0.17	0.33	**0.81†**	0.52	1.00‡	-0.12
ERRα/ERα	**-0.76†**	**0.71***	**-0.85‡**	**-0.88‡**	-0.17	0.14	-0.12	1.00‡

Table 1. Spearman's Rank Correlation Coefficients (ρ_s) for breast carcinoma properties. Statistical significance at ($P \leq 0.10$) *, ($P \leq 0.05$) †, and ($P \leq 0.01$) ‡. Significant correlation coefficients involving receptor mRNAs, other than with themselves, are bolded.

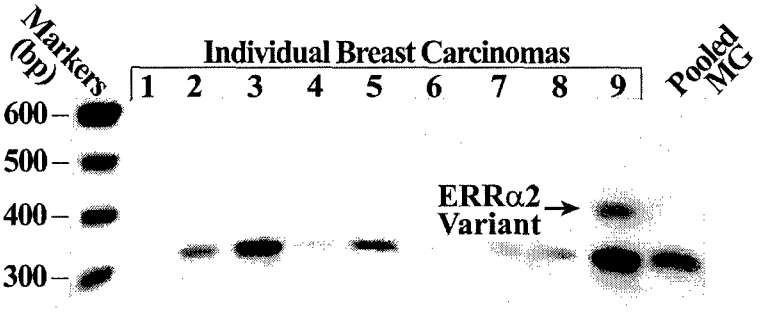


Fig. 5. ERRα2 PCR products after 1 round of amplification in nine random primary breast carcinomas and a pool of eight normal mammary glands.

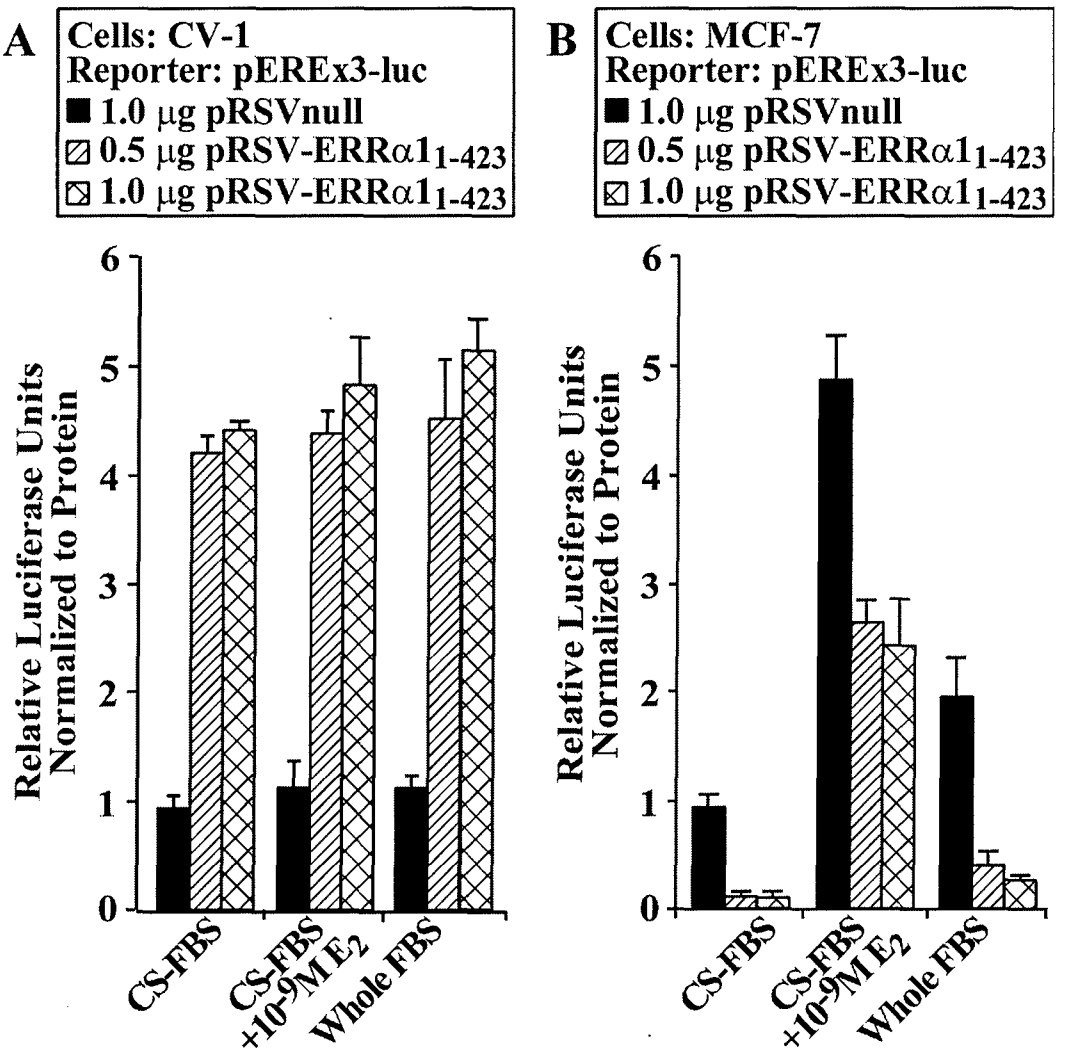
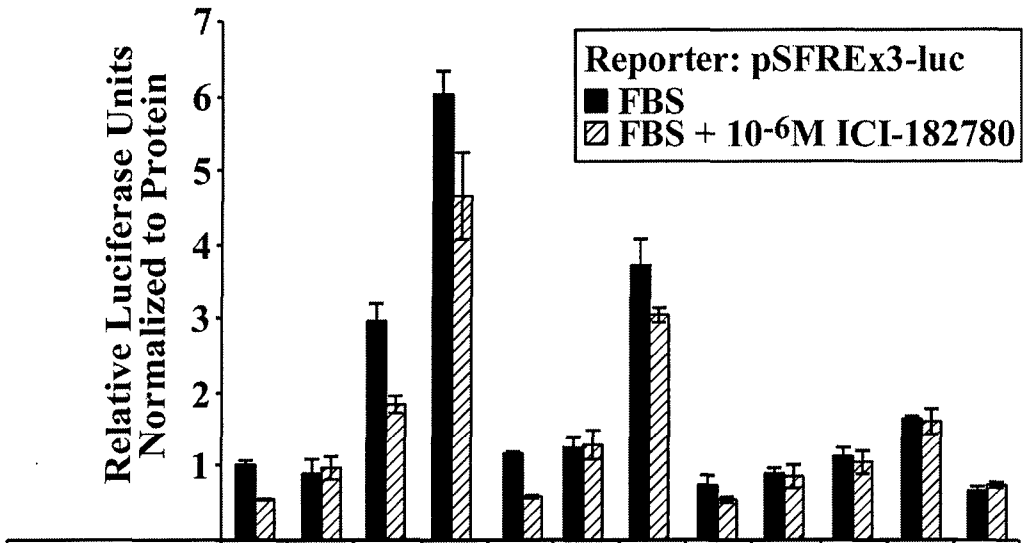


