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GRANT NUMBER DAMD17-94-J-4219

TITLE: Effect of HSP27 on Human Breast Tumor Cell Growth and Motility

PRINCIPAL INVESTIGATOR: Eileen Hickey, Ph.D.

CONTRACTING ORGANIZATION: University of Nevada, Reno
Reno, Nevada 89557-0015

REPORT DATE: September 1997

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Effect of HSP27 on Human Breast Tumor Cell Growth and Motility

5. FUNDING NUMBERS
DAMD17-94-J-4219

6. AUTHOR(S)
Eileen Hickey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nevado, Reno
Reno, Nevada 89557-0015

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

This award is a Predoctoral Fellowship to support the training of Donna Egender (Housley). The goal of the research is to investigate the effects of the small heat shock protein, Hsp27, on growth and motility characteristics of human mammary tumor cell lines. Since Hsp27 regulates actin microfilament dynamics, we hypothesize that cells expressing high levels of Hsp27 will show increased motility and altered chemotactic properties, in addition to increased resistance to hyperthermic killing and to certain antitumor drugs. The development of stable transfected MB-MDA231 cell lines that express significantly elevated levels of Hsp27 has proven to be daunting. Down regulation of Hsp27 levels in MCF7 cells using antisense technology has also not been achieved because of toxic effects. In order to overcome these limitations, Donna spent much of the past year working with the adenovirus vector system. This approach was also found to give inadequate expression levels in mammary tumor cells. She has, therefor, returned to her original approach of selecting stable transformed cell lines by using plasmids containing high level constitutive promoters driving *hsp*27 expression. She has begun to analyze the 3 high level expressing cell lines already in hand with respect to stress resistance, growth, and motility.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer , Hsp27, .estrogen receptor, gene activation	22
	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

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Eileen Hickey 10/24/97

PI - Signature / Date

TABLE OF CONTENTS

INTRODUCTION

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender.

The goal of this research is to investigate the effects of the small stress protein, Hsp27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Preliminary clinical studies indicate that elevated levels of Hsp27 in breast tumor cells correlates with aggressive metastasis and poor prognosis (1,2). We have shown that Hsp27 overexpression confers resistance to killing by hyperthermia and by certain anti-tumor drugs (3,4). Phosphorylation of Hsp27 increases rapidly in cells treated with heat, cytokines or mitogens (5,6,7,8). In rodent cells overexpressing human Hsp27, the actin cytoskeleton is resistant to damage caused by hyperthermia or cytochalasin D treatment (9,10). High levels of Hsp27 also correlate with increased accumulation of cortical actin, suggesting a possible effect on cellular motility. We have recently demonstrated that overexpression of Hsp27 in rodent fibroblasts results in increased cell motility (11). In contrast, cells expressing a non-phosphorylatable form of Hsp27 show inhibition of processes depending on cortical microfilament dynamics (10).

This study is based on the hypothesis that Hsp27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We propose that breast tumor cells overexpressing Hsp27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and to certain drugs. Overexpressing cells may respond more vigorously to chemotactic agents, or may respond to different signaling molecules than the parent cell type. We predict that the cells expressing antisense *hsp*27 sequences, or those expressing the unphosphorylatable mutant will show responses antagonistic to those shown by cells overexpressing normal Hsp27.

We proposed to 1) prepare human mammary cell lines expressing either increased levels of Hsp27, unphosphorylatable Hsp27, or antisense sequences that reduce endogenous Hsp27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs. These goals were to be achieved through the following approaches:

a. Plasmids were constructed that allow expression of the *hsp*27 gene independently of estrogen under control of a metallothionein promoter in human mammary cell lines. Constructions expressing normal Hsp27, non-phosphorylatable mutants of Hsp27, and antisense sequences for down-regulation of Hsp27 expression were prepared.

b. *hsp*27 gene expression was to be: a) down regulated in ER positive MCF7 cells by transformation with antisense gene constructions, and b) expressed independently of estrogen in ER negative MDA-231 cells. Because of difficulties in obtaining satisfactory stable cell lines (Progress Report, 1996), this was to be achieved using the adenovirus infection/expression system. Cells

expressing different levels of Hsp27 were to be compared with respect to proliferation rate, general motility, chemotactic properties, and resistance to general stress or anti-proliferative drugs.

c. The importance of Hsp27 phosphorylation for the effects observed in Aim 2 was to be studied by transforming MDA-231 cells with plasmid encoding non-phosphorylatable variants of the hsp27 gene.

Since much of the work in the early part of this project involves long periods of hands-off cell culture, Donna initiated a side project in which she has been studying the structural and functional properties of the *hsp*27 promoter. Her goal is to identify the control elements that regulate gene expression in response to stress and estrogen treatment. This study was initiated during the second year of the fellowship and has continued as time has allowed. These studies are an extension of the plasmid construction work that Donna completed during the first year of the fellowship. Although these experiments were not proposed in the original fellowship application, they have provided her with an opportunity to develop additional skills in molecular biology.

PROGRESS REPORT

I. ACADEMIC: Donna has completed all requirements for her Ph.D. except her dissertation. During the past year, Donna focused entirely on her research. She presented her results at the annual CMB Program Research Meeting at Granlibakken, CA, in November, 1996.

II. RESEARCH:

During the first year of fellowship support, Donna successfully engineered plasmids that expressed the human Hsp27 and mouse Hsp25 proteins under control of the constitutive SV40 and β-actin promoters and a metal-inducible sheep metallothionein promoter. A plasmid expressing the human *hsp*27 cDNA in the antisense orientation was also constructed. During the second year of the fellowship, she isolated a total of 88 independent clonal cell lines by co-transfection with a gene conferring resistance to puromycin. However, the fraction of puromycin resistant clones that expressed Hsp27 at levels that were significantly different from the parent cell lines was very small. Only 8 out of 35 MB-MDA 231 clones co-transfected with the *hsp*27 gene under control of the β-actin promoter overexpressed Hsp27. And only 2 of these clones showed more than a two-fold increase over the parent cell line. Only 1 out of 35 clones co-transfected with the *hsp*27 gene under control of the SV40 early promoter showed increased amounts of the protein. Only 6 out of 35 cell lines co-transfected with the *hsp*27 gene under control of the metallothionein promoter showed increased Hsp27 content following treatment with 3 uM cadmium chloride. Unfortunately, the maximum level of induction was only about 3-fold over control. Donna was unable to demonstrate any difference in the level of Hsp27 in the 3 MCF7 clones co-transfected with the antisense *hsp*27 plasmids relative to controls.

