

UNCLASSIFIED

AD NUMBER
ADB259015
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Aug 99. Other requests shall be referred to US Army Medical Research and Materiel Command, Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 1 Apr 2003

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8098

TITLE: The Role of a First Intron Negative Regulatory Element in
the Repression of EGFR Expression in Hormone-Dependent
Breast Cancer

PRINCIPAL INVESTIGATOR: Melissa A. Wilson

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: August 1999

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government
agencies only (proprietary information, Aug 99). 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.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

THIS DOCUMENT IS UNCLASSIFIED 4

20001019 106

NOTICE

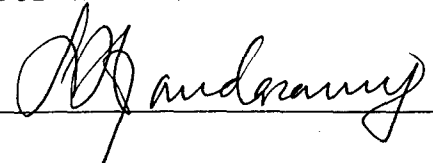
USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

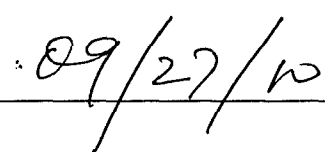
LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8098
Organization: Georgetown University

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.





REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4 and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1999		3. REPORT TYPE AND DATES COVERED Annual Summary (1 Aug 98 - 31 Jul 99)	
4. TITLE AND SUBTITLE The Role of a First Intron Negative Regulatory Element in the Repression of EGFR Expression in Hormone-Dependent Breast Cancer				5. FUNDING NUMBERS DAMD17-98-1-8098	
6. AUTHOR(S) Melissa A. Wilson					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057 E-MAIL: Wilsome@gunet.georgetown.edu				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 authorized to U.S. Government agencies only proprietary information, Aug 99). 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 CO
13. ABSTRACT (<i>Maximum 200 Words</i>) Breast cancer progression from a hormone-dependent, anti-estrogen sensitive to a hormone-independent, anti-estrogen insensitive phenotype involves the loss of estrogen receptor (ER) expression and the up-regulation of a number of growth factor receptors and/or their ligands, including the epidermal growth factor receptor (EGFR). EGFR has been demonstrated to be overexpressed in breast cancer and is inversely correlated with ER status in a majority of breast tumors. EGFR overexpression, independent of ER status, is associated with a more aggressive phenotype and predicts for poor response to endocrine therapy, suggesting that up-regulation of EGFR is involved in the progression to a more aggressive, hormone-independent phenotype. Experiments were designed to characterize the mechanism by which negative regulatory elements present within the EGFR gene first intron specifically repressed EGFR gene expression in estrogen-dependent breast cancer cells. Results have indicated that a 305bp fragment repressed EGFR gene expression in ER+ breast cancer cells. Furthermore, a 96bp fragment demonstrated differential factor binding in ER+ vs. ER- breast cancer cell lines. Results suggest that known as well as novel factors are involved in the interaction between nuclear factors and the 96bp intron negative regulatory element. As a result, we hypothesize that cis-elements within the EGFR gene first intron are involved in the repression of EGFR gene expression in hormone-dependent breast cancer.					
14. SUBJECT TERMS Breast Cancer, Epidermal Growth Factor Receptor, Gene Regulation, Hormone-Dependence, Transcriptional Repression, Intron Elements				15. NUMBER OF PAGES 20	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABST Limited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

MAW For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

MAW In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

MAW In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Melissa Ann Wilson 9/29/99
PI - Signature Date

**THE ROLE OF A FIRST INTRON NEGATIVE REGULATORY ELEMENT IN THE REPRESSION OF
EGFR EXPRESSION IN HORMONE-DEPENDENT BREAST CANCER**

TABLE OF CONTENTS

	<u>Page</u>
1) Front Cover	
2) SF298 Report Documentation Page	2
3) Foreword	3
4) Table of Contents	4
5) Introduction	5
6) Body	5
7) Appendices	18
Key research accomplishments	18
Reportable outcomes	18
Manuscripts and abstracts	19

THE ROLE OF A FIRST INTRON NEGATIVE REGULATORY ELEMENT IN THE REPRESSION OF EGFR EXPRESSION IN HORMONE-DEPENDENT BREAST CANCER

INTRODUCTION

The progression of breast cancer from an estrogen receptor positive (ER+), hormone-dependent to an estrogen receptor negative (ER-), hormone-independent phenotype involves the loss of ER expression and the up-regulation of a number of growth factor receptors and/or their ligands. The EGFR is one particular that has been demonstrated to be overexpressed in breast cancer. There is a noted inverse correlation between the overexpression of ER and EGFR in a majority of breast tumors, being either ER+/EGFR- or ER-/EGFR+. However, a significant number of tumors have been observed to co-express both receptors, being ER+/EGFR+, while the absence of both receptors (ER-/EGFR-) is a rare occurrence. Individual cells within the population of ER+/EGFR+ tumors overexpress one of the receptors, ER or EGFR, but not both, emphasizing the inverse overexpression of ER vs. EGFR in breast cancer. The expression of EGFR, independent of ER status, is indicative of a more aggressive phenotype and predicts for poor response to endocrine therapy and poor survival rate, suggesting that up-regulation of EGFR is involved in the progression of breast cancer to a more aggressive, hormone-independent phenotype. Such overexpression of EGFR in breast cancer cells has been shown to be primarily due to transcriptional control. Both positive and negative regulatory factors appear to play an important role in the regulation of EGFR gene expression in other cell lines and are suspected to do so in breast cancer cell lines. The main focus of our laboratory is the investigation of the differential regulation of the EGFR gene in ER+ vs. ER- breast cancer cell lines. Experiments performed both *in vivo* and *in vitro* have indicated that regions of the EGFR gene first intron are involved in the enhancement and repression of EGFR gene expression. The overall goal of this project is to identify negative regulatory elements within the EGFR gene first intron and characterize the mechanism by which EGFR gene expression is repressed in estrogen-dependent breast cancer.

BODY

Research Accomplishments

The main focus of our laboratory is the investigation of the differential regulation of the EGFR gene in ER+ vs. ER- breast cancer cells. Experiments performed in the lab have indicated regions within the EGFR gene first intron are involved in both the enhancement and repression of EGFR gene expression, and in particular, a 730 bp fragment located in the 5' region of the EGFR gene first intron (Fig. 1A) may be involved in transcriptional repression. To investigate the role of intron 1 regions in the repression of the EGFR gene in ER+ vs. ER- breast cancer cells and to identify the minimal *cis*-element(s) demonstrating repressive activity, transient transfections were performed utilizing CAT reporter constructs with both homologous and heterologous promoters. The homologous promoter constructs, designated pJFEC, address the ability of intron fragments to regulate transcriptional activity of the EGFR promoter, while the heterologous promoter constructs, designated promCAT, investigate their ability to control transcriptional activity of the SV40 promoter.