Fig. 6. ERRα activates transcription in CV-1 cells **(A)** and represses transcription in MCF-7 cells **(B)**. Cells were seeded in 60-mm dishes in estrogen-free media two days prior to co-transfection with a vector encoding full-length ERRα1 (pRSV-ERRα1) and the reporter plasmid pEREx3-luc. Following co-transfection, cells were treated as indicated for 72 h and harvested for luciferase and protein assays. CS (charcoal-stripped), FBS (fetal bovine serum). Luciferase units were plotted relative to reporter gene activity in pRSVnull-transfected cells cultured in CS-FBS.



	1	2	3	4	5	6	7	8	9	10	11	12
ERRα1$_{1-423}$	-	0.1	-	0.1	-	0.1	0.1	-	0.1	-	0.1	0.05
ERRα1$_{1-376}$	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.15
c-*erb*B2$_{Onc}$	-	-	0.1	0.1	-	-	0.1	-	-	0.1	0.1	0.1
H*ras*$_{DN}$	-	-	-	-	0.1	0.1	0.1	-	-	-	-	-

Fig. 7. Modulation of ERRα-transactivation potential through the MAPK pathway. MCF-7 cells were seeded in 22-mm dishes and co-transfected with pSFREx3-luc and the indicated amounts (in μg) of expression vectors encoding N-terminally "Express"-epitope tagged (Invitrogen), full-length ERRα1 (pExpERRα1$_{1-423}$), C-terminal truncated ERRα1 (pExpERRα1$_{1-376}$), c-*erb*B2$_{Onc}$ (pJRneu), and dominant-negative H*ras* (pJRH*ras*$_{DN}$). Immediately following co-transfection, cells were treated with or without 10^{-6} M ICI-182780 and harvested after 48 h for luciferase and protein assays. Luciferase units were plotted relative to reporter gene activity in cells cultured without ICI-182780 (column 1).