Our previous results with rodent fibroblastic cell lines transfected with the human *hsp*27 gene indicated a significant amount of clonal variation in the physiological properties of cell line overexpressing Hsp27, even among lines containing similar amounts of the protein (4,9). We also found that there was a threshold for Hsp27 expression below which no physiological effects such as increased stress resistance or motility could be detected. We therefore had hoped to be able to isolate at least 10 independent clonal breast tumor cell lines with Hsp27 content between 5 and 10 fold higher than control. To date we have only 3 clones that meet that criteria, 2 driven by the β-actin promoter and 1 by the SV40 early promoter. Fig. 1 illustrates the Hsp27 content of these clonal cell lines as well as several metallothionein promoter driven clones and control cell lines transfected with empty vector.

Since it takes approximately 4 months to establish, isolate, screen and expand stable cell lines, Donna decided to focus on the replication defective adenovirus system for overexpressing Hsp27 in mammary cell lines. Much of her time during the third year of the fellowship was devoted to testing the adenovirus system for transient expression of sense and antisense hsp27 in MCF7 and MB-MDA 231 cells. As discussed below, this approach was not successful. Her results show that MCF7 cells are not efficiently infected by the ade5 vector and while MB-MDA 231 cells can be infected by virus carrying a reporter gene, little increase in Hsp27 content is detected following infection with virus containing the hsp27 gene.

Donna has continued to work on the second project to study the structural/functional properties of the human *hsp*27 gene promoter. Her results to date indicate that stimulation of *hsp*27 gene expression following estrogen administration is a more complex process than simple transactivation by activated estrogen receptor.

Figures and Figure Legends are in the Appendix section of this report.

A. Establishment of the replication deficient adenovirus system for overexpressing Hsp27 in mammary cell lines:

The advantages of the adenovirus system are several. Infection results in rapid transient induction of the protein with maximum levels typically achieved within 48 hr. In non-permissive cells, no other adenovirus genes are expressed. Unlike other transient gene expression systems, a very high uniform population of expressing cells (greater than 90%) can be achieved. In a collaborative project with Jodie Martin and Wolfgang Dillman at UC San Diego, we have constructed recombinant adenovirus containing the wild type human *hsp*27 gene in the sense and anti-sense orientation with respect to the powerful CMV promoter. In addition, we have also introduced various mutations that affect phosphorylation into the viral-contained gene. The purpose of constructing these vectors was to study the effects of Hsp27 expression in cardiac tissue with respect to resistance to ischemic damage. Infection of rat cardiomyocytes with these recombinant adenoviruses at multiplicities of infection (moi) between 3 and 10 produces high levels of human Hsp27 expression; typically 1 - 2% of total cell protein.

The identical preparations of ade5-hsp27 virus used successfully to program high levels of Hsp27 expression in cardiomyocytes were used to infect MCF7, MDA-MB-231, and a control rat fibroblast cell line (Rat1). As can be seen in Fig. 2, Western blot analysis of the 3 cells lines infected at a multiplicity of 3, 10, or 30 plaque forming units per cell produced no detectable increase in Hsp27 content in MCF7 cells over a period of 4 days. A small increase in Hsp27 content was detected in MB-MDA-231 cells at the highest m.o.i. after 3 days. However, the increase was only 2-fold over the endogenous level of the protein. The Rat1 cells, which contain no endogenous human Hsp27, show a time and infectious dose dependent accumulation of the protein indicating that the viral preparations were capable of infection and expression. Thus, it would appear that the human mammary tumor lines do not support either infection by the ade5 virus or efficient expression of the recombinant *hsp27* gene.

In order to further investigate the problem, Donna obtained a recombinant adenovirus that contained a luciferase reporter gene under control of the identical promoter used to drive expression of the human hsp27 gene (CMV early). Infection of the same cell lines with the ade5-lux virus revealed a time and infectious dose dependent accumulation of luciferase activity in all 3 cell types (Fig. 3). However, the maximum level of luciferase expression in MCF7 cells was only 5% of that seen in Rat1 cells. The MB-MDA231 cells expressed greater amounts of luciferase, but only approximately 20% of the level shown by the Rat1 cells. Large differences in the ability of ade5 to infect different cell types have been observed which are related to the abundance of cell surface receptors and other factors (11). Unfortunately, human mammary cells do not appear to be suitable hosts for this viral gene delivery vector. It is clear from these results that a different approach will be needed to generate cells that over and under express Hsp27.

B. Characterization of hsp27 promoter activity in mammary tumor cell lines.

During the second year of the fellowship, Donna initiated studies to define the promoter elements of the *hsp*27 gene that are responsible for the observed difference in expression of the protein in estrogen receptor positive cells such as MCF7 and estrogen receptor negative cells such as MDA MB-231. Using constructions in which truncated segments of the *hsp*27 promoter drive expression of a luciferase reporter gene in a transient expression assay, Donna found that the *hsp*27 promoter is more efficiently expressed in MCF7 cells than in MB-MDA231 cells, both constitutively and following heat stress. Deletion of the single consensus heat shock element (GAANNTTC) essentially eliminates heat inducibility. However, the constitutive transcription from the promoter, which is 20-fold greater in MCF7, is unaffected by deletion of the heat shock element. Thus, other sequences contained in the *hsp*27 promoter are responsible for increased expression in ER+ cells.

