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

The goal of this research is to study association of the estrogen receptor (ER) with the nuclear matrix, and to identify nuclear matrix acceptor sites for ER. We constructed an ER fusion protein (GFP-ER) with a $His_6$ tag, HA tag, and the green fluorescent protein. Using fluorescent imaging techniques we studied the cellular localization of GFP-ER in the presence of various ER ligands in human breast cancer cell lines transiently transfected to express the GFP-ER protein. Striking differences in the patterns of distribution were observed. Isolation of GFP-ER associated proteins requires a large number of cells expressing GFP-ER. Therefore, three stable cell lines expressing GFP-ER under the control of an inducible promoter were constructed. Characterization of GFP-ER expression in one of the stable cell lines demonstrated that we can control levels of GFP-ER expression. We characterized the effect of expressing different levels of GFP-ER on regulation of the endogenous *c-myc* gene, to determine what levels of expression caused aberrant effects. We demonstrated that GFP-ER association with nuclear matrix increases with the length of exposure to estrogen, and that ER associated to the nuclear matrix is also binding to DNA, suggesting that nuclear matrix bound ER is transcriptionally active.

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Aug 13, 1998

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## INTRODUCTION

In the last year, we have published three papers and one review article. We have also submitted another manuscript for publication. They are as follows:

1. Holth, L.T., D.N. Chadee, V.A. Spencer, S.K. Samuel, J.R. Safneck and J.R. Davie. (1998). Chromatin, nuclear matrix and the cytoskeleton: Role of Cell structure in neoplastic transformation. In press, International Journal of Oncology.

2. Htun, H.*, L.T. Holth* **(*contributed equally to this work)**, D. Walker, J.R. Davie and G.L. Hager. (1998). Direct Visualization of Unoccupied and Occupied Human Estrogen Receptor $\alpha$ Revels a Role for Ligand in the Nuclear Distribution of the Receptor. Submitted to Mol. Biol. of the Cell.

3. Samuel, S.K., V.A. Spencer, L. Bajno, J.-M. Sun, L.T. Holth, S. Oesterreich and J.R. Davie. (1998). *In Situ* Cross-Linking by Cisplatin of Nuclear Matrix-bound Transcription Factors to Nuclear DNA of Human Breast Cancer Cells. Cancer Research, *in press*. (expected in vol 58, July 15).

4. Walia, H., H.Y. Chen, J.-M. Sun, L.T. Holth and J.R. Davie. (1998). Histone acetylation is required to maintain the unfolded nucleosome structure associated with transcribing DNA. Journal of Biological Chemistry 273:14516-14522.

5. Davie, J.R., S. Samuel, V. Spencer, L. Bajno, J.-M. Sun, H.Y. Chen, and L.T. Holth. (1998). Nuclear matrix: application to diagnosis of cancer and role in transcription and modulation of chromatin structure. Gene Therapy and Molecular Biology 1:509-528.

While all of the above publications are relevant to this project, the most directly related ones are the review article (number 1, above), and the manuscript submitted to Molecular Biology of the Cell (number 2, above). Therefore, these have been appended as Appendix A and B respectively, and will be referred to frequently in this report.

## BACKGROUND

### A. Rationale

At presentation only 30 to 40 percent of human breast cancers are hormonally dependent, that is, they will respond to some form of endocrine manipulative therapy, e.g., antiestrogen therapy. However, most human breast cancers originate as hormonally dependent tumors as ovariectomized, non-estrogenized women (Turner's Syndrome) and males rarely get breast cancer [1]. While the mechanisms responsible for hormone dependence in human breast cancer are complex and mostly unresolved, the hormonally-dependent phenotype correlates strongly with the presence of estrogen receptors (ER) in the tumor [2]. Thus, about 60% of ER+ human breast tumors respond to antiestrogen or other

endocrine therapies [3]. However, most of these tumors will eventually develop resistance to endocrine therapies, despite continued ER expression.

ER is a nuclear matrix bound transcription factor that binds to nuclear matrix proteins (acceptors) [4-8]. There is evidence that the ER acceptors differ in antiestrogen-sensitive and -resistant breast cancer cells [8]. Identification of these proteins may be informative in revealing whether an ER+ breast cancer is sensitive to antiestrogen therapy. We have designed a novel strategy to isolate and characterize the nuclear matrix acceptors for ER.

**B. Nuclear Matrix and Transcription Domains**

The nucleus is highly organized, with transcribed genes being found in discrete foci [9-14]. Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus, with transcriptionally active chromatin being selectively bound to the nuclear matrix [15-19]. The nuclear matrix is a three-dimensional nuclear network that provides the structural support for enzymes and proteins (e.g., transcription factors) involved in nuclear processes such as transcription, replication and RNA processing (see Fig. 1 and 2 of Appendix A). The nuclear matrix, a RNA-protein structure, is visualized following removal of chromatin from nuclease digested nuclei [20-23]. Actively transcribed chromatin regions are attached to the nuclear matrix by multiple dynamic attachment sites [16]. The transcription machinery, specific transcription factors (e.g., ER), and nuclear enzymes are thought to mediate the dynamic attachments between transcriptionally active chromatin and nuclear matrix [24-30].

The nuclear matrix is selective about which transcription factors it binds, and this selectivity varies with cell type [25,31,32]. It has been postulated that the nuclear matrix has a role in the expression of genes by concentrating a subset of transcription factors at specific nuclear sites [28,33]. Transcription factors associated with the nuclear matrix include ER, YY1, AML-1, Sp1, Oct1, mutant p53, and Rb [25,28,31,34-40].

Appendix A, part 4 reviews alterations in nuclear matrix composition in cancer, while part 5 of appendix A discusses transcription factors and the nuclear matrix.