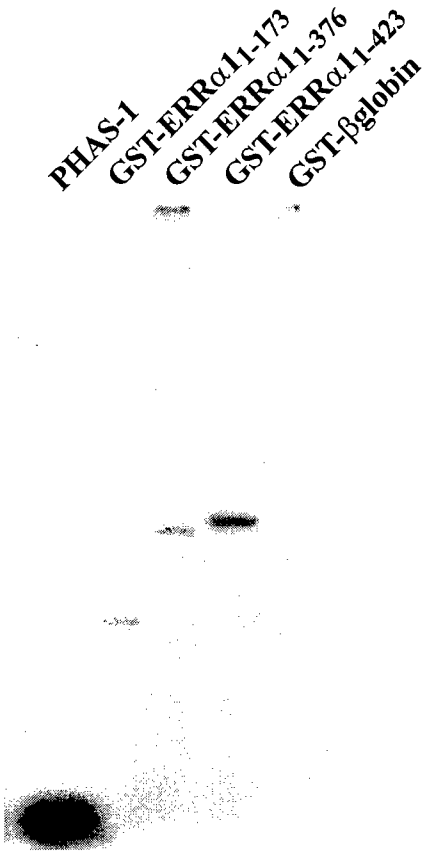


Fig. 8. Autoradiogram of SDS-PAGE of products of MAPK phosphorylation reactions. Equimolar amounts of the indicated purified proteins were incubated with activated MAPK and [γ-^{32}P]ATP.

Gene	Prognostic Status Reference(s)	E_2 Response Reference(s)	Potential ERRα-binding Sequence	ERRα Bound†
Aromatase	-	-	cctgagactctaCCAAGGTCAgaaatgctgcaa	yes
BRCA1	(83-86)	(87-92)	gtaattgctgtaCGAAGGTCAgaatcgctacct	yes
BRCA2	(85, 86)	(92)	agaacatcccttTTAAGGTCAgaacaaaggtat	yes
cathepsin D	(93, 94)	(95-101)	tggcatattgggTGAAGGTCAagggagtggctt	yes
cyclin D1	(102-106)	(107-109)	gcgaggaaagcgTGAAGGTGAtttcagttaatt	yes
ERα	(1, 2, 110, 111)	(17, 18, 20, 112-	atgtttggtatgAAAAGGTCAcattttatattc	yes
ERβ	(37, 47)	(24)	ggtgctcccactTAGAGGTCAcgcgcggcg	yes
EGF	(115-117)	-	caaataatgggcTGAAGGTGAactatctttact	ND
FGF-3/*int*-2	(103, 118, 119)	-	acaggaaagcccCAAAGGACAgcagcaagagga	ND
c-*erb*B2/HER-2/*neu*	(110, 120-124)	(125-128)	aaaggaactttcCCAAGGTCAcagagctgagct	yes
IGF1	(129, 130)	(131)	aaaagaaaaaatTCAAGGTCCaggttatttcca	no
IGF2 site 1 (IGF2-1)	(132, 133)	(134, 135)	tgaggagcatttGAAAGGTGCctgtctgcaaac	no
IGF2 site 2 (IGF2-2)			ctgtcggcaggaACAAGGTCAccccttggcgtt	yes
IGF2 site 3 (IGF2-3)			ggtggacgctgcTGAAGGTGAgcgagaccccgg	yes
IGF1R	(130, 136-139)	(140)	tcgcctcggctgTGACCTTCAgcgagccggagc	yes
MDM2	(141-143)	(144)	gggagttcagggTAAAGGTCAcggggccgggggc	yes
PgR site 1 (PgR-1)	(2, 110, 111, 145, 146)	(147, 148)	aaaattgttttgTCTAGGTCAtttgcattttca	yes
PgR site 2 (PgR-2)			tccttgctaaacCCAAGGTCAtaaatcttttct	ND
PgR site 3 (PgR-31)			tcctaaggactgTCAAGGTCAtcaaatacaagg	ND
Prl site 1 (Prl-1)	(149, 150)	(151)	tgtccattttctTCTAGGTCAaccccaatggta	yes
Prl site 2 (Prl-2)			caaatttgaaacTAAAGGTCAcaggctgcttta	yes
Prl site 3 (Prl-3)			cctcagagtggcTCAGGGTCAgagaaggtagag	yes
Prl site 4 (Prl-4)			tgagagaaatgaTGAAGGTGAgatctgagacta	no
pS2 site 1 (pS2-1)	(152-154)	(155-161)	tcccttccccctGCAAGGTCAcggtggccaccc	yes‡
pS2 site 2 (pS2-2)			gtaggacctggaTTAAGGTCAggttggaggaga	ND
RARα	(91)	(162-164)	gaagtgacttggTCAAGGTCAcacagctctcag	ND
TERT	(165-174)	(175)	ccagctccttcaGGCAGGACAcctgcgggggaa	yes