Initially, results from seven transient transfections of SV40 promoter-EGFR intron constructs in MCF-7 cells, which are ER+/low EGFR expressors, demonstrated the a 730 bp region of the EGFR gene first intron repressed transcription 51% ($p < 0.001$), and that a 305 bp portion of this region retained repressive activity, repressing transcription 42% ($p < 0.001$) (Fig. 1B). Results from twelve transfections of EGFR promoter-intron constructs demonstrated that this 305 bp fragment exhibited 67% ($p < 0.001$) repression of transcriptional activity in the forward orientation in MCF-7 cells, but no significant effect in the reverse orientation (Fig. 1B). In BT549 cells, which are ER-/high EGFR expressors, results from seven transient transfections of SV40 promoter-EGFR intron constructs, as well as fourteen

transfections of EGFR promoter-intron constructs, demonstrated that neither the 730 bp region nor the 305 bp fragment exhibited repressive activity (Fig. 1C). The results obtained were in accord with previously documented reports that documented the variability of silencer activity in regards to orientation dependence. We believe the variability of the CAT data obtained in BT549 breast cancer cells was not a reflection of technique inconsistency, but rather the regulatory mechanisms involved. It has been documented by a number of labs that when intron repressor elements are taken out of their native context, or cell-type, in which it is not participating in repression, the elements demonstrate variable activity. These results indicated that I had identified a 305 bp fragment within the EGFR gene first intron that exhibited repressive activity with both homologous and heterologous promoter constructs in transient transfection assays in ER+/low EGFR MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells.

We performed *in vitro* DNase I footprinting analysis on the 305 bp intron negative regulatory element to investigate factors interacting with this element which may be involved in mediating its transcriptional repressive activity, and to identify sequences involved in the DNA-protein interactions. DNase I digestion of the 305 bp probe incubated with 40 ug of MCF-7 nuclear extract produced an altered digestion pattern, as compared to the 305 bp probe alone, without the presence of nuclear extract (Fig. 2). Ten regions protected from DNase I digestion were identified; furthermore, a number of sites at the boundaries of these footprints demonstrated increased sensitivity to DNase I digestion. These protected regions and hypersensitive sites are indicative of factor binding.

In order to investigate proteins which interacted with the 305 bp negative regulatory element directly, Southwestern analysis (or DNA-protein blotting) was performed. Nuclear extracts from a panel of ER+ vs. ER- breast cancer cell lines were separated by SDS-PAGE, transferred to nitrocellulose membranes, and hybridized with probes corresponding to intron negative regulatory elements. The 305 bp intron negative regulatory element interacted with two proteins around 130 KDa and 35 KDa in size (Fig. 3). The expression pattern and amounts of the two proteins relative to each other suggest that the ratios of particular factors may be involved in the transcriptional regulation of EGFR gene expression mediated through intron elements, as opposed to the complete presence and/or absence of a specific nuclear factor. It should be noted that although the footprint indicated numerous factors interacting with the 305 bp fragment, Southwestern analysis only identified a subset of these factors. The reason for this could be due to the inherent limitations of Southwestern analysis. Some proteins may need to be in their native conformation or in a complex in order to interact with the DNA probe; if this is the case, these proteins will not be detected by this method.

Based on results from *in vitro* DNase I footprinting, numerous factors were determined to interact throughout the entire 305 bp negative regulatory element. To further identify the minimal intron element within the 305 bp region responsible for transcriptional repression of EGFR gene expression and differential factor binding in ER+ vs. ER- breast cancer cell lines, the 305 bp negative regulatory element was divided into smaller, overlapping fragments using convenient restriction sites and oligonucleotides. To evaluate the transcriptional activity of these smaller intron elements, fragments were subcloned into the EGFR promoter construct, pJFEC, and transient transfections were performed. Electrophoretic mobility shift assays, or gel shifts, were performed to investigate factor binding within the 305 bp intron negative regulatory element, and to define the minimal protein-binding element. The use of overlapping DNA probes allowed for the correlation of function and factor-binding.

Digestion of the 305 bp intron negative regulatory element with the enzymes *Pml* I and *Alu* I resulted in three fragments, 56 bp, 96 bp, and 150 bp in size (Fig. 4). Results from six transient transfections with the 56 bp fragment and five transient transfections with the 150 bp fragment in MCF-7 cells demonstrated that these fragments repressed EGFR transcriptional activity 51% ($p < 0.001$) and 60% ($p < 0.001$), respectively (Fig. 4). Furthermore, results from four transient transfections demonstrated that the 96 bp fragment repressed EGFR transcriptional activity to a much greater extent, 93% ($p < 0.001$) repression of EGFR promoter activity (Fig. 4). In BT549 cells, results from transient transfections demonstrated that the 150 bp and 56 bp fragments did not have a significant effect on transcriptional activity of the EGFR promoter. Moreover, the 96 bp fragment appeared to enhance transcriptional activity 2.7-fold

($p=0.05$) (Fig. 4). Like the 305 bp intron negative regulatory element, these sub-fragments demonstrated similar activity, repressing EGFR transcriptional activity in ER+/low EGFR expressing MCF-7 cells, but not in the ER-/high EGFR expressing BT549 cells. Moreover, results suggested that the 96 bp intron fragment contained the major negative regulatory element responsible for exhibiting differential repressive activity.

Gel shift assays were performed with overlapping fragments representing the 305 bp intron negative regulatory element to investigate factor binding in nuclear extracts from a panel of ER+ vs. ER- breast cancer cell lines. Similar shift patterns were obtained with all probes corresponding to the 305 bp intron element, with the exception of the 96 bp fragment. In contrast to the other probes, the 96 bp fragment demonstrated a differential shift pattern in ER+ vs. ER- nuclear extracts (Fig. 5). Incubation of the 96 bp probe with MCF-7 nuclear extract resulted in one distinct DNA-protein complex, as compared to probe alone. BT474 and HeLa (a human cervical carcinoma cell line) nuclear extracts also exhibited a similar shifted DNA-protein complex. Complex formation was greatly diminished in BT549 nuclear extracts, as compared to MCF-7 nuclear extracts. While factors interacting with the 96 bp intron element did not appear to be breast-specific since DNA-complexes were observed in HeLa nuclear extracts, complex formation appeared to be estrogen-regulated. Treatment with the anti-estrogen ICI 182,780 or estrogen-depletion through a process known as "stripping" resulted in decreased complex formation with MCF-7 and BT474 nuclear extracts. These results demonstrated that the 96 bp intron fragment demonstrated differential binding activity in ER+ vs. ER- breast cancer cells which directly correlated with functionality data. These results further substantiate the localization of the major negative regulatory element within the 96 bp intron fragment.

To further delineate the minimal negative regulatory element responsible for exhibiting transcriptional repressive activity, the 96 bp region was subdivided into smaller fragments representing the entire 96 bp negative regulatory element. The transcriptional activity of these fragments was determined utilizing transient transfections and CAT assays. Gel shift assays were performed in order to examine factor binding to these intron sites. Digestion of the 96 bp negative regulatory element with *Hae* II resulted in two smaller fragments, a 37 bp fragment and a 59 bp fragment (Fig. 6). Results from eight transient transfection assays with EGFR promoter constructs containing the 59 bp intron element demonstrated that the 59 bp fragment repressed EGFR transcriptional activity 50% ($p<0.001$) in MCF-7 cells and 62% ($p<0.001$) in BT549 cells (Fig. 6). Results from six transient transfections with EGFR promoter constructs containing the 37 bp fragment demonstrated a 3-fold ($p=0.04$) increase in EGFR transcriptional activity in MCF-7 cells and no significant effect in BT549 cells (Fig. 6). These results suggested that the 59 bp intron region may contain the negative regulatory element and the 37 bp intron region may contain the regulatory element responsible for mediating cell type-specificity with respect to repressive activity. Experiments are currently being performed utilizing oligonucleotides representing the 96 bp intron element in order to confirm these results, as well as to narrow down the minimal negative regulatory element.