**C. Estrogen Receptor and Nuclear Matrix**

The first step in the mechanism by which estrogens stimulate growth and antiestrogens inhibit growth of many ER+ human breast cancer cells is the direct binding of ligand to ER [41]. Unliganded ER can also be activated indirectly by phosphorylation, a process involving the MAP kinase signal transduction pathway [42], or directly by binding to cyclin D1 [43]. Steroid hormone receptors are DNA-binding proteins that can transcriptionally regulate target genes by directly interacting with the gene's promoter elements [44,45]. A consensus estrogen response element (ERE) consisting of a palindromic DNA sequence that binds ER as a monomer or dimer has been identified [44,45]. ER can interact

synergistically with several transcription factors and bind to a half palindromic ERE [44-51]. Studies show that hormone receptors can activate transcription without binding to DNA [52,53]. This indirect regulation appears to require other *trans*-acting factors to mediate the hormone response. Recently, a novel ER, ER β , which is expressed at low levels in breast cancer cells has been identified [54-56]. ERβ and ERα (called ER in this report) have a high conservation of amino acid sequence in hormone binding and DNA binding domains, but the structures of these proteins differ elsewhere.

The nuclear matrix has a central role in steroid hormone action [57-60]. ER is associated with the nuclear matrix of estrogen responsive tissues [4,5,61-63]. In *in vitro* reconstitution studies with nuclear matrices and hormone receptors (e.g., ER and androgen receptor), it has been shown that nuclear acceptor sites for the hormone receptors are associated with the nuclear matrix [57,62,64]. The binding of the ER to the nuclear matrix was saturable, of high affinity, target tissue specific, and receptor specific [62]. Acceptor proteins for ER have been identified in a variety of estrogen-responsive tissues, including human breast cancer cell line MCF-7 [7,8,57,65,66]. In human breast cancer cells, some nuclear matrix acceptors for ER are ligand dependent. Protein acceptors for estradiol-ER differ from those for antiestrogen [4-(N,N-diethylaminoethoxy)4'-methoxy-α-(*p*-hydroxyphenol)α-ethyl-stilbene]-ER. Importantly, antiestrogen-resistant MCF-7 cells had a lower level of acceptors for antiestrogen-ER than did antiestrogen-sensitive MCF-7 cells [8]. Part 7 of appendix A reviews how the nuclear matrix bound ER affects chromatin structure in a ligand dependent manner (Fig. 5 in Appendix A).

**D. Purpose**

We propose that nuclear matrix acceptors for ER direct ER to specific sites in the nucleus, and that there exist nuclear matrix acceptors that bind ER in a ligand-dependent manner. An objective of this research is to isolate the nuclear matrix acceptors for ER in human breast cancer cells. To achieve this goal, we have designed an expression vector that directs the synthesis of GFP-ER fusion protein. Advantages of using this fusion protein (e.g., GFP-ER) are that it can be seen in living cells, and that it has a $His_6$ tag and HA tag to simplify purification and detection. The GFP-ER protein will provide the means to isolate the nuclear matrix acceptors for ER. The nuclear matrix acceptor proteins may be useful diagnostic and prognostic markers. The function and mechanism of action of the ER depends on its' associated proteins and therefore identifying these proteins is a critical step in understanding these processes. Since it is now known that transcriptionally active chromatin is selectively bound to the nuclear matrix [15-19], those proteins associated with both the ER and the nuclear matrix are likely to be important in modulating ER activity.

## MATERIALS AND METHODS

### A. Immunoblot Analysis

Total cell lysates electrophoresed on SDS-polyacrylamide gels were transferred to nitrocellulose (Bio-Rad). The nitrocellulose filters were stained immunochemically with either antibody H222 to human ER (gift from Geoffrey Greene) or with an antibody to the HA tag (YPYDVPDYA) (from BabCo, Berkeley, CA), followed respectively by either goat anti-rat (Bio-Rad) or goat anti-mouse (Bio-Rad) conjugated to horseradish peroxidase. The immunochemcal staining was detected using the enhanced chemiluminescence detection system (Amersham, Mississauga, Ontario, Canada).

### B. Northern Analysis

Northern blot analysis was done as previously described [69].

### C. Isolation of Nuclear Matrix

Protocol for preparation of nuclear matrix in suspension was derived from references 20-23. Details of the protocol are outlined in the Materials and Methods section of Appendix B.

## RESULTS AND DISCUSSION

### A. Localization of the Estrogen Receptor Fusion Protein

In the previous annual report we reported that we had constructed a vector pCI-nGFP-ER, that when transiently transfected in human breast cancer cell lines directs the expression of an ER fusion protein we call GFP-ER. This ER fusion protein (GFP-ER) has three N-terminal tags that simplify its detection and isolation. The first, is the $His_6$ tag which will facilitate the isolation of the fusion protein using metal chelating columns or beads. Second, is an HA epitope tag which can be detected on western blots with anti-HA antibodies. Third, is the Green Fluorescent Protein (GFP) which makes it possible to observe GFP-ER in living cells using fluorescent and confocal microscopy

We further reported that the GFP-ER was a functional protein, which was determined by transient transfection assays performed in the ER negative MDA MB 435A breast cancer cell line using the ERE-tk-CAT reporter vector.

As reported in the previous annual report, the initial experiments examining the ligand dependent localization of GFP-ER were done in MCF-7 (ER+) and MDA MB 231 (ER-) human breast cancer cells cultured in either the presence or absence of 17β-estradiol. Transiently expressed GFP-ER protein was found only in the cells nucleus with exclusion from the nucleoli. In the transiently transfected MCF-7 cells cultured in phenol red free media with 5% twice charcoal stripped fetal bovine serum (i.e. no added ligand), a punctate pattern of nuclear localization was seen, while in MDA MB 231 cells the pattern was more diffuse. When 17β-estradiol was added to the media, the GFP-ER pattern in MCF-7 cells remained the same as when no ligand was added, while in MDA MB 231 cells the

pattern became more punctate or speckled. The same effects were observed when 4-hydroxytamoxifen was added to the media.

Because of the dramatic differences that were observed between the MCF7 and MDA MB 231 cell lines, these experiments were repeated in two additional human breast cancer cell lines, the ER- line MDA MB 435A, and the ER+ cell line T47D. Both of these cell lines gave patterns similar to that reported above for the MDA MB 231 cell line.