During this past year Donna continued to investigate why ER+ cell lines and tumors contain higher levels of Hsp27 than cells and tumors lacking functional estrogen receptor. The simplest explanation for the association of high Hsp27 expression with the presence of the ER would be to postulate direct regulation of the promoter by the activated receptor. Following activation by ligand, the ER binds

to a specific palindromic sequence (ERE, estrogen response element) that is found proximal to the transcription start site of a number of estrogen sensitive genes. The human *hsp*27 promoter contains a number of half-ERE sequences which could potentially be involved in direct stimulation of the gene by activated ER. In order to delineate the sequence elements that are responsible for estrogen regulation, a series of luciferase reporter gene plasmids were constructed that contained different amounts of the *hsp*27 promoter region.

To test for estrogen induction of luciferase driven by the intact *hsp*27 promoter, plasmid pBg27Exlux was transfected into MCF7 cells for a transient gene expression assay. This plasmid contains 1 kb of *hsp*27 upstream sequence fused with a luciferase reporter gene, the complete hsp27 coding region, and 500 bp of 3'- flanking sequence (Fig. 4). However, no increase in expression was detected following estrogen administration to transfected MCF7 cells (Fig. 5). A large increase in luciferase activity was detected following a mild heat shock, indicating that the cells have been transfected efficiently and that the promoter construction was functional. This experiment was repeated several times using both early and late passage MCF7 cells and different concentrations of estrogen. In addition, constructions containing only the 5'-promoter region fused with luciferase which had previously been shown to be expressed at higher levels in ER+ than in ER- breast tumor cell lines (12) were also tested. In no case was induction of luciferase activity by estrogen detected (data not shown).

The MCF7 cells used in these experiments still contain functional estrogen receptor and are capable of transactivating estrogen sensitive genes. Evidence for this is shown in Fig. 6, where cells were transfected with pBg27ExLux (*hsp*27 promoter) or pERELux, which contains a consensus palindromic ERE enhanced promoter. It can be seen that the ERE-driven gene is induced more than 10-fold following estrogen stimulation while expression of the *hsp*27-driven gene is unaffected. Further experiments in which cells were co-transfected with pBg27ExLux and a second plasmid expressing a constitutive mutant ER that does not require ligand for activity also failed to demonstrate stimulation of the hsp27 promoter by ER. As shown in Fig. 7, expression of the mutant ER in either HeLa cells or ER- MDA-MB231 cells had no effect on *hsp*27 promoter activity while expression of the consensus ERE driven luciferase gene was stimulated 2 - 3 fold. While a 2 - 3 fold increase in *hsp*27 mRNA content following estrogen treatment of MCF7 cells has been well documented by others and is observed consistently in our own laboratory, direct assays of promoter activity fails to detect increased transcription in the presence of hormone.

These negative results suggest that the ER does not regulate *hsp*27 gene expression via a simple transactivation mechanism. Porter et al have recently reported that a chimeric promoter containing -108 to +23 bp of the *hsp*27 gene fused upstream from the Herpes virus promoter linked to a CAT reporter gene can be stimulated by estrogen in MCF7 cells (13,14). Promoter-reporter constructions containing only the -108 to -84 region of the *hsp27* gene were also estrogen responsive. This gene fragment contains an ERE half-palindrome and an Sp1 site. These authors have obtained further evidence that this fragment binds a complex of Sp1 and ER in an in vitro gel mobility shift assay. Since our pBg27ExLux construction contains an additional 900 bp of upstream sequence, it is possible that inhibitory elements contained therein might be responsible for the observed estrogen

insensitivity. To test for this effect, MCF7 cells were transfected with a series of promoter deletion plasmids including one construction (Eco27Lux) that contained the -108 to +23 region fused to the luciferase reporter gene (Fig. 8). As shown in Fig. 9, estrogen treatment increased expression of all of the hsp27 promoter plasmids by only 10 - 20%. However, this same low level of stimulation was also seen for the control plasmid pLuxF3, which contains an SV40 early promoter driving expression of luciferase. Thus, in this experiment estrogen treatment appeared to cause a small general increase in transcription that was not specific to the *hsp*27 promoter. Deletion of the *hsp*27 promoter sequence up to -108 did not significantly affect gene expression in the presence of estrogen nor did deletion of the heat-shock element. The positive control plasmid ERELux showed an 8 - fold increase in expression following estrogen treatment indicating that the ER was activated as expected. Again, we found no evidence for involvement of the activated estrogen receptor in regulation of the *hsp*27 gene. Estrogen might possibly stimulate hsp27 gene expression at a post-transcriptional level such as stimulating splicing or by stabilizing the mRNA in the cytoplasm. At this time we have no explanation for the discrepancy between our results and those of Porter et al. We should point out that our studies were done using a reporter gene splice into the intact *hsp*27 gene containing both 5'- and 3'- flanking regions rather than a chimeric promoter construction.

CONCLUSIONS AND FUTURE DIRECTIONS

This has been a frustrating year for Donna. Her efforts to use the ade5 vector system to modulate Hsp27 content in mammary tumor cells and her studies on regulation of the hsp27 gene by estrogen have both reached dead ends. She is clearly at a critical stage of her graduate career where she must decide where to take her dissertation project. After consultation with her advisors and her members of her thesis committee, Donna has decided to continue working on this project as it was originally proposed. She will proceed to characterize the 3 MB-MDA231 overexpressing clonal lines she has in hand with respect to growth rate, motility, and stress resistance. While these experiments are in progress, additional transfections and screenings will be conducted to obtain additional cell lines expressing wild type and non-phosphorylatable mutant forms of Hsp27 by cotransfection as was originally proposed. While this is admittedly a "brute force" approach, Donna has demonstrated that she can obtain stable overexpressing cell lines using this method. She has come to understand that it is often best to use workable existing technology to approach a problem. She realizes that she has invested a large amount of time unsuccessfully trying to improve the methodology. Donna has learned the hard way that she should have focused on the scientific question that was posed rather than to become seduced by the technology of molecular biology. Many bright students fall into this trap and often become completely discouraged and lose interest. However, Donna has shown remarkable perseverance and she is now totally committed to completing her degree. She is now in the final year of fellowship support and she understands that she must concentrate on collecting useful data and interpreting her results. Her committee feels that once she is able to obtain suitable cell lines, her work ethic will allow her to complete the characterization within a year.