In order to further identify the minimal negative element containing both transcriptional repressive activity and differential factor binding, the 59 bp and 37 bp fragments were used as probes in gel shift assays. Incubation of the 59 bp fragment with 5 ug MCF-7 nuclear extract resulted in a single-shifted complex, relative to probe alone, which was reduced in BT549 nuclear extracts (Fig. 7). Incubation of the 37 bp fragment with 5 ug MCF-7 nuclear extract also resulted in a single-shifted complex, relative to probe alone, that was reduced in BT549 nuclear extracts (Fig. 7). These sub-fragments retained the differential binding pattern originally displayed by the 96 bp intron negative regulatory element. Gel shift assays utilizing the oligonucleotides representing the 96 bp intron element are currently being performed to investigate factor binding to the intron negative regulatory element.

Sequence comparison of regions identified by *in vitro* DNase I footprinting identified a number of putative binding sites for transcription factors which had been demonstrated to be involved in the regulation of EGFR transcriptional activity in other cell lines. In order to investigate the role of specific factors in the regulation of EGFR gene expression in breast cancer cells and to identify their presence within the DNA-protein complex formed with

specific intron fragments, competition gel shift assays and antibody supershifts were performed. Two putative ETR, or EGFR gene transcriptional repressor, sites were identified within the 96 bp intron negative regulatory element. Initially, the ETR was identified in the distal promoter of the EGFR gene and was shown to demonstrate repressive transcriptional activity in various non-breast human cell lines (Hou *et al.*, 1994). Incubation of the 96 bp probe with 5 ug MCF-7 nuclear extract produced a distinct DNA-protein complex (Fig. 8). Addition of increasing amounts of cold competitor ETR oligo resulted in a faster migrating DNA-protein complex, as compared to the initial DNA-protein complex formed with MCF-7 nuclear extract (Fig. 8). These results suggested that the ETR may be a component of the complex which interacts with the 96 bp negative regulatory element.

Results from experiments previously performed in our lab have suggested that the first intron of the EGFR gene is organized in a different chromatin configuration in ER+ vs. ER- breast cancer cell lines. Recently, attention has focused on histone acetylation as a mechanism for transcriptional regulation. Hyperacetylation of nucleosomes has been demonstrated to result in a looser, more open chromatin conformation, and increased transcriptional activity. To investigate the role of chromatin conformation in the negative regulation of EGFR gene expression in ER+ vs. ER- breast cancer cells, we utilized the histone deacetylase inhibitor, Trichostatin A (TSA), and examined the levels of endogenous EGFR mRNA levels in MCF-7 vs. BT549 breast cancer cells.

RNA was harvested from MCF-7 and BT549 breast cancer cells which were untreated or treated for 24 hours with 3uM TSA. RNA was hybridized to probes corresponding to EGFR and an internal control, 36B4. Unhybridized, single-stranded RNA was digested with RNase A, and the protected RNA fragments were run on 6% denaturing polyacrylamide gels containing urea. The visualized RNA is representative of expression levels; EGFR levels were normalized to the internal control RNA, 36B4. Effects of TSA on the levels of EGFR were compared to levels of EGFR in untreated cells. Treatment with 3uM TSA resulted in a 2.5-fold increase in EGFR message levels in MCF-7 breast cancer cells, while a 45% decrease in EGFR levels were observed in BT549 breast cancer cells (Fig. 9). Results from the TSA experiments suggested that the EGFR gene adopted a more open chromatin conformation in MCF-7 breast cancer cells which increased EGFR transcriptional activity, which is what we had anticipated. The decrease in EGFR levels in BT549 breast cancer cells, however, was a little surprising. We hypothesize that this decrease was an indirect effect; treatment with TSA, globally affects transcriptional activity of gene expression, and it is not confined to the EGFR gene. We suspect that TSA treatment resulted in the induction of gene expression of a repressor that now interacts with the EGFR gene and resulted in its decreased transcriptional activity.

Experiments are in progress to identify the minimal negative regulatory within the EGFR gene first intron which demonstrates repressive transcriptional activity and displays differential factor binding with nuclear extracts from ER+ vs. ER- breast cancer cell lines. Furthermore, the role of estrogen in the transcriptional repression exhibited by the intron negative regulatory element is being investigated. In conclusion, I have identified a 96 bp element within the EGFR gene first intron which represses EGFR transcriptional activity in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells. Furthermore, the 96 bp intron negative regulatory element demonstrated differential factor binding in ER+ vs. ER- nuclear extracts which appeared to be estrogen-dependent.

Training

This project is providing Ms. Wilson with training in the areas of gene regulation and breast cancer. I am in the process of writing my thesis dissertation, as well as a manuscript.

PROPRIETARY DATA

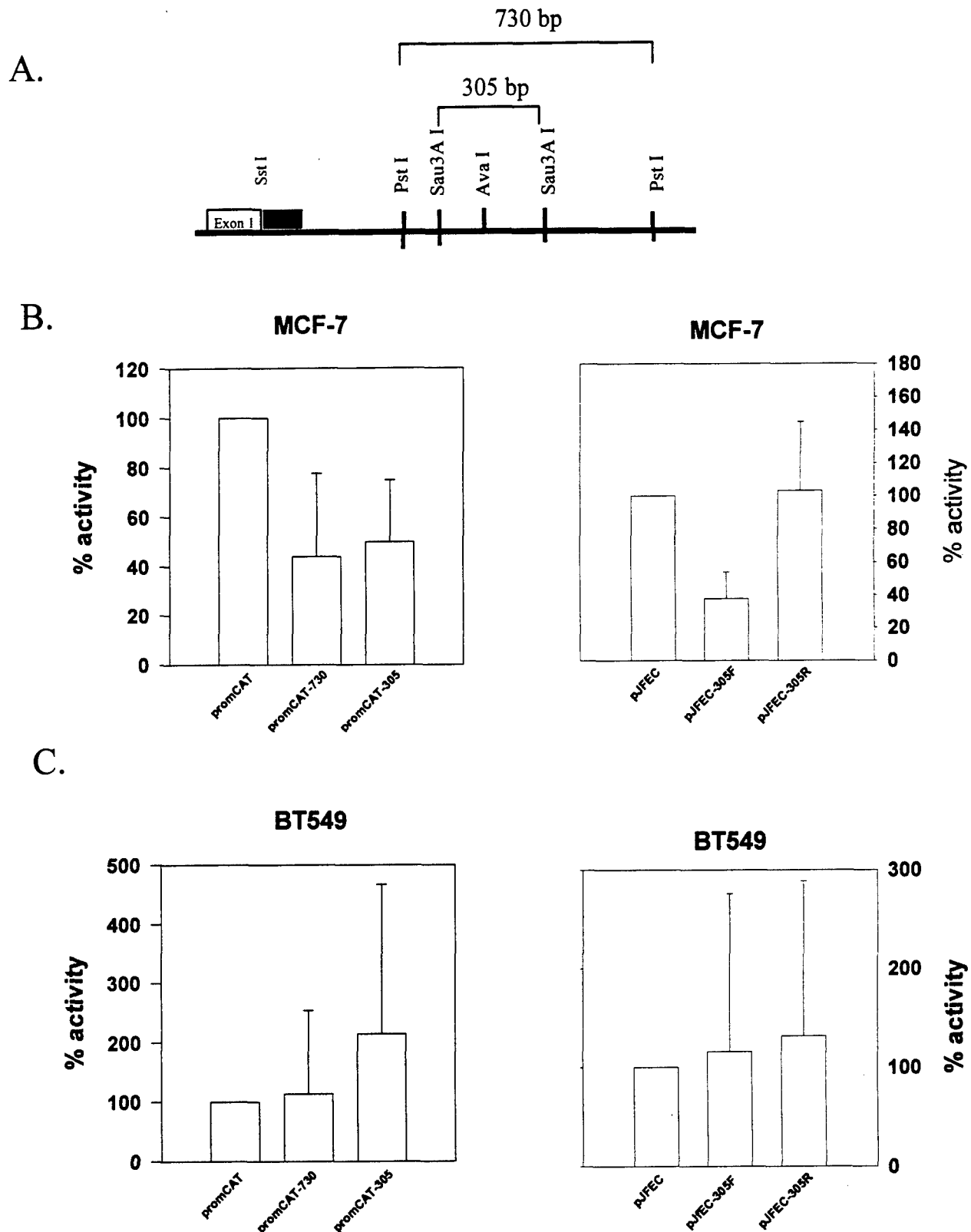


Figure 1. *A.* Map of the EGFR first exon and approximately 1 Kb of the first intron indicating intron fragments for subcloning into reporter constructs. *B.* CAT activity of promCAT and pJFEC constructs in MCF-7 cells. *C.* CAT activity of promCAT and pJFEC constructs in BT549 cells. F and R refer to the fragments in the forward and reverse orientation, respectively.

PROPRIETARY DATA

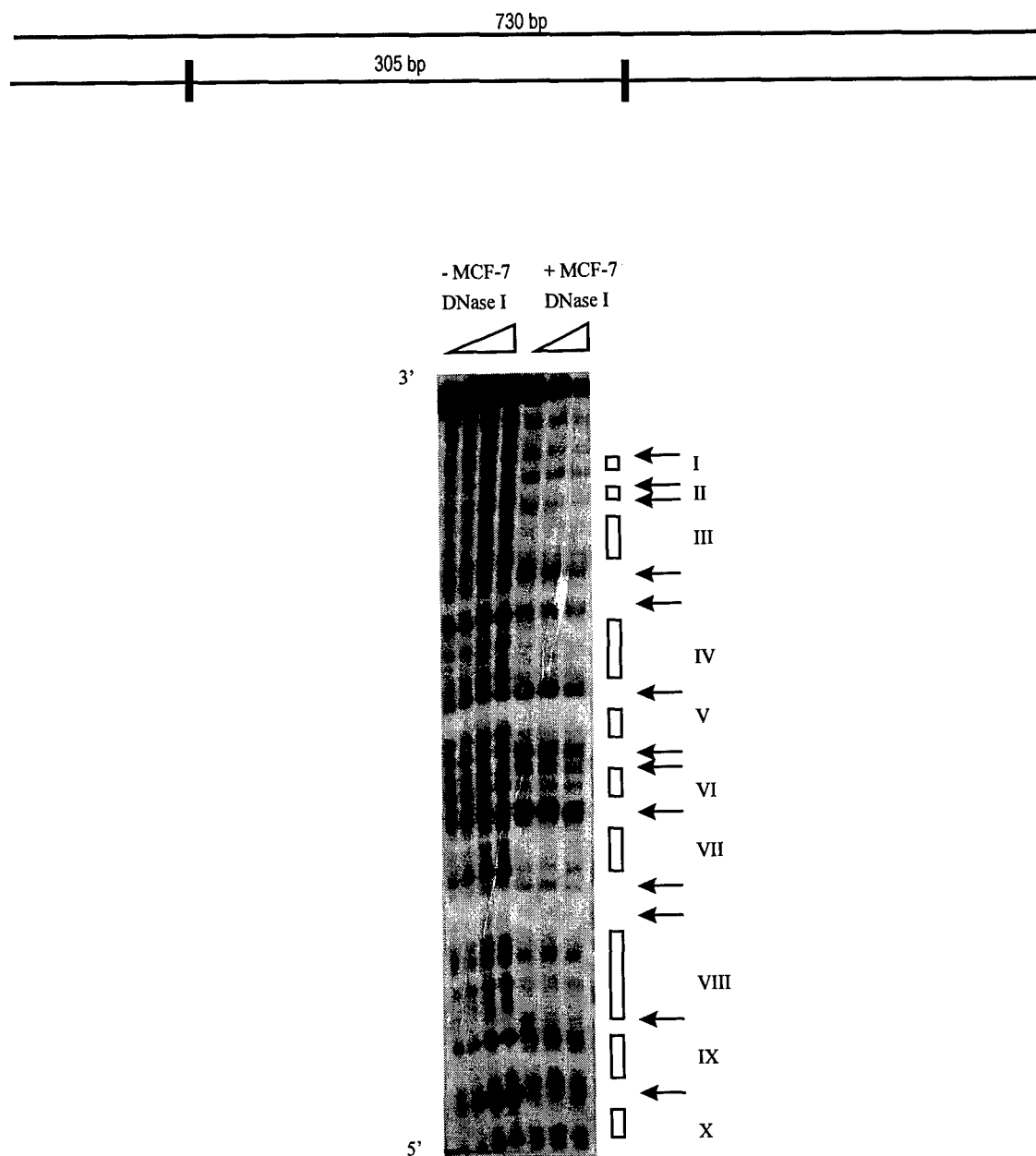


Figure 2. *In vitro* DNase I footprinting of the 305 bp intron negative regulatory element. Map of the 305 bp intron element depicting the footprint probe used to define specific regions of DNA-protein interaction and to delineate the protein-binding sequences. The 305 bp probe was labeled on one strand at the 5' end and incubated with increasing

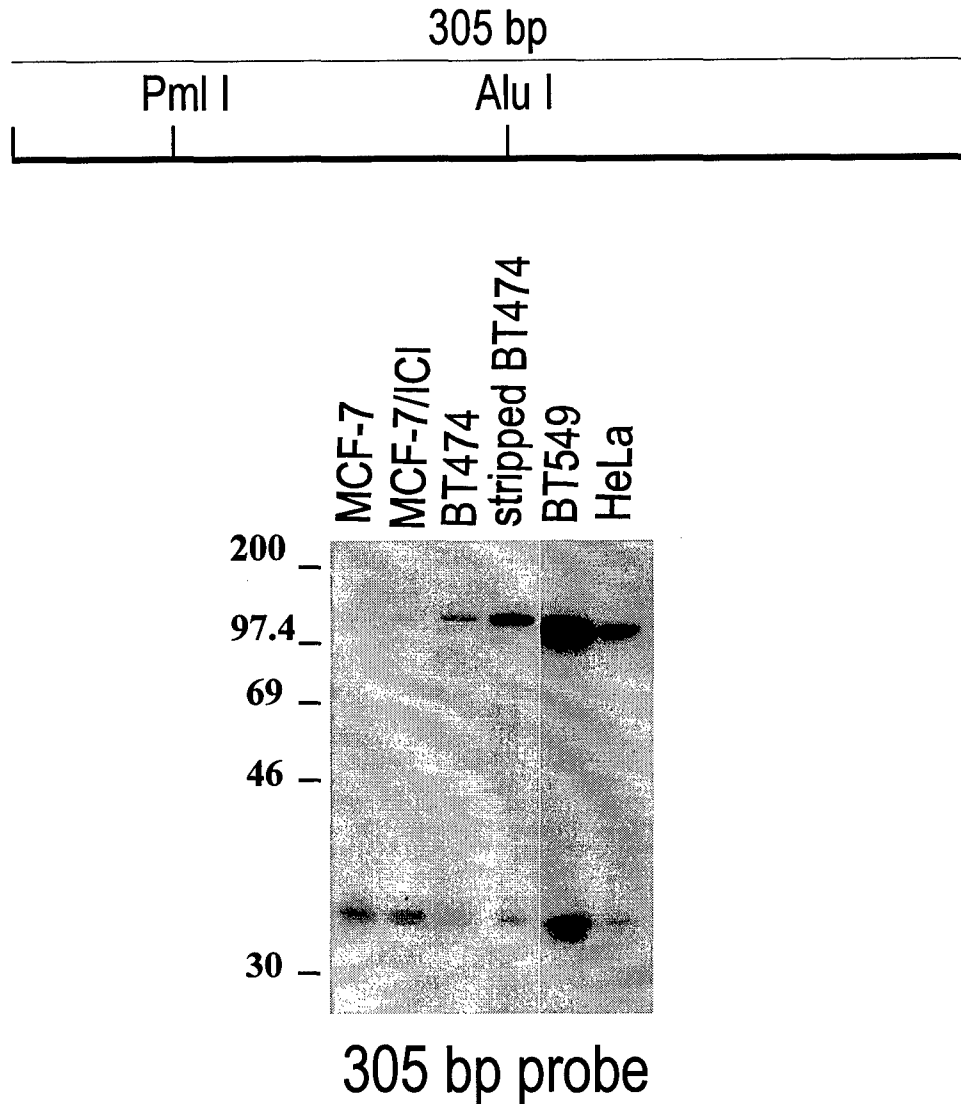


Figure 3. Southwestern analysis of a panel of ER+ vs. ER- breast cancer cells. Southwestern analysis (DNA-protein blotting) was performed to examine factors interacting with the 305 bp intron negative regulatory element that exhibited repressive activity. Nuclear extracts from a panel of breast cancer cell lines were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the ^{32}P -labeled 305 bp DNA fragment. BT474 are ER+ breast cancer cells which express higher levels of EGFR than MCF-7 cells. To investigate the effect of estrogen on DNA-protein interaction, nuclear extracts were made from MCF-7 cells treated with the anti-estrogen 182,780 and BT474 cells depleted of estrogen by a process known as "stripping." HeLa cells are human cervical carcinoma cells.

CAT Assay Results

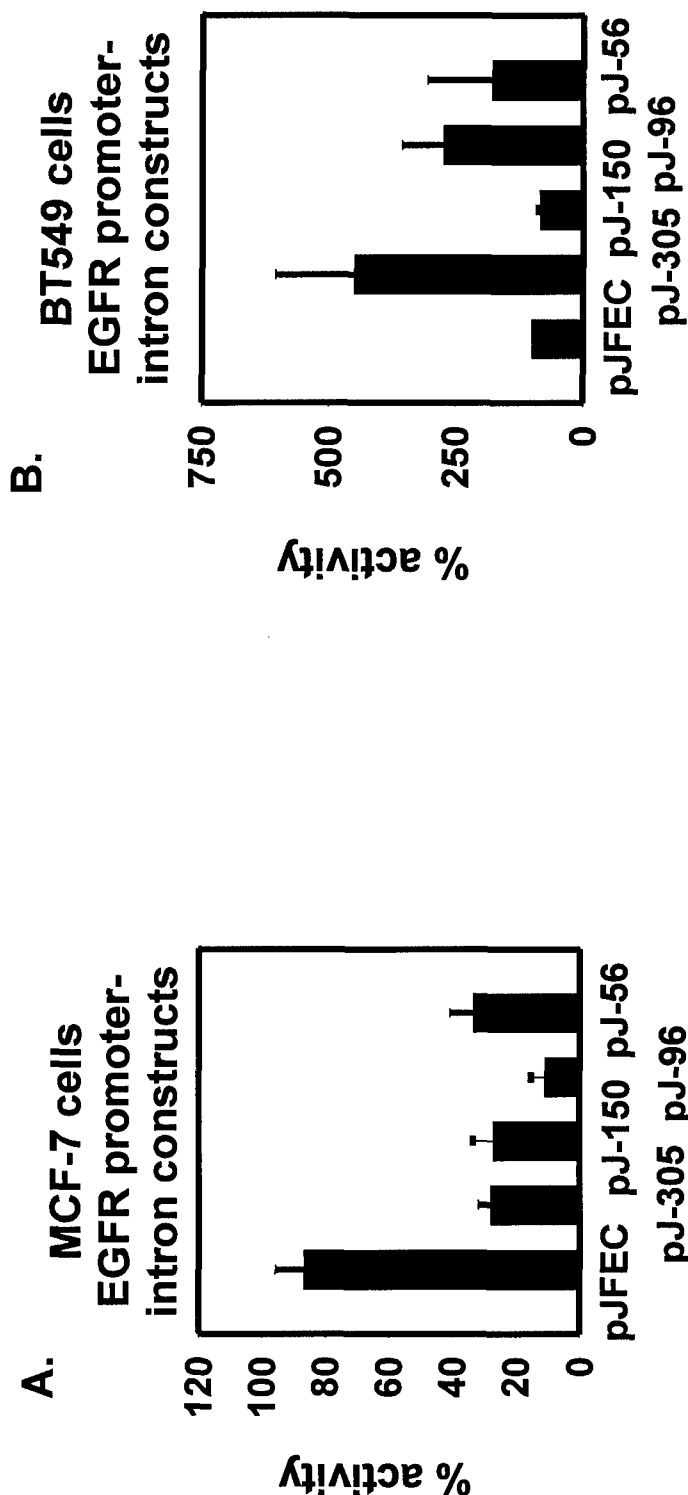
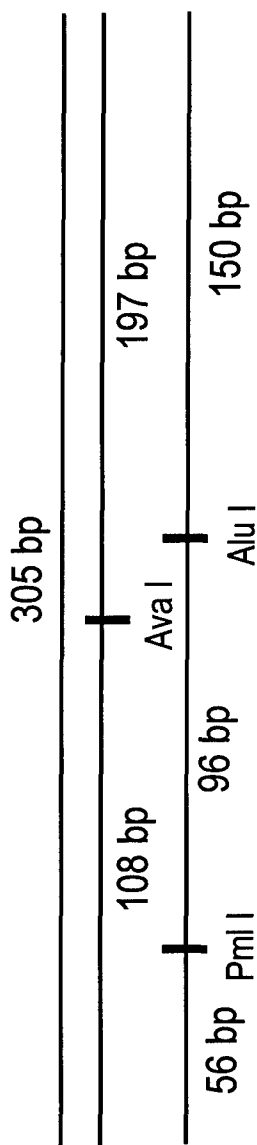


Figure 4. CAT assay results in MCF-7 and BT549 breast cancer cells with sub-fragments of the 305 bp intron negative regulatory element. The map depicts the fragments relative to one another.

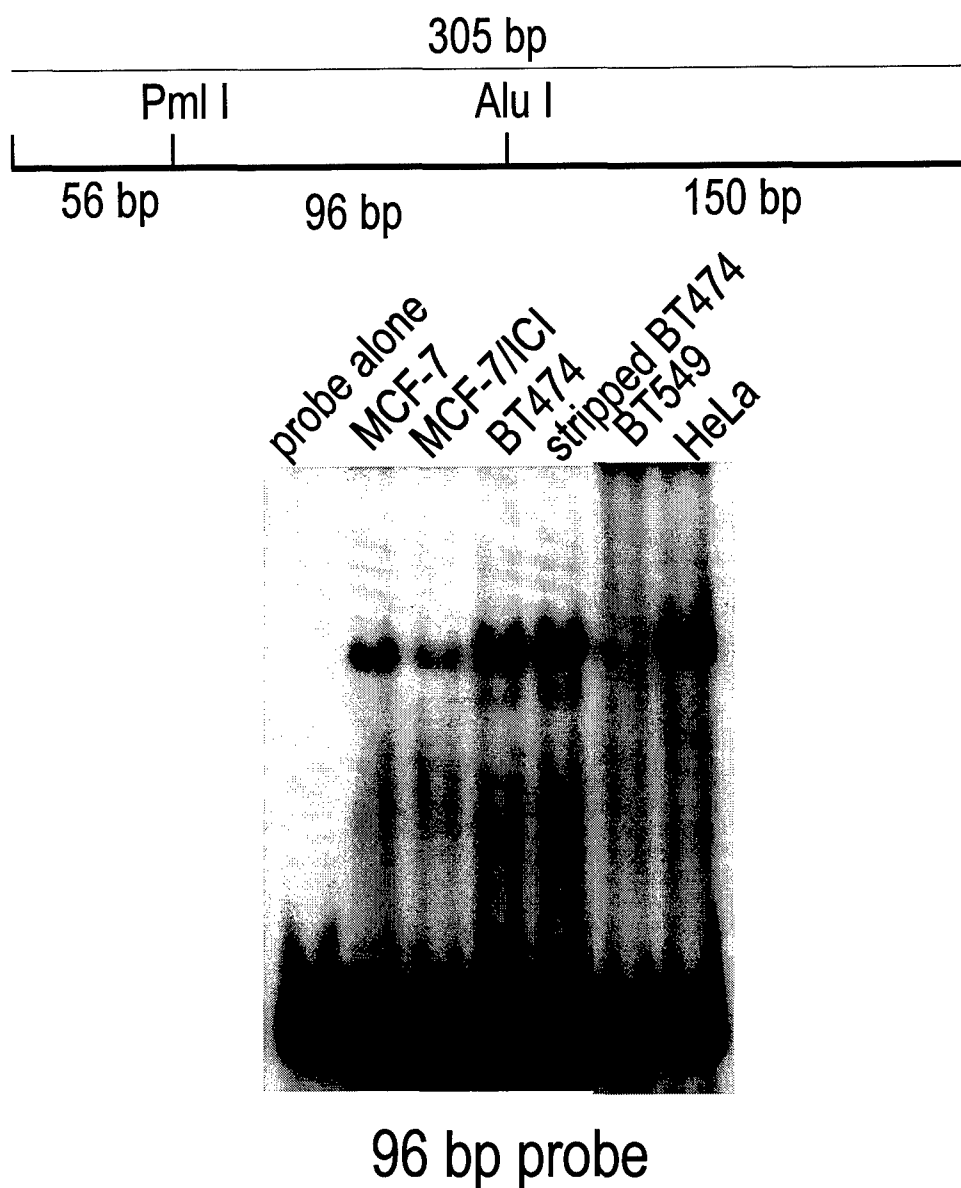


Figure 5. DNA-protein complex formation with the 96 bp intron fragment. The 96 bp probe was incubated with 5ug of nuclear extracts from a panel of breast cancer cell lines, as well as from HeLa cells. The 96 bp intron fragment demonstrated differential shift patterns in ER+ vs. ER- nuclear extracts. The DNA-protein complex was dramatically reduced in BT549 nuclear extracts. Furthermore, complex formation with the 96 bp element appeared to be estrogen-regulated; treatment with ICI or "stripping" reduced complex formation.

CAT Assay Results

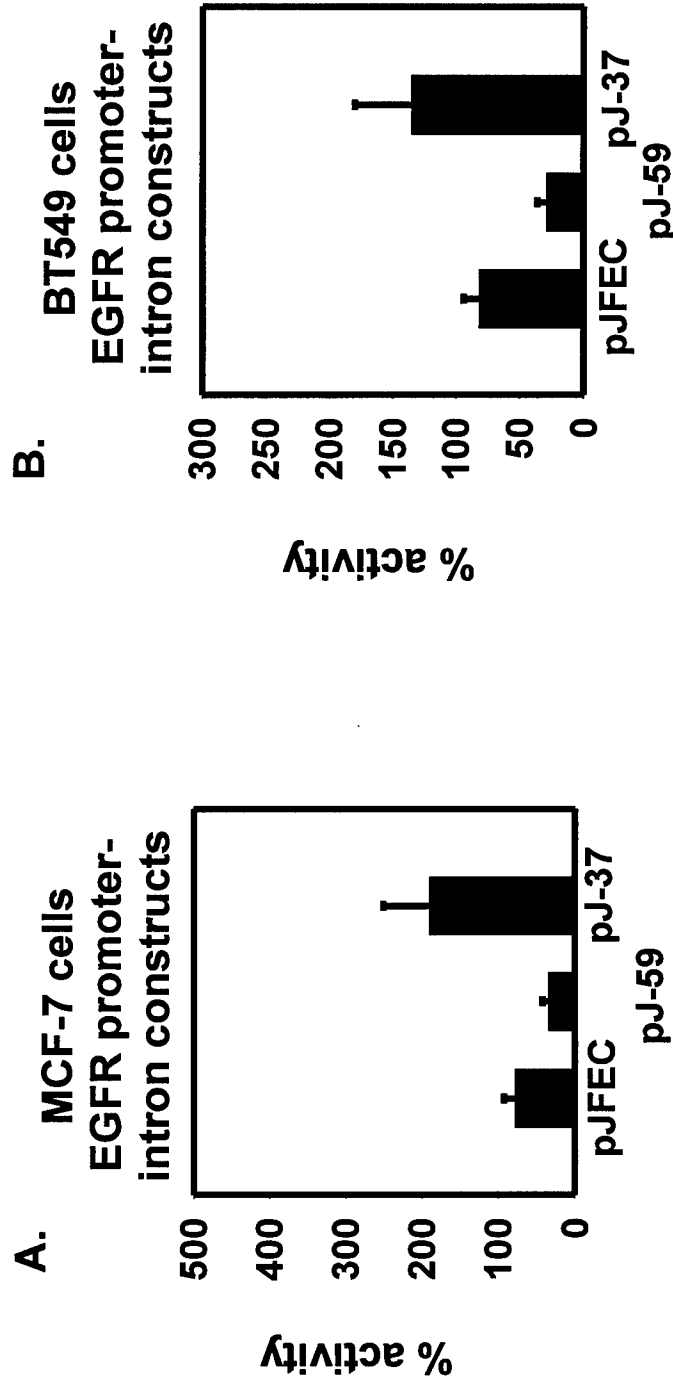
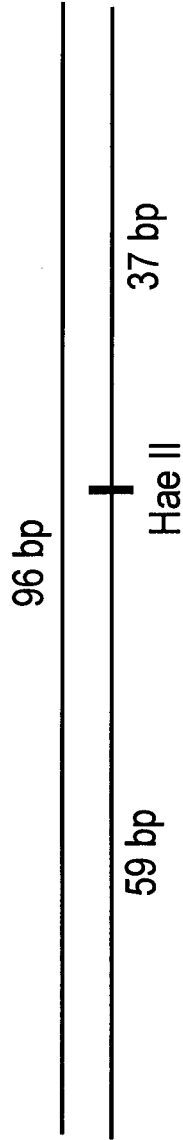
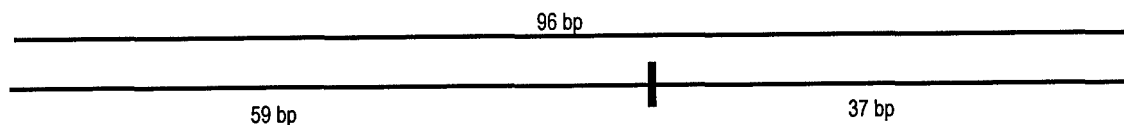


Figure 6. CAT assay results in MCF-7 and BT549 breast cancer cells with sub-fragments of the 96 bp intron negative regulatory element. The map depicts the fragments relative to one another.



proprietary data

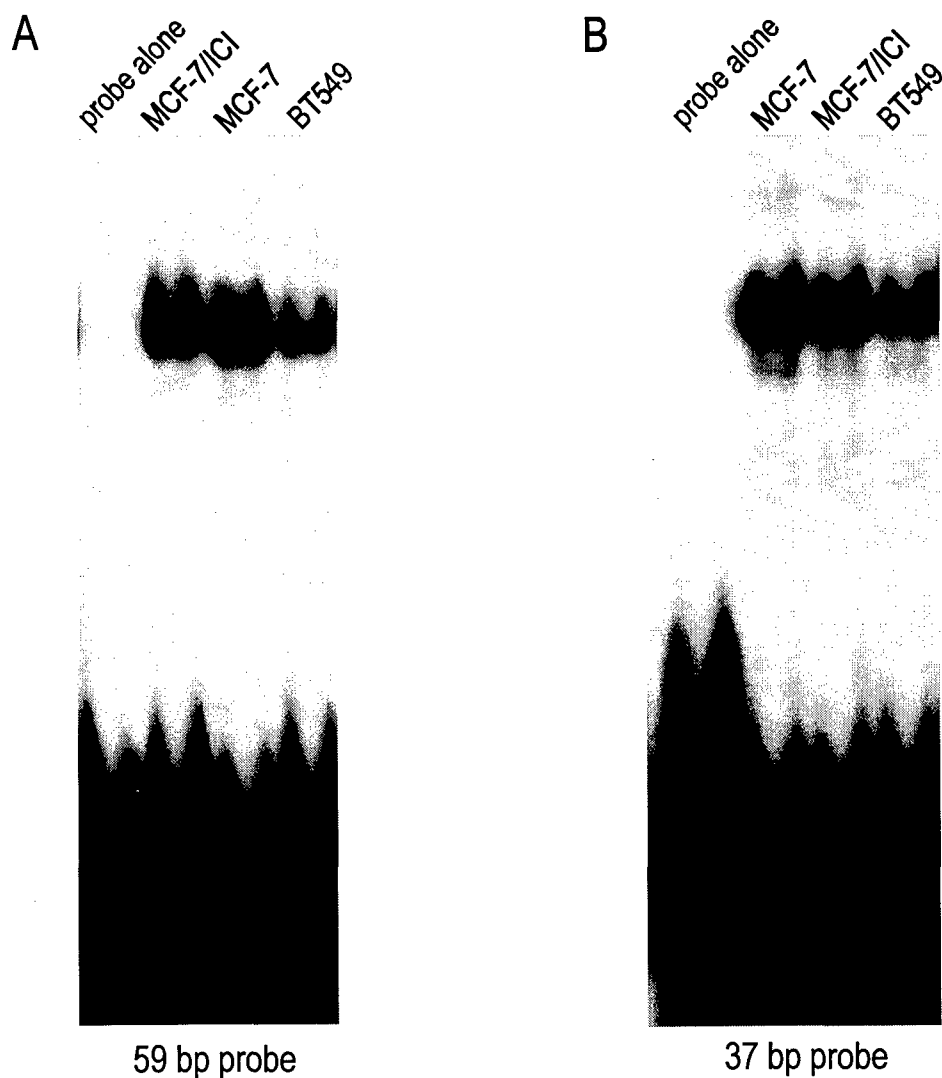
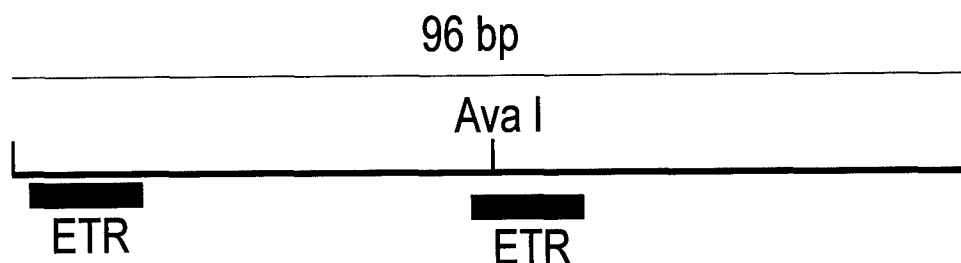


Figure 7. DNA-protein complex formation with the 59 bp and 37 bp intron fragments. 5ug of indicated nuclear extracts were incubated with A.) the 59 bp probe and B.) the 37 bp probe. The 59 bp and 37 bp intron elements formed DNA-protein complexes with MCF-7 nuclear extracts that were dramatically reduced in BT549 nuclear extracts.



probe alone 5 ug MCF-7
 - 10X 50X 100X ETR



Figure 8. Competition gel shift assay between the 96 bp probe and the EGFR gene transcriptional repressor (ETR). Incubation of the 96 bp probe with 5ug MCF-7 nuclear extract resulted in the formation of a distinct DNA-protein complex that was competed away with the addition of increasing concentrations of cold competitor ETR oligo. Addition of the competitor ETR DNA resulted in a faster migrating complex, suggesting the ETR was involved in the DNA-protein complex formed between the 96 bp intron negative regulatory element and MCF-7 nuclear extract.

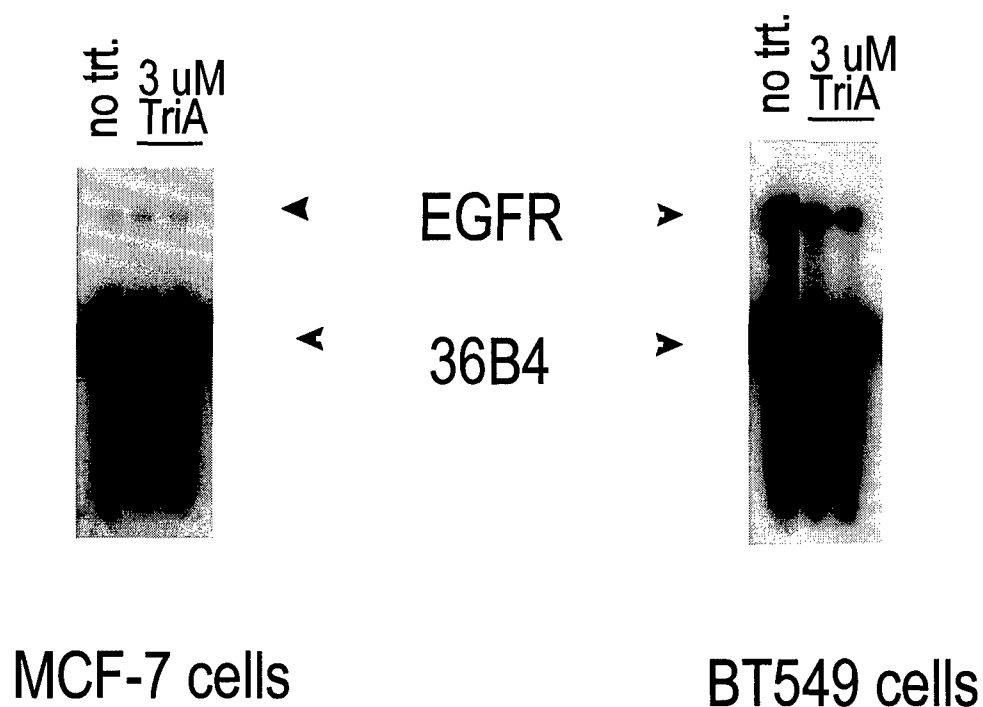


Figure 9.. Effect of Trichostatin A (TSA) on EGFR message levels in breast cancer cell lines. Representative RNase protection assay in MCF-7 and BT549 breast cancer cell lines untreated and treated with 3uM TSA.

APPENDICES

KEY RESEARCH ACCOMPLISHMENTS

- ◆ A 305 bp region within the EGFR gene first intron demonstrates repressive activity with both homologous and heterologous promoter/CAT constructs in the ER+/low EGFR expressing MCF-7 breast cancer cells, but not in the ER-/high EGFR expressing BT549 breast cancer cells.
- ◆ Multiple factors within MCF-7 nuclear extracts interact with the 305 bp intron negative regulatory and may be involved in the transcriptional activity displayed by this intron element.
- ◆ A 96 bp sub-fragment appears to contain the major negative regulatory element responsible for transcriptional repressive activity. Furthermore, this element demonstrates a differential binding pattern with nuclear extracts from ER+ vs. ER- breast cancer cells.
- ◆ The EGFR gene transcriptional repressor, or ETR, appears to be involved in the DNA-protein complex formed between factors in MCF-7 nuclear extracts and the 96 bp probe.
- ◆ Chromatin conformation may play a role in the negative regulation of EGFR gene expression in hormone-dependent breast cancer cells.

REPORTABLE OUTCOMES

- ◆ Third Place in Student Research Days Poster Competition. Wilson, M.A., McInerney, J.M., and Chrysogelos, S.A. Negative Regulation of EGFR Gene Expression in Hormone-Dependent Breast Cancer Cells."
- ◆ Wilson, M.A., McInerney, J.M., and Chrysogelos, S.A. Negative Regulation of EGFR Gene Expression in Hormone-Dependent Breast Cancer Cells. 90th Annual American Association for Cancer Research Meeting, Philadelphia, PA, April 10-14, 1999.
- ◆ Manuscript: Wilson, M.A. and Chrysogelos, S.A. Identification and characterization of a negative regulatory element within the epidermal growth factor receptor gene in hormone-dependent breast cancer cells. manuscript in preparation.

MANUSCRIPTS AND ABSTRACTS ATTACHED

- ◆ Wilson, M.A., McInerney, J.M., and Chrysogelos, S.A. Negative Regulation of EGFR Gene Expression in Hormone-Dependent Breast Cancer Cells. Student Research Days, Georgetown University, February 23, 1999, and the 90th Annual American Association for Cancer Research Meeting, Philadelphia, PA, April 10-14, 1999.

NEGATIVE REGULATION OF EGFR GENE EXPRESSION IN HORMONE-DEPENDENT BREAST CANCER CELLS Melissa A. Wilson, Jane M. McInerney, and Susan A. Chrysogelos *Lombardi Cancer Center, Department of Biochemistry/Molecular Biology (M.A.W., S.A.C.); Dartmouth Medical School, Departments of Medicine and Pharmacology/Toxicology (J.M.M.)*

The overall objective of this study is to understand the role the first intron of the epidermal growth factor receptor (EGFR) gene plays in the negative regulation of EGFR gene expression in hormone-dependent breast cancer. EGFR, which has been demonstrated to be overexpressed in breast cancer, is inversely correlated with estrogen receptor (ER) status in a majority of tumors; however, a significant number of tumors co-express the two receptors. EGFR expression, independent of ER status, is associated with a more aggressive phenotype and predicts for poor response to endocrine therapy, suggesting that up-regulation of EGFR is involved in the progression to a more aggressive, hormone-independent phenotype. Experiments were designed to characterize the mechanism by which negative regulatory elements present within the EGFR gene first intron specifically repress EGFR gene expression in estrogen-dependent breast cancer cells. We have identified a 305bp region of the EGFR gene first intron that represses transcriptional activity of the EGFR gene in ER+/low EGFR expressing MCF-7 breast cancer cells, but not in ER-/high EGFR expressing BT549 breast cancer cells. Furthermore, gel shift assays with various fragments representing the 305bp region have revealed differences in DNA-protein complexes between MCF-7 and BT549 cells; incubation of MCF-7 nuclear extracts with a 96bp fragment results in the formation of a distinct DNA-protein complex which is absent in BT549 nuclear extracts. Results from *in vitro* DNaseI footprinting and southwestern analysis suggest that multiple protein factors may be involved in regulating EGFR gene expression. Preliminary results from RNase protection assays demonstrate that treatment with Trichostatin A, a histone deacetylase inhibitor, results in an increase in EGFR message levels in MCF-7 cells, while a decrease in EGFR levels is observed in BT549 cells. Based on our experimental results, we hypothesize that there are multiple forms of transcriptional repression acting on the EGFR gene, and that the 305bp region within the EGFR gene first intron is involved in transcriptional repression observed in hormone-dependent breast cancer cells. By studying the role of transcriptional repression in EGFR gene expression, we hope to elucidate the molecular mechanisms by which EGFR expression is altered during breast cancer progression.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 Apr 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 document numbers be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

A handwritten signature in cursive script, reading "Phyllis Rinehart", is positioned above the typed name.

PHYLLIS M. RINEHART

Deputy Chief of Staff for
Information Management

ADB277986
ADB263450
ADB267669
ADB277564
ADB261754
ADB257280
ADB283722
ADB249627
ADB282841
ADB266235
ADB283529
ADB283519
ADB256683
ADB262564
ADB271045
ADB283537
ADB257204
ADB283513
ADB281571
ADB262777
ADB270818
ADB283748
ADB274588
ADB283788
ADB259015
ADB266031