However, as we had changed serum lots for tissue culture since the initial experiments were done in the MCF7 cell line, the MCF7 cell line experiments were repeated with the new lot of serum. In the absence of ligand the distribution of GFP-ER was now found to be very diffuse, becoming punctate when either 17 β-estradiol or 4-hydroxytamoxifen were added to the media. Thus the results for the MCF7 cells follow the same trends as observed for the other three cell lines used in this study. The reason for the different results between serum lots is assumed to be due to incomplete stripping of estrogens from the first lot of serum, or some other source of estrogen contamination. Incomplete stripping of estrogens, and substances having estrogenic activity is a common problem encountered when studying estrogen effects. Therefore, it is important to have control experiments, such as transient transfection reporter assay, or monitoring of endogenous gene expression. These control experiments were not working during the initial experiments examining GFP-ER localization in the MCF7 cell line, however this problem has been remedied.

The results of these experiments are given in Appendix B, a manuscript entitled "Direct Visualization of the Human Estrogen Receptor α Reveals a role for Ligand in the Nuclear Distribution of the Receptor" which has been recently submitted to the journal Molecular Biology of the Cell. In addition to the experiments discussed above, this manuscript also includes examination of the effect of the antiestrogen ICI 182780 on GFP-ER localization. Finally the manuscript includes data on the association of GFP-ER with the nuclear matrix.

**B. Stable Cell Lines Expressing GFP-ER**

In order to isolate the nuclear matrix proteins associated with GFP-ER it was necessary to prepare stable cell lines expressing this protein. In the last annual report I reported that we chose to regulate the expression of GFP-ER by introducing the tetracycline-on system [67] into the desired cell lines. To use this system cells lines must first be stabily transfected with the plasmid pUHD172-1neo that directs the expression reverse tetracycline-controlled transactivator (rtTA, a fusion between reverse Tetracycline repressor and activating domain of VP16 protein) and neomycin resistance gene. This was followed by stabily transfecting cells with GFP-ER cloned into the pUHD10-3 plasmid, placing GFP-ER under the control of a tetracycline inducible operon. In the absence of doxycycline, rtTA does not activate the expression of GFP-ER, but when doxycycline is added, there is doxycycline concentration dependent activation of

GFP-ER expression. In our previous annual reported we reported that we had selected several stable MCF7(rTet) GFP-ER clones, and were in the process of selecting MDA MB 435A (RTA-1) GFP-ER stable clones. MCF7 cells are ER+, while MDA MB 435A cells are ER-. At the time these stable cell lines were created we were observing very different patterns of GFP-ER distribution between these cell lines in the absence of ligand, which we hypothesized were due to a basic difference in cellular components and possibly related to the difference in their endogenous ER status. Subsequent experiments visualizing the distribution of GFP-ER expression in T47D cells (an ER positive line) indicated that their GFP-ER distribution pattern was similar to the ER- lines, so that this distribution was not related to the cells endogenous ER status. We therefore decided that a stabily transfected T47D cell line which could inducibly express GFP-ER was also needed, so that GFP-ER associated proteins could be isolated from both ER+ cell lines (MCF7 and T47D). This would have allowed us to determine if the proteins associated with GFP-ER changed with its cell specific distribution pattern. However, after the T47D stable cell line expressing GFP-ER was created we realized that the gross differences in GFP-ER distribution patterns in the absence of ligand between MCF7 cells and the other cell lines were due to the serum lot (see previous section). Subsequently at this time, although there were cell line specific variations in the distribution pattern of GFP-ER, it seemed unnecessary and inefficient to proceed with more than one cell line in subsequent experiments. Therefore, we have only been working with the MCF7 stable cell line.

**C. Characterization of the Stabily Transfected MCF7 Cell Line which Inducibly Expresses GFP-ER**

As we are interested in isolating nuclear matrix proteins associated with GFP-ER as a method of identifying the nuclear matrix proteins normally associated with the endogenous ER, it is important that we do not over express GFP-ER to such an extent that it is causing abnormal cellular events, as our results would then be prone to artifact. It is therefore important to characterize the expression of GFP-ER in the stabily transfected MCF7 cell line which inducibly expresses GFP-ER in the presence of doxycycline. Figure 1 shows a doxycycline dose response of GFP-ER expression 48 hours after the addition of doxycycline to the cells. No expression of GFP-ER is detected in the absence of doxycycline (Fig. 1, lane 1). An increase in GFP-ER expression is observed with increasing doxycycline concentrations, with this effect plateauing at 1 ug of doxycycline per ml of media. From these results it is apparent that we can control the expression levels of GFP-ER in these cells. Figure 2 shows the induction of GFP-ER expression at 1 ug/ml for various times after the addition of doxycycline. Seven hours after doxycylin is added GFP-ER expression is barely detectable (Fig. 2 lane 2), expression continues to increase up to the 48 hour time point (Fig. 2, lane 4), after which it seems to have reached equilibrium levels. This result suggests that experiments to isolate the nuclear matrix proteins associated with GFP-ER should be done at the least three days after the addition of doxycycline to allow cells to go through one cell cycle after GFP-ER expression has reached

equilibrium levels. The reasoning behind allowing at least one round of the cell cycle after reaching equilibrium values is that many DNA binding proteins only have access to DNA during S phase, and we want the GFP-ER to establish the same associations as the endogenous ER.

In order to determine if estrogen induction of endogenous genes was perturbed by the expression of GFP-ER, time courses looking at the induction of endogenous genes with estrogen were done. Figure 3 is a Northern blot prepared with RNA from these time course experiments and probed with the cDNA for *c-myc*. The first six lanes show the induction time course for *c-myc* done on the parent MCF7 cell line which has not been transfected with GFP-ER. Lanes 7-12 show the same time course done on the MCF7 cells which are stably transfected with GFP-ER, but with out it being induced, while in lanes 13-18 the time course of estrogen induction is done in the presence of 0.1 ug/ml doxycycline. No significant differences are observed in the patterns of induction between these three sets, However, when the time course with estradiol is done in the presence of 1.0 ug/ml of doxycycline (Fig. 3 lanes 19-24) the pattern of the induction of *c-myc* changes, the induction of the message at 30 min. (Fig. 3, lane 20) is much greater than observed in the other three sets (compare lane 20 to lanes 2, 8, and 14). These data suggest that at 1.0 ug/ml doxycycline induction of *c-myc* expression is altered. Therefore experiments to look at the GFP-ER associated nuclear matrix proteins should be done below these levels. We are in the process of repeating these experiments with doxycycline concentrations between 0.1 and 1.0 ug/ml, as it is desirable to have the highest level possible of GFP-ER expression with out effecting normal cellular processes as the more GFP-ER expressed, presumably the easier it will be to isolate. These experiments will also be repeated for different estrogen inducible genes such as *pS2* and the progesterone receptor.