Table 2. Potential ERRα-binding sites located in the transcriptional regulatory regions of genes implicated in breast cancer. †ERRα-binding sites were confirmed using EMSAs as shown in Fig. 9. ‡ Data not shown. ND (not determined), BRCA (breast and ovarian cancer susceptibility gene), FGF (fibroblast growth factor), IGF (insulin-like growth factor), IGF1R (IGF1 receptor), MDM2 (human p53-binding protein murine double minute 2), Prl (prolactin), RARα (retinoic acid receptor α), TERT (telomerase catalytic subunit gene).

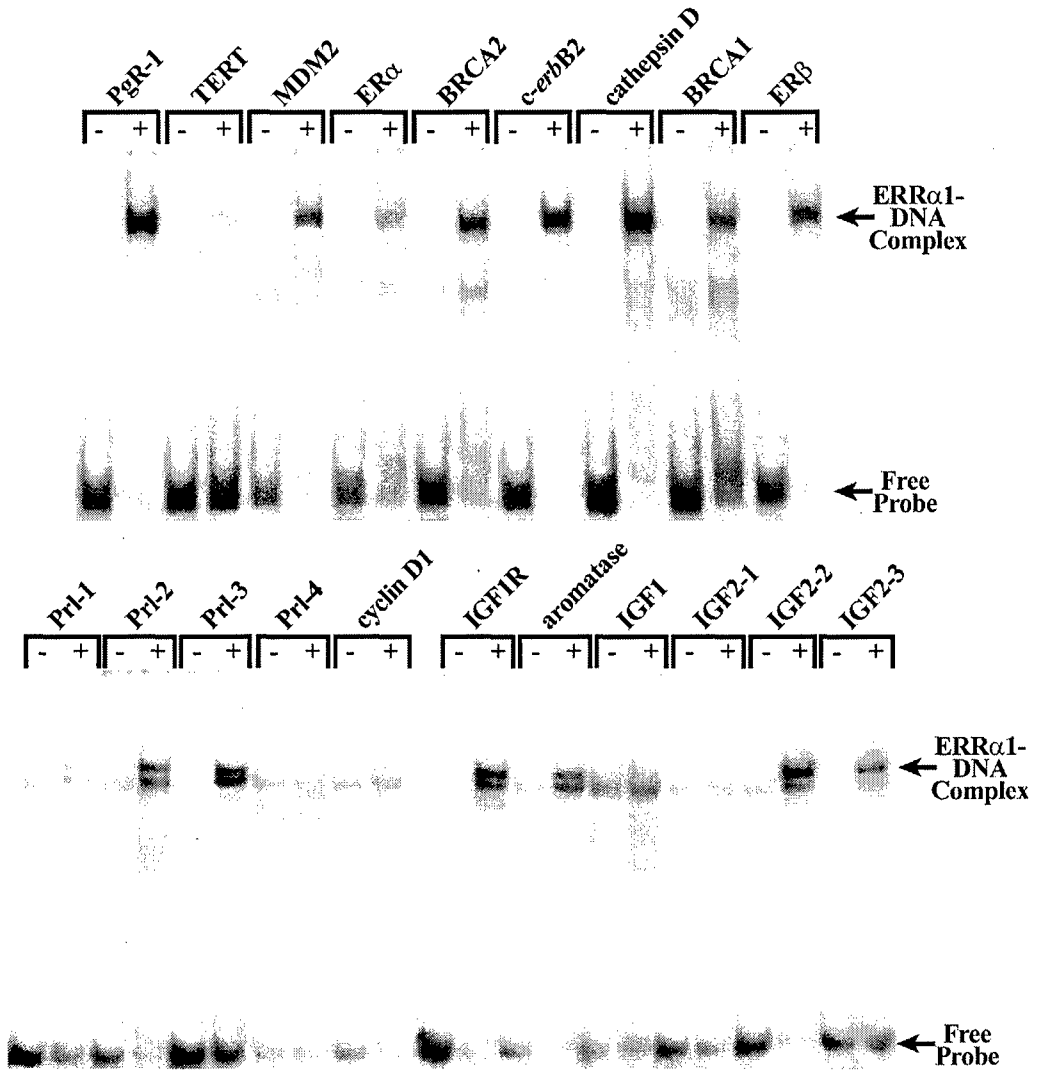


Fig. 9. EMSAs showing ERRα binding to SFREs located in the transcriptional regulatory regions of genes implicated in breast cancer listed in Table 2. Whole-cell extracts of COS cells transfected with pExpERRα1 (+) or mock-transfected (-) were used as protein sources.

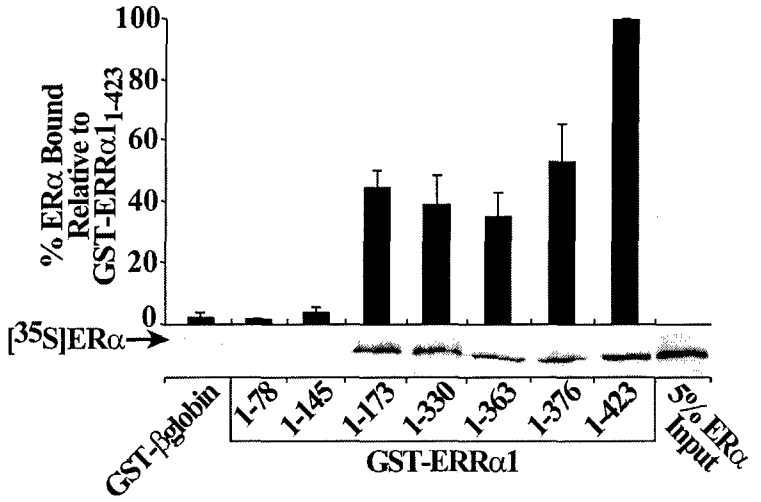


Fig. 10. GST pull-down assays of GST-ERRα1 truncation variants interacting with [^{35}S]-ERα. GST-ERRα1 variants were expressed and purified from *E. coli*. [^{35}S]-ERα was synthesized *in vitro* with rabbit reticulocyte lysates.

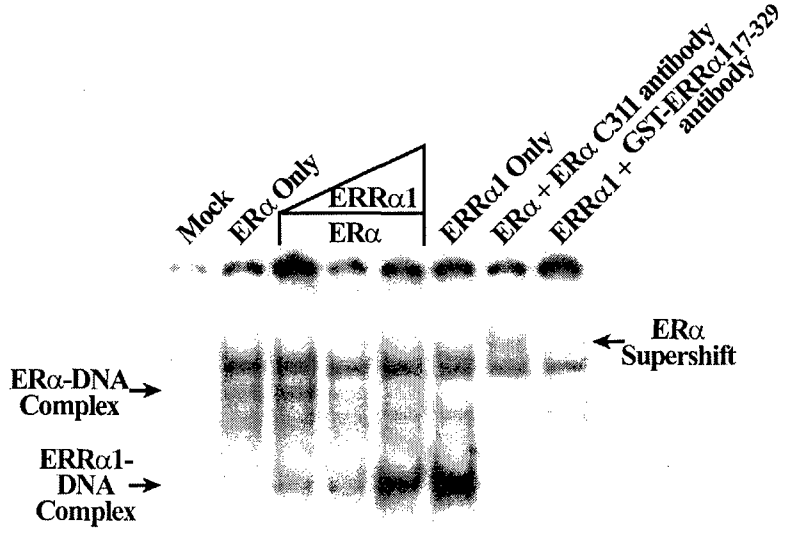


Fig. 11. Competition EMSAs between ERRα1 and ERα for binding a vitERE probe DNA. Lysates of COS cells transfected with ERRα1 and ERα expression plasmids were used as protein sources.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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