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APPENDIX

Figures 1-9 and legends

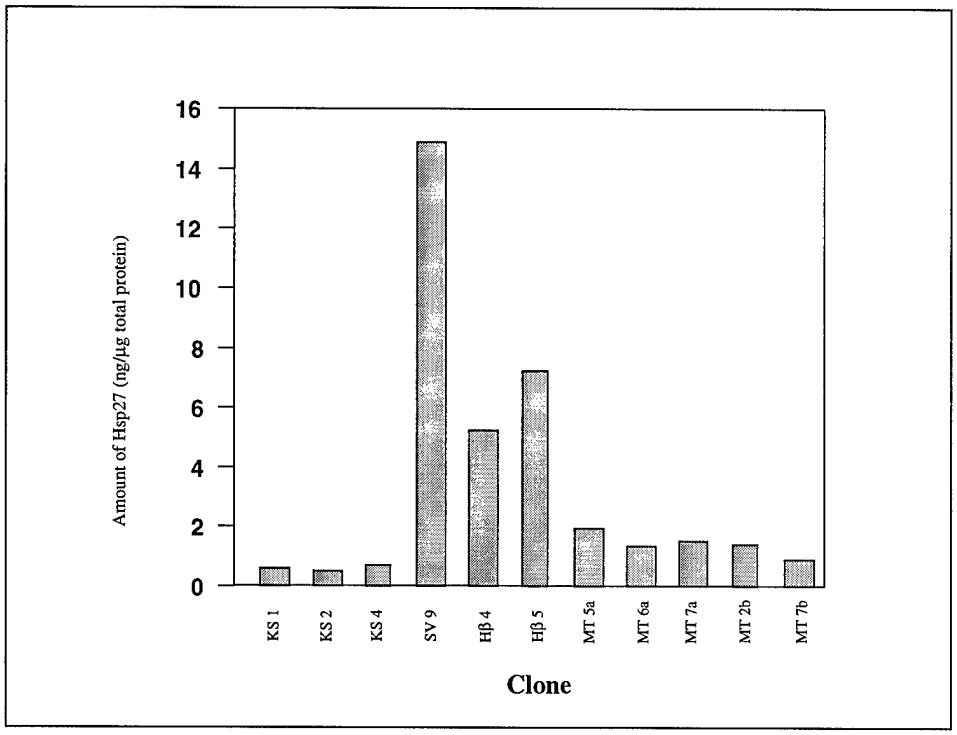

Fig. 1 *Hsp27 content of MB-MDA231 cell lines transfected with the hsp27 gene.* Aliquots of each clonal cell line containing 10 ug of total protein were resolved by SDS-PAGE along with known amounts of recombinant human Hsp27 (StressGen Biotechnologies). Hsp27 was measured by Western blot analysis using anti human Hsp27 antibody and an alkaline phosphatase conjugated second antibody, developed with CDP-star (TROPIX), and imaged on a BioRad Phosphoimager. KS cells are control cell lines co-transfected with empty Bluescript KS+ plasmid along with pPUR, which allowed selection for puromycin resistance. SV, Hβ, and MT clones were co-transfected with the human *hsp*27 gene driven by the SV40 early, β-actin, and sheep metallothionein promoters, respectively.

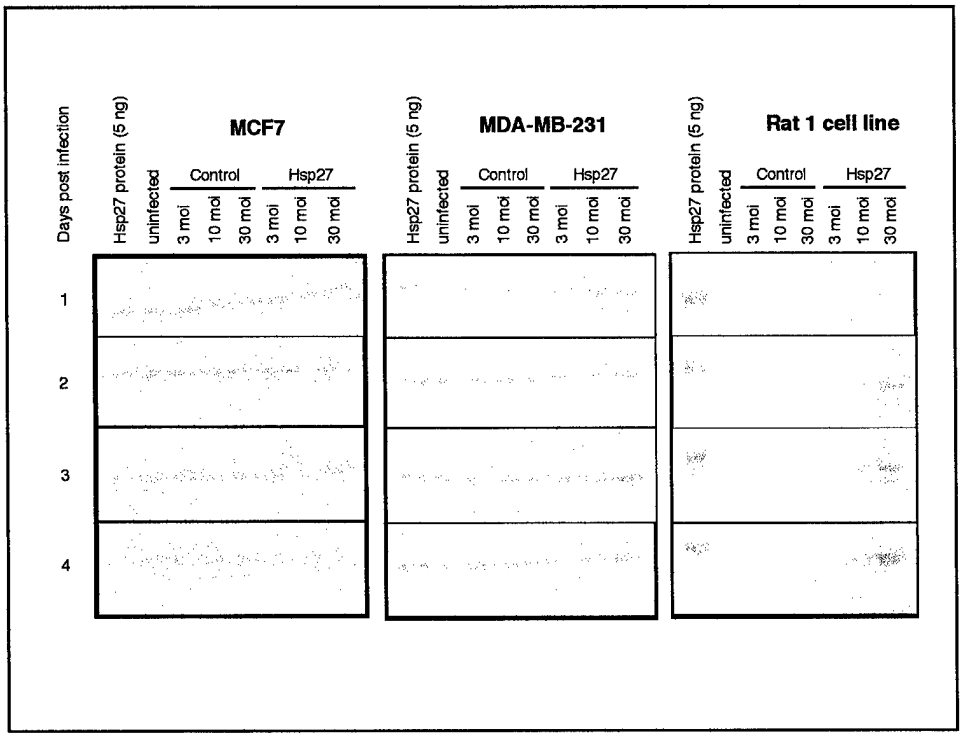

Fig. 2 *Expression of Hsp27 in cells infected with Hsp27 expressing adenovirus vectors.* The indicated cell lines were plated in 12-well plates at 7 x 10^4 cells/ well, and infected with adenovirus vectors containing no insert (control) or hsp27 coding sequences (Hsp27) at multiplicities of 3, 10, or 30, as indicated. At time zero, and at 24 hour intervals for 4 days subsequently, cells were harvested for analysis of expression. On the days indicated at the left of the figure, cells were lysed in SDS -containing buffer, and Hsp27 expression was measured by quantitative Western blotting as described in Fig. 1. Purified recombinant Hsp27 was loaded into the far left lane of each gel as a positive control.

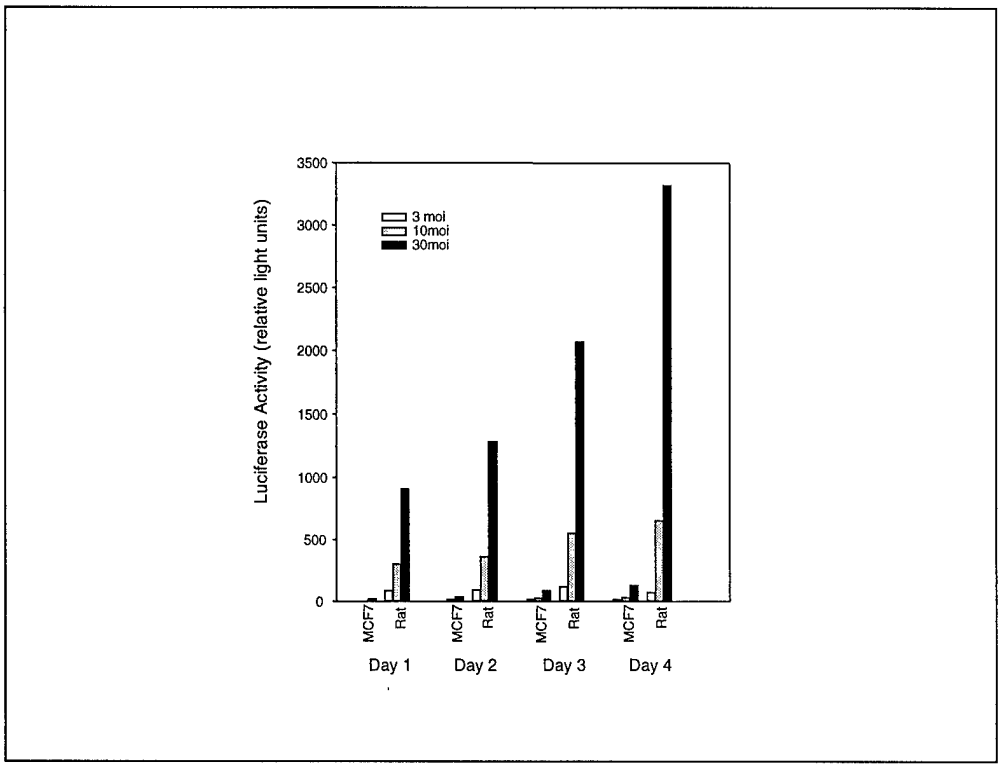

Fig. 3 *Expression of luciferase expressing adenovirus vectors in MCF7 and Rat 1 Cells.* The indicated cell lines were plated in 12-well plates at 7 x 10^4 cells/well, and infected with adenovirus vectors containing no insert or luciferase at multiplicities of 3, 10, or 30, as indicated. At time zero, and at 24 hour intervals for 4 days subsequently, cells were harvested in reporter lysis buffer and luciferase activity was determined with the Promega Luciferase Assay System using a Turner luminometer. Luminometer units are plotted versus time for the different treatments as indicated.

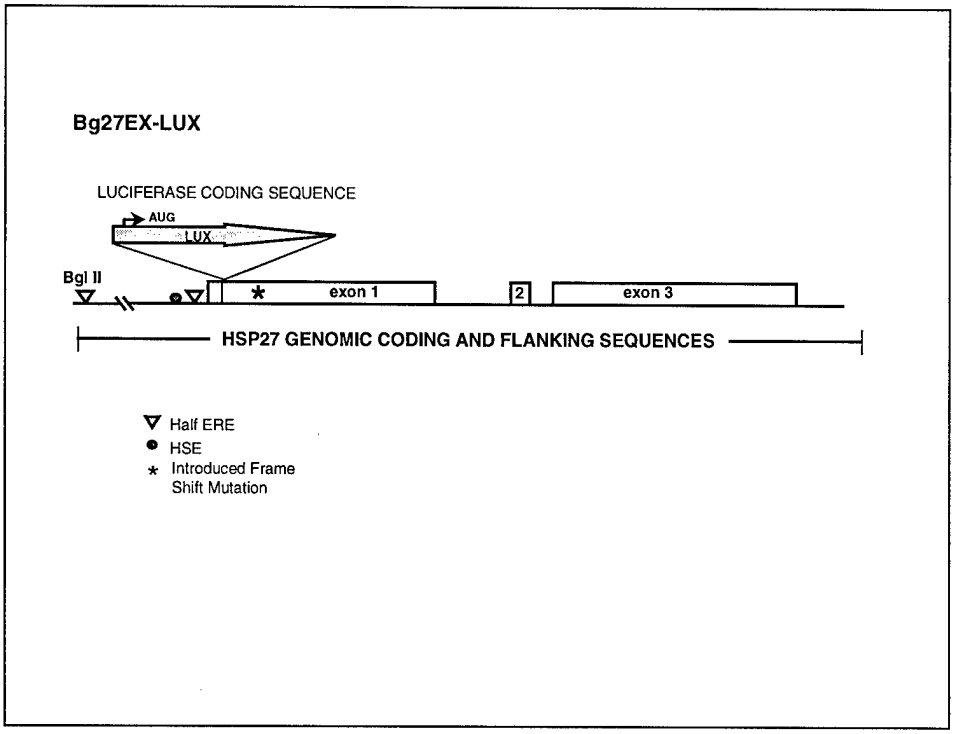

Figure 4. *The Hsp27 promoter-Luciferase reporter plasmid.* The initial reporter gene construct consists of the coding regions of the firefly Luciferase gene from plasmid pLUX-F3 (provided by D. Lloyd), introduced into plasmid Bg2711EX, to give Bg27EX-LUX. Bg2711 EX is a subclone in Bluescript KS (Stratagene) of the human *hsp27* genomic sequences between the 5' Bgl II site and the 3' Hind III site. This subclone has been modified by introduction of a Hind III site into the 5' untranslated leader of the transcribed region, as cloning site for genes to be expressed. The 3' Hind III site has been destroyed. The plasmid therefore contains, in addition to the introduced gene, about 1Kb 5' promoter sequences of *hsp27,* the complete *hsp27* coding sequences, as well as all introns and 3' termination and processing signals. A frame shift has been introduced at codon 22 of the human *hsp27* gene, to assure that the *hsp27* regions of the transcript can not be translated into functional Hsp27 protein.

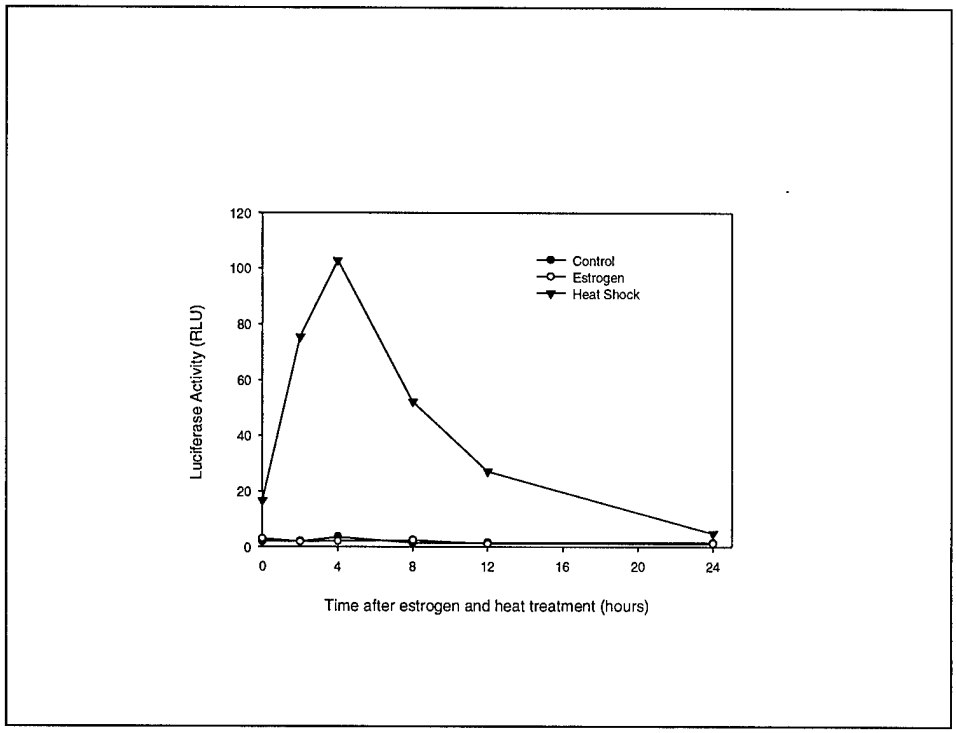

Fig. 5 *Effect of estrogen and heat treatment on luciferase activity expressed from the* hsp27 *promoter.* Estrogen depletion was initiated in MCF7 cells by rinsing twice in DME base without phenol red, and replacing the culture medium with DME base supplemented with 5% dextran coated charcoal treated bovine calf serum (DCC - BCS) and 6 ng/ml insulin. Twenty-four hours subsequently, cells were plated into 4 T25's at 5 x 10^5 cells/flask in estrogen depletion medium and cultured for two more days. The cells in each flask were then transfected with 4 µg of plasmid containing the luciferase coding sequences under control of 1kb of 5' flanking promoter sequence from Hsp27 (Bgl27ExLux) in 40 µl LipofectIN (Life Technologies) in 2 ml of phenol red free Optimem for 6 hours, and the estrogen depletion medium replaced. Two days after transfection, cells were trypsinized, pooled, and replated into three 6-well plates. Following attachment of the cells, each plate was treated by feeding with depletion media containing either 0.1% EtOH or 10^{-7} M estradiol, or by incubation at 42°C for 1.5 hours. One well each was sampled by lysis at 0, 2, 4, 8, 12, or 24 hours following treatment. Luciferase activity was measured as described previously.

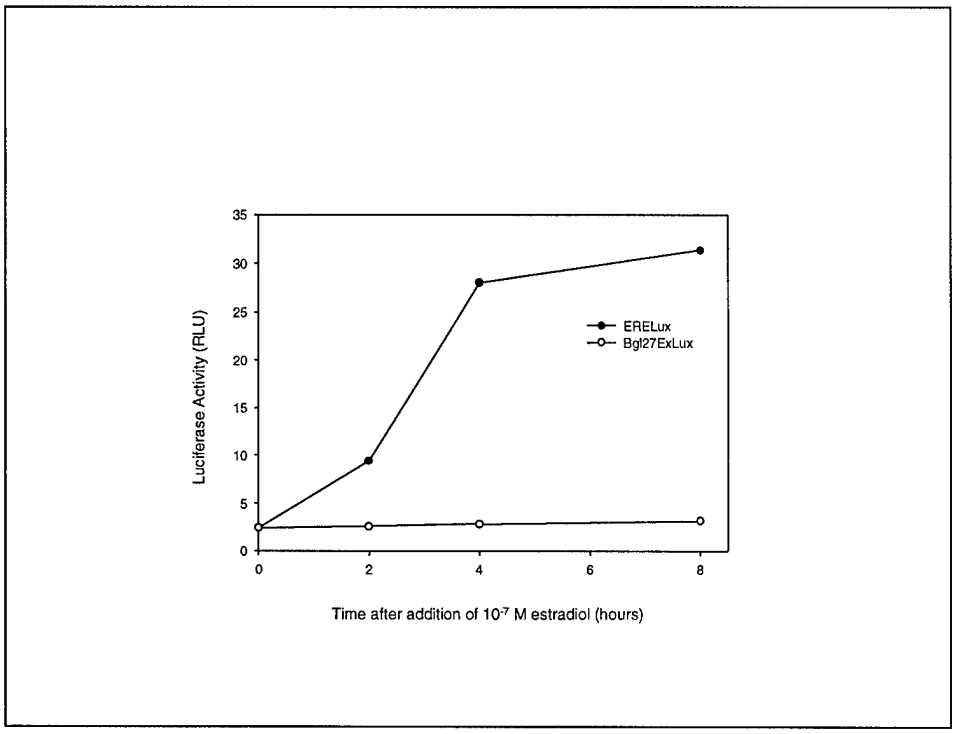

Fig. 6 *ERE and hsp27 promoter driven expression of luciferase in MCF7 cells following estrogen treatment.* MCF7 cells were plated at a density of 8 x10^5 cells per T25 flask in DMEM supplemented with 10% bovine calf serum and 6 ng/ml insulin. After 24 hours, the cells were rinsed twice in DME base without phenol red, and fed with estrogen depletion medium as in Figure 4. Following 24 hours, the cells were transfected with 4 μg of DNA (0.8 μg pSVβGal and 3.2 μg of luciferase reporter plasmid) and 40 μl LipofectIN as in Figure 3. Reporter plasmids tested were ERELux (containing luciferase coding sequences under control of a consensus ERE enhanced promoter) or Bgl27ExLux. After 6 hours the transfection mixture was replaced with estrogen depletion medium for 48 hours. At this time, 10^{-7} M β estradiol, or ethanol carrier were added. At the indicated times, cells were lysed and analysed for expression of luciferase as described previously. Transfection efficiencies were normalized by correcting for β-galactosidase expression, which was detected using the β-galactosidase Assay System (Promega).

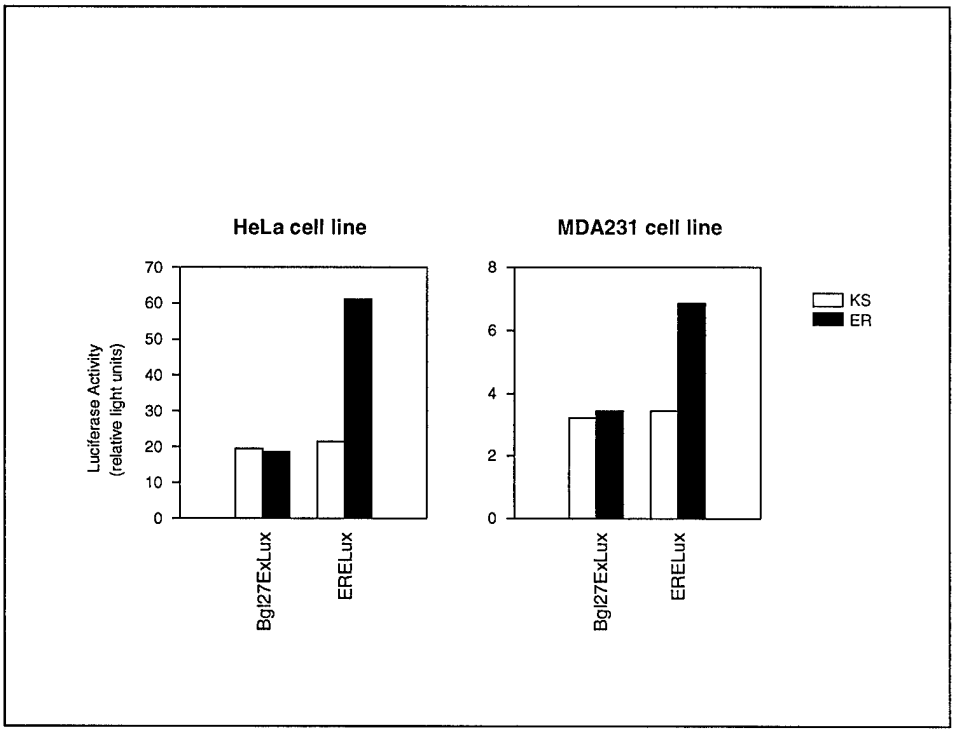

Fig. 7 *Effect of constitutively active estrogen receptor on expression of Bgl27ExLux and ERELux.* HeLa cells and the MDA-MB-231 estrogen receptor (ER) negative breast tumor cell line were each cotransfected with one of the two luciferase expression plasmids (Bgl27ExLux or ERELux) along with pCMV-exon5, a plasmid that expresses an estrogen independant mutant form of the human ER driven by a CMV promoter. After 2 days in complete medium, cells were transfected with 2 µg DNA per well using 20 µl Lipofectin per well for 6 hours. Control wells were transfected with Bluescript KS plasmid in place of pCMV-exon5. Following 48 hours, cells were lysed in reporter lysis buffer and luciferase activity was determined as was described.

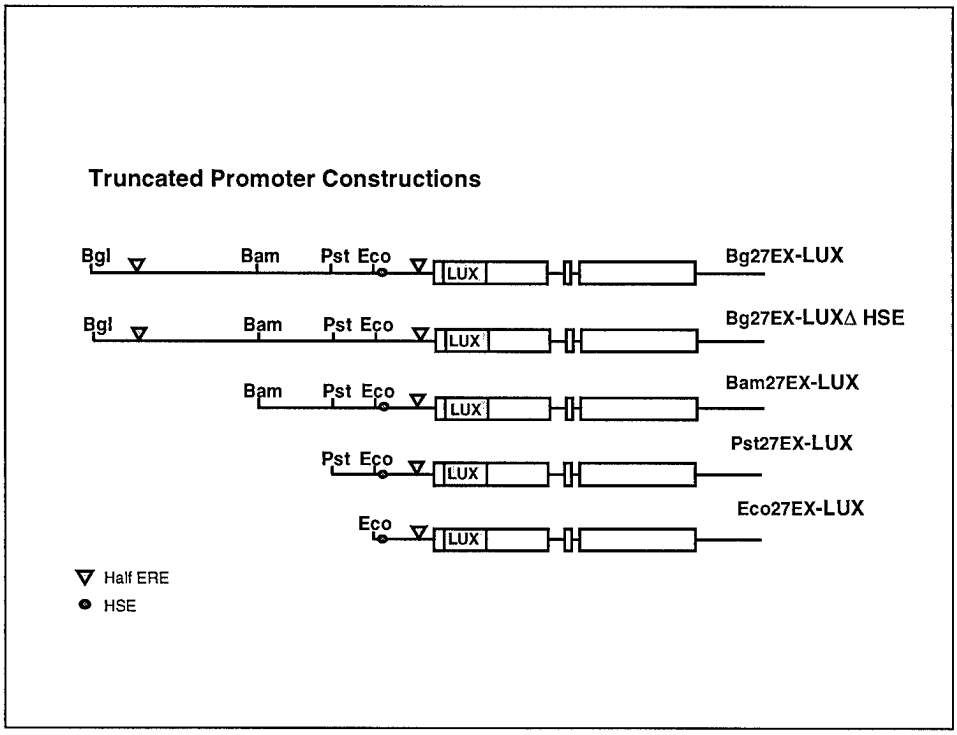

Figure 8. *Luciferase reporter plasmids with truncated or mutated Hsp27 promoter sequences.* The promoter deletion constructions consist of the original Bg27EX-LUX plasmid in which the 5' promoter region has been progressively shortened by using conveniently located restriction sites found in this region, as shown. This gives 5' promoter sequences starting at -1037 for Bg27EX-LUX, at -665 for Bm27EX-LUX, at -392 for Ps27EX-LUX, and at -157 for Eco27EX-LUX. This gene uses two transcription start sites, and the first nucleotide of the longer transcript is counted as #1. Bg27EX-LUX DHSE has had the nucleotides between -120 and -133 that contain the heat shock response element (HSE) removed by oligonucleotide directed mutagenesis.

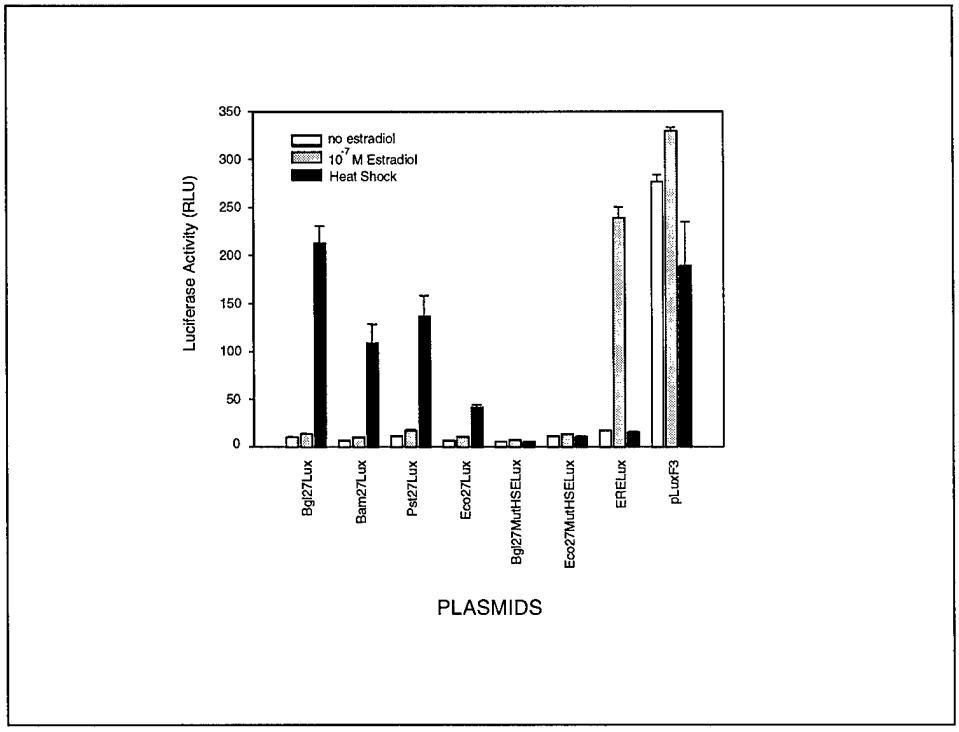

Fig. 9 *Effect of hsp27 promoter modifications on expression of luciferase activity.* Plasmids containing the luciferase coding sequences under control of wild type, truncations, or mutagenized forms of the Hsp27 promoter sequences were transfected into MCF7 cells using 4 μg of DNA (0.8 μg pSVβGal and 3.2 μg of luciferase reporter plasmid) as described previously. Control MCF7 cultures were transfected with plasmids containing the luciferase gene under control of the consensus ERE (ERELux) or a constitutive SV40 promoter (pLUX F3). Forty-eight hours following transfection, cultures were either treated with 10^{-7} M β estradiol or ethanol vector, as indicated, for 24 hours, or treated at 42° C for 90 min. and allowed to recover at 37° C for 4 hours. Cells were then lysed and luciferase activity was measured as before. Transfection efficiencies were normalized by correcting for β-galactosidase expression.