**D. Estrogen Increases Association of GFP-ER with Nuclear Matrix**

In appendix B, we report that transiently expressed GFP-ER associates with the nuclear matrix. As we are planning to use our stable cell lines to isolate the nuclear matrix acceptor for the ER, using the GFP-ER protein, it was important to verify that GFP-ER was also associating with the nuclear matrix in the stably transfected MCF7 cell line. We were further interested in the effect of estrogen on GFP-ER association with the nuclear matrix. Figure 4 is a western blot of nuclear matrix proteins isolated at various times after the addition of estrogen. The blot is probed with HA antibody which identifies the N-terminus of GFP-ER. In the absence of exposure (Fig. 4, lane 1) some GFP-ER is found associated with the nuclear matrix. As the length of exposure to GFP-ER is increased so does the amount of GFP-ER found in the nuclear matrix. Thus estrogen very clearly affects the association of GFP-ER with the nuclear matrix. We expect to find that at least some of the nuclear proteins associated with the ER will change depending on the ligand associated with the ER.

## Fig 1

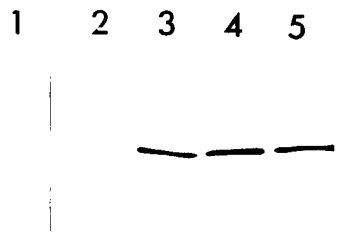


**Figure 1. Doxycycline dose response.** Stably transfected MCF7 cell which inducibly express GFP-ER when exposed to doxycycline were induced with different concentrations of doxycycline for 48 hours. Lane 1, no doxycycline. Lane 2, 0.1 ug/ml doxycycline. Lane 3, 0.5 ug/ml doxycycline. Lane 4, 1.0 ug/ml doxycycline. Lane 5, 5.0 ug/ml doxycycline. 20 ug of total cellular protein was run in each lane on an 8% SDS-PAGE gel. Immunoblotting was performed with the HA antibody which recognizes the N-terminal tag on the GFP-ER protein.

## Fig 2

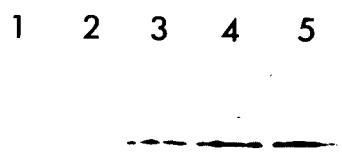


**Figure 2. Time course of response to doxycycline induction.** Stably transfected MCF7 cell which inducibly express GFP-ER when exposed to doxycycline were induced with 1 ug/ml of doxycycline for various lengths of time. Lane 1, 0 hours. Lane 2, 7 hours. Lane 3, 23 hours. Lane 4, 48 hours. Lane 5, 72 hours. 20 ug of total cellular protein was run in each lane on an 8% SDS-PAGE gel. Immunoblotting was performed with the HA antibody which recognizes the N-terminal tag on the GFP-ER protein.

## Fig 3


**Figure 3. Northern blot of time course of *c-myc* gene induction by estrogen.** The parent MCF7 cell line that has been stably transfected with the plasmid pUHD172-1neo that directs expression of the reverse tetracycline-controlled transactivator, but not the GFP-ER expression plasmid was used as a control for the induction of *c-myc* (lanes 1-6). The stably transfected MCF7 cell which inducibly express GFP-ER when exposed to doxycycline were induced with various concentrations of doxycycline for 7 days prior to addition of 10 nM 17β-estradiol. Lanes 7-12, no doxycyline. Lanes 13-18, 0.1 ug/ml doxycycline. Lanes 19-24, 1.0 ug/ml doxycycline. 10 nM 17β-estradiol was added for 0 min. (lanes 1, 7, 13, 19), 30 min. (lanes 2, 8, 14, 20), 1 hour (lanes 3, 9, 15, 21), 4 hours (lanes 4, 10, 16, 22), 7 hours (lanes 5, 11, 17, 23), 24 hours (lanes 6, 12, 18, 24). All lanes contain 10 ug of total RNA. Blot was probed with the *c-myc* cDNA.

## Fig 4

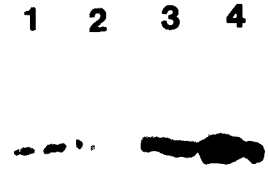


**Figure 4. Immunoassay with the HA Antibody to GFP-ER on nuclear matrix preparations isolated from stably transfected MCF7 cells expressing GFP-ER that have been exposed to 10 nM 17β-estradiol for various lengths of time.** Cells were grown in the presence of 1 ug/ml of doxycycline for 7 days prior to the addition of 17β-estradiol. Each lane contains 50 ug (which was isolated form equivalent numbers of cells) of total nuclear matrix proteins run on a 7.5% SDS-PAGE gel. Cells for sample in lane 1 were not exposed to 17β-estradiol. Cells for samples in lanes 2-4 were exposed to 10 nM 17β-estradiol for 30 min. (lane 2), 1 hour (lane 3) and 4 hours (lane 4)

**E. Purification of GFP-ER.**

The main goal of this project is to find the nuclear matrix acceptor(s) for the ER. This will be done by isolating nuclear matrix and then crosslinking it, followed by isolation of GFP-ER and its associated crosslinked proteins. Therefore, of primary importance to this project is the isolation and purification of GFP-ER.

In preliminary studies reported in our previous annual report, we showed isolation of GFP-ER from MCF-7 cells transiently transfected with GFP-ER expression plasmid, was possible with the Talon metal affinity resin. Metal affinity resins can be used to isolate proteins with $His_6$ tags. Further purification of GFP-ER was planned by additionally doing immunoprecipitation with anti-HA antibodies and protein A [68].

As the initial results on purification of GFP-ER from the transiently transfected cells were very positive, we hoped that the purification from the stable cell lines expressing GFP-ER would be fairly straight forward. Unfortunately the Talon metal affinity resin purification was much less efficient on the stable cells expressing GFP-ER. One plausible reason for this was the isolation of GFP-ER from the transiently transfected cells was done 24-48 hours after transfection with the transients while with the stable lines GFP-ER had been expressing for at least three days, and that this difference in time may have resulted in it becoming more tightly associated with other components of the cell. A second reason for the differences is that the expression levels on an individual cell basis are lower in the stable cell lines than in the transients (although more cells are expressing GFP-ER in the stabily transfected cells than in the transient transfected cells), the GFP-ER over expression in the transients may have resulted in some GFP-ER that was only loosely associated with cellular structures and therefore more soluble. We are currently in the process of trouble shooting and optimizing the isolation of GFP-ER by altering buffer conditions for the extractions and trying metal affinity resins from other sources.

**F. ER binds DNA while associated with the Nuclear Matrix**

We recently published (paper 3, listed in introduction) that the endogenous nuclear matrix bound ER of MCF-7 cells was crosslinked to DNA with cisplatin (an antitumor drug). The results of this paper are summarized in section 5 of appendix A. This paper provides evidence that the nuclear matrix is not merely a storage site for inactive transcription factors, but that the nuclear matrix associated ER is functional in the sense that it is bound to DNA.

## SUMMARY AND CONCLUSIONS

While the goals of this project have not changed, the methods of achieving these goals have. It is therefore not possible to summarize the progress of this grant in relation to the statement of work.

To date we have created an ER tagged with the HA epitope tag, the $His_6$ tag for metal affinity purification and with the green fluorescent protein. The triple tagged ER is called GFP-ER. Transient transfection experiments in the ER- cell line MDA MB 435A demonstrated that GFP-ER has functional activity and is associated with the nuclear matrix. Transient transfection studies have demonstrated that the nuclear distribution of GFP-ER is ligand dependent indicating that it's association with nuclear structures changes with ligand. In addition we demonstrated that the ER antagonist ICI 182780 alters the nucleocytoplasmic compartmentalization of the receptor, causing partial accumulation in the cytoplasm in a process requiring continued protein synthesis. See Appendix B.

Three stable cell lines (MCF7, MDA MB 435A and T47D) expressing GFP-ER under the control of an inducible promoter system have been created for the purpose of isolating the nuclear matrix acceptor for ER, using crosslinking techniques.

Characterization of the MCF7 cell line stably transfected with GFP-ER under control of an inducible expression system showed that the level of expression can be regulated depending on the concentration of the inducer (doxycyline), added to the cells, and that it took 48 hours after the addition of doxycycline to the media for the expression levels to reach equilibrium. We further characterized the stable cell line by studying the effect of different levels of GFP-ER expression on the regulation of endogenous genes. We found that at high levels of GFP-ER expression the timing of induction of the *c-myc* gene was altered, but at the lower levels of expression of GFP-ER normal regulation was not perturbed. These experiments will be repeated for other genes. In addition we will be investigating if there is an effect of GFP-ER on the cell cycle using FACS analysis. The main goal of these experiments is to determine the maximal level of GFP-ER expression possible without altering the normal ER activity.

In addition we have demonstrated that GFP-ER association with the nuclear matrix increases with the length of time it is exposed to estrogen. This suggests the nuclear matrix proteins associated with ER change with ligand, and the association with the nuclear matrix plays a role in the activation of genes by the estrogen when ligand is present. Further evidence for the role of the nuclear matrix in the regulation of genes by ER comes from our demonstration that endogenous ER that is bound to the nuclear matrix can be cross-linked to DNA *in vivo.*

## ACKNOWLEDGMENTS

I would like to acknowledge the following individuals for their contributions to this work. The head of my lab and my mentor, Dr. James Davie, our collaborators on the construction of the GFP-ER expression vector, Dr. Gordon Hager and Dr. Han Htun (N.I.H.), and Dr. Leigh Murphy (Univ. of Manitoba) for helpful discussions.

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# APPENDIX A

# Chromatin, nuclear matrix and the cytoskeleton: Role of cell structure in neoplastic transformation (Review)

LAUREL T. HOLTH[1], DEBORAH N. CHADEE[1], VIRGINIA A. SPENCER[1], SHANTI K. SAMUEL[1], JANICE R. SAFNECK[2] and JAMES R. DAVIE[1]

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**Abstract.** Aberrant nuclear and cellular structures are hallmarks of malignant transformation. Thus it is not surprising that the three-dimensional structure of the cell both affects and is affected by changes in gene expression. Here we review the role of the cytoskeleton, nuclear matrix, and chromatin structure in the genesis of cancer. The shape of a cell is governed by a dynamic tissue matrix, which includes extracellular matrix, cytoskeleton and nuclear matrix. Mechanical and chemical signals are transmitted to the nucleus, resulting in alterations in the three-dimensional chromatin organization of genes. The signal transduction pathways affect histone modifications, such as acetylation and phosphorylation, resulting in a relaxed chromatin structure observed in oncogene-transformed cells.

**Contents**



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*Abbreviations:* AF, activating function; ER, estrogen receptor; ERE, estrogen responsive element; $E_2$, estradiol; HAT, histone acetyltransferase, HDAC, histone deacetylase; MAR, matrix attachment region; NMBCs, nuclear matrix proteins in breast cancer; N-CoR, nuclear receptor corepressor; Rb, retinoblastoma protein

*Key words:* cytoskeleton, intermediate filaments, nuclear matrix, chromatin structure, histone acetylation and phosphorylation, breast cancer, oncogene-transformed fibroblasts

## 1. Introduction

Pathologists have long used aberrant appearing nuclei as a diagnostic marker for cancer. Cellular transformation is accompanied by alterations in both nuclear and cytoplasmic organizations (1-6). Although extensive evidence exists based on transformation parameters such as viral and oncogenic products, much less is known regarding changes occurring in the nuclear structure that relate to the transformed phenotype. The shape of a cell is governed by a dynamic tissue matrix system that links together the three-dimensional skeletal networks from the nuclear matrix, cytoskeleton and extracellular matrix (7,8) (Fig. 1). The nuclear matrix binds to DNA regions along the chromatin fiber referred to as matrix attachment regions (MARs) (9), and, therefore, organises DNA into loop domains (10) (Fig. 2). Thus, the tissue matrix system forms a structural and functional connection between the cell periphery and the DNA, stabilizing nuclear form and integrating cell and nuclear structure (8,11,12).

Changes in the shape of the nucleus and the cytoskeleton most likely alter chromatin structure (13) and perturb the nuclear matrix (14). Changes in nuclear shape contribute to alterations in DNA synthesis and gene expression (15,16). The ß-casein gene is an excellent example of the importance of cell shape and structure in gene regulation (17). The extracellular matrix and prolactin activate the BCE-1 enhancer of this gene through at least three transcription factors binding to the enhancer and, perhaps, by altering the acetylation state of the histones. The chromatin context of the enhancer is critical because BCE-1 on a non-integrated template will not respond to extracellular and prolactin signals (18). The authors proposed two mechanisms by which the extracellular matrix induces the expression of the ß-casein gene. Changes in the three-dimensional architecture of the cell by the extracellular matrix could alter the three-dimensional structure of the nucleus and the structure and/or composition of the nuclear matrix. Perturbation in the nuclear matrix could reposition nuclear matrix-associated histone acetyltransferases (HATs) and/or histone deacetylases (HDACs) (19), resulting in the

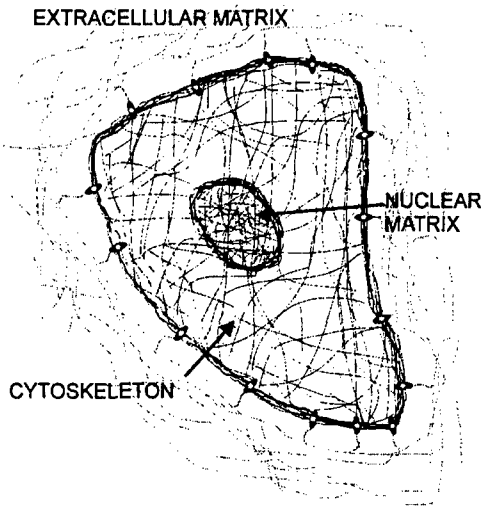


Figure 1. A model of the tissue matrix system. The tissue matrix consists of the extracellular matrix, cytoskeleton and nuclear matrix. It is important to note that the three fiber systems are interconnected and are dynamic structures.

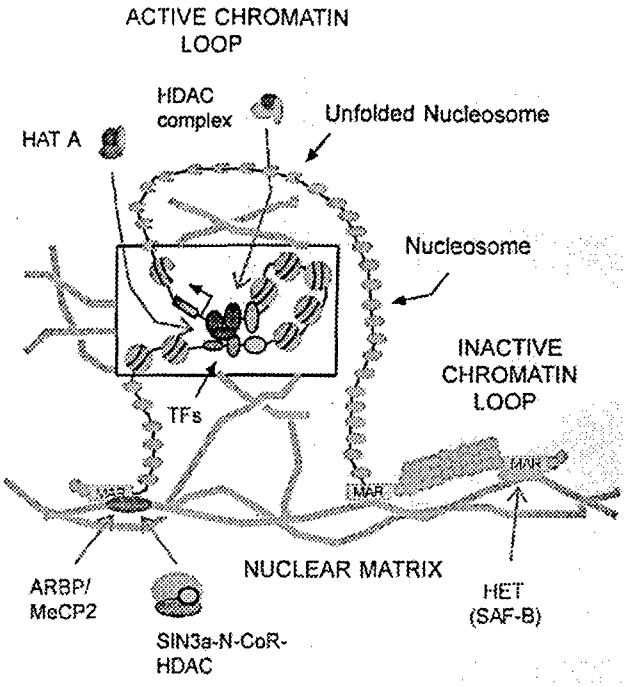


Figure 2. Organization of nuclear DNA. The inset is meant to show the multiple dynamic interactions between the nuclear matrix and regulatory and transcribed DNA sequences. TFs are transcription factors, some of which are associated with the nuclear matrix. HET/SAF-B is a MAR-binding protein that also acts as a repressor of the hsp27 gene. ARBP/MeCP2 is also a MAR binding protein that is a repressor. The nuclear matrix associated ARBP/ MeCP2 is shown recruiting the HDAC1 complex to a MAR.

remodelling by histone acetylation and transcriptional activation of the ß-casein chromatin template. Alternatively, the extracellular matrix could induce or modify cofactors, which have HDAC or HAT activity (20). Thus it can be seen from this example how the structure of a cell can play a role in the regulation of gene expression.

Cellular transformation is a result of altered gene expression, which in turn causes the expression of other genes to be altered. Here we look at the role of cell structure on the aberrant regulation of genes and in cellular transformation. Tumorigenesis involves a series of poorly understood morphological changes that lead to the development of hyperplasia, dysplasia, *in situ* carcinoma, invasive carcinoma, and in many instances finally metastatic carcinoma (21). Nuclei from different stages of disease progression exhibit changes in shape (22) and the reorganization of chromatin, which appears to correlate with malignancy (23). Thus it can be concluded that cell and nuclear structure are both affected by and affect gene expression within the cell, and therefore are likely to play a role in transformation.

## 2. Chromatin structure and histone modifications

Alteration in expression of specific genes involved in growth regulation can result in malignant transformation. There is an increasing awareness of the role of chromatin structure in the regulation of gene expression and in the genesis or suppression of cancer (20,24). The basic repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octamer. The histone octamer contains two each of the core histones H2A, H2B, H3 and H4 (Fig. 3). Histone H1 associates with the outer surface of the nucleosome. The H1 histones are a group of several subtypes that differ in amino acid sequence (25). H1 has a tripartite structure consisting of a central globular core and lysine rich N- and C-terminal domains. These domains interact with linker DNA to stabilize the compaction of chromatin. It has been demonstrated that both H1 and the N-terminal domain of H3 are necessary for proper chromatin folding (26-28). Due to their role in chromatin compaction, H1 histones are considered as general repressors of transcription; however, recent evidence demonstrates that H1 can function as a positive or negative gene-specific regulator of transcription (29). For example, H1b binds a regulatory element within the sequence of the replication-dependent mouse H3.2 gene, and therefore H1b may play a specific role in regulating the expression of this gene (30).

The tail domains of the core histones are involved in transcriptional regulation, replication, and chromatin condensation. The tails can undergo a context-dependent rearrangement in chromatin (31). The N-terminal domains of the core histones can be post-translationally modified by acetylation, methylation, phosphorylation and ADP-ribosylation, and the C-terminal domains of histones H2A and H2B can be ubiquitinated (10,32) (Fig. 3). Acetylated core histones are associated with transcriptionally poised and active genes (reviewed in ref. 20). The acetylation of the N-terminal tails may disrupt interactions with non-histone chromosomal proteins (33-37). Further, histone acetylation maintains the altered structure of the unfolded transcribing nucleosome (38,39) and has a role in the disruption of higher order chromatin packaging (40-43).

The chromatin fiber is organized into loops where the base of the loop (MAR) is attached to nuclear matrix proteins (10,44,45). The chromatin loop may have one or several genes. Chromatin loops containing expressed genes have a decondensed configuration that is sensitive to DNAse I

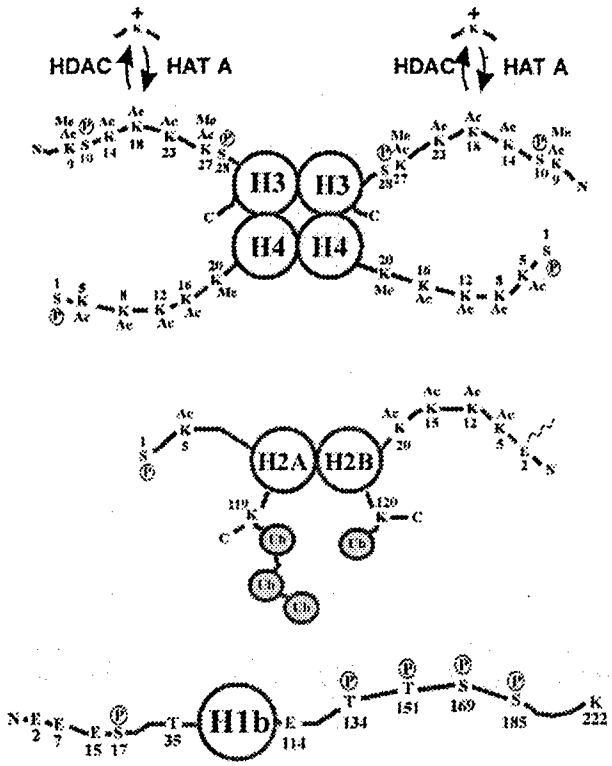


Figure 3. Post-synthetic modifications of the core and H1 histones are shown. The core histones are shown as an H2A-H2B dimer and $(H3\text{-}H4)_2$ tetramer. H3 and H4 are modified by acetylation (Ac), methylation (Me), and phosphorylation (P). H2A and H2B are modified by acetylation, phosphorylation, ubiquitination (Ub), multiubiquitination, and ADP-ribosylation (the stepladder structure). The enzymes catalyzing reversible acetylation are shown (HAT A, histone acetyltransferase; HDAC, histone deacetylase). Mouse H1b is shown in the lower part of the drawing. The potential phosphorylation sites are shown.

digestion, while loops with repressed genes have a condensed structure (Fig. 2). The boundaries of the DNAse I sensitive loop domain coincide with the position of MARs (10). These MARs delineate the loop domain in different cell types regardless of the transcriptional activity of the gene(s) within the domain. A comparison of the DNA sequences of MARs shows that they do not share extensive sequence homology; however, MAR-DNA sequences have high bending potential and may act as topological sinks (9,46-48).

Transcribing chromatin is selectively bound to the nuclear matrix (10,19,49). Multiple dynamic MARs attach transcribed chromatin regions to the nuclear matrix (see inset in Fig. 2); these MARs are different from those found at the base of loops (19). Nuclear matrix proteins, including nuclear matrix bound transcription factors (e.g., YY1 and AML), the transcription machinery, and histone modifying enzymes (e.g., HATs and HDACs) are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix (19,49-51).

### 3. Role of the cytoskeleton in cancer

In eukaryotic cells, the cytoskeleton is composed of actin-containing microfilaments, tubulin-containing microtubules and intermediate filaments that are composed of keratins, desmins, and vimentin (52). The cytoskeleton is physically associated with molecules involved in chemical signalling events (11). The cytoskeleton is also associated with the nuclear matrix and may influence directly or indirectly nuclear matrix-DNA interactions, including interactions between the nuclear matrix and transcribed DNA sequences.

Intermediate filaments extend from the nucleus to the plasma membrane (53), and evidence collected from several studies suggests that intermediate filaments are enmeshed with (7,54) and penetrate the nuclear lamina (55,56). Furthermore, *in vitro* intermediate filaments bind to DNA fragments that had either MAR sequences, transcription factor motifs or structural properties important in recombination and gene expression (56). The most compelling evidence that intermediate filaments are associated with nuclear DNA *in vivo* comes from the studies of Hnilica and colleagues (55,57). They demonstrated that intermediate filaments (cytokeratins) were cross-linked to DNA *in vivo* by *cis*-diamminedichloroplatinum, an agent that preferentially cross-links nuclear matrix proteins to DNA (58). These results contradict the view that intermediate filaments are found only in the cytoplasm, and strengthen the hypothesis that intermediate filaments exist in the nucleus (56), where they may influence DNA organization and gene expression (59). It is conceivable that intermediate filaments communicate signals from the extracellular matrix to nuclear DNA, resulting in changes in gene expression. Perturbations in intermediate filament composition or structure may alter chromatin organization and lead to aberrant gene expression and the development of a malignant phenotype.

The composition of the intermediate filaments can be radically altered in cancer cells. For example, the intermediate filaments profile of breast carcinoma cells is significantly altered when compared to profiles of normal breast epithelial cells. In breast tumours, the principal cytokeratins are K8, K18 and K19 (60), whereas normal breast epithelial cells predominantly express cytokeratins K4, K5, K6, K14, and K17 (61). Likewise, the progression of rat hepatocyte nodules to a state of malignancy is accompanied by the heightened expression of cytokeratin K19 (14).

In addition to cytokeratins, the intermediate filament protein vimentin may also be involved in breast cancer development. Several studies have shown that vimentin is only expressed in breast cancer cells with a hormone independent phenotype (62,63). The overexpression of vimentin leads to the formation of aberrant vimentin structures, the co-collapse of cytokeratin K8/K18 intermediate filaments, a decrease in cytokeratin protein levels, and a distortion of nuclear shape (64). The co-expression of cytokeratins with the intermediate filament protein vimentin increases the mobility and invasiveness of human breast cancer cells (65). Thus, it is possible that vimentin is involved in the development of a metastatic phenotype in breast cancer.

The exact mechanisms responsible for altering cytoskeletal protein expression in cancer cells are unknown. Oncogenic products from the *ras* signal transduction pathway stimulate the transcription of cytokeratin 18 in a mouse embryonal carcinoma cell line (66), and cytokeratins 8 and 18 in *ras*-transformed mouse epidermal keratinocytes (67). In addition to oncogenic products, hormones regulate the expression of

intermediate filament proteins. Treatment of rat vaginal epithelium with estrogen increases the synthesis of cytokeratins (68), while androgen represses cytokeratins K8 and K18 levels in the rat ventral prostate (69). Moreover, we demonstrated that the levels of cytokeratins 8, 18 and 19 associated with the nuclear matrix were drastically reduced in an estrogen receptor (ER) positive, hormone-dependent breast cancer cell line grown in acutely estrogen-depleted conditions, while treatment of these estrogen-starved cells with estrogen restored the levels of nuclear matrix associated cytokeratin (70). This study also showed that cells chronically depleted of estrogen developed a hormone-independent phenotype and overexpressed cytokeratins K8, K18 and K19 compared to levels observed in the control cell line grown in the presence of estrogen (70).

**4. Alterations in nuclear matrix composition in cancer**

Important cellular processes such as DNA replication, transcription, RNA processing and transport are associated with the nuclear matrix (50,71-78). The nuclear matrix is the nuclear structure that is present following the salt extraction of nuclease-digested nuclei (79-81). It is the dynamic structural framework of the nucleus comprised of a meshwork of core filaments linked to the nuclear lamina proteins. The core filaments of the internal matrix are similar in diameter to cytoskeletal intermediate filaments (79,82,83). The nuclear matrix is thought to provide the structural support from which several nuclear processes such as DNA replication and transcription occur (84).

Several studies have shown that the protein composition of the nuclear matrix is both tissue (85) and cell type specific (86), and undergoes changes with differentiation (87-89) and transformation (86,90,91). Nuclear matrix proteins that are specific for the cell type and/or state of differentiation have been identified (92-94). These observations attest to the cell type specificity of nuclear matrix proteins.

We are interested in how the profiles of nuclear matrix proteins changed with the metastatic progression of cancerous cells. Therefore, using single oncogene transformed mouse fibroblast cell lines, we examined whether nuclear matrix protein profiles are altered in cells with increasing metastatic potential (95). Two sets of cell lines were used. The first set was derived from 10T½ fibroblasts (96). These lines were transfected with H-*ras* and the transformed foci gave rise to the cell lines CIRAS-1, -2, -3. Using criteria such as experimental metastasis assays, tumour latency, anchorage independent growth and frequency of spontaneous metastasis (97,98), it was determined that CIRAS-1 was poorly metastatic, CIRAS-2 had intermediate metastatic properties, and CIRAS-3 was highly metastatic. The second panel of cell lines was derived from NIH 3T3 fibroblasts transfected with oncogenes encoding kinases (serine/threonine or tyrosine) (99). Criteria for selection were similar to that of the 10T½ derived fibroblast cell lines. From this, it was determined that 3T3/*raf* was poorly metastatic, while 3T3/*fes* was highly metastatic. Furthermore, the highly metastatic cell lines had an altered nuclear structure (100). Using these cell lines, we found that the highly metastatic cell lines (CIRAS-3, 3T3-*fes*) had similar nuclear matrix profiles to each other that were different from the poorly metastatic cell lines (CIRAS-1, 3T3-*raf*). Clearly, this data suggests that there is a unique nuclear matrix profile for each stage of malignancy regardless of transformation agent; therefore, we decided to pursue this line of research to identify nuclear matrix biomarkers in human breast cancer.

Breast carcinoma is the most common significant cancer in women. While it is the second leading cause of cancer mortality among females, the pathogenesis of the disease remains unclear (101,102). As breast carcinoma progresses, tumour cells typically change from estrogen dependent growth to a more aggressive phenotype characterised by estrogen independent growth, resistance to endocrine therapy and a high metastatic potential (103,104). Although the most influential prognostic marker for human breast carcinoma is metastasis to the axillary lymph nodes, the presence of ERs in breast tumours is generally acknowledged as an important factor in determining the type of treatment a patient is offered (105-108). Studies have shown that both *in vivo* and *in vitro*, human breast cancer cells that lack the ER are poorly-differentiated, express vimentin, invasive, estrogen independent, and resistant to anti-estrogen therapy. Conversely, cells that express the ER are typically well differentiated, poorly metastatic, lack vimentin, and, especially at the early stages of progression, are sensitive to anti-estrogen therapy (62,108-110).

Of potential importance is the demonstration that nuclear matrix proteins can be detected in the serum and urine of cancer patients (94,111,112). Therefore it was of interest to determine if unique nuclear matrix proteins could be identified for use as diagnostic and prognostic indicators for breast cancer. In a recent study by our lab, the nuclear matrix protein profiles of a variety of human breast cancer cell lines were examined by two-dimensional gel electrophoresis (113). These human breast epithelial cancer cell lines were MCF-7, ZR-75 and T47D ($ER^+$/hormone dependent), MDA MB 231 and BT-20 ($ER^-$/hormone independent), and an $ER^+$/hormone independent strain of MCF-7 cells called T5-PRF. MCF-10A1, a spontaneously, immortalized human breast epithelial cell line was used as a control. Using these cell lines, we identified specific nuclear matrix proteins exclusive to ER status. These proteins were present in each of the relevant preparations (either $ER^+$ and/or $ER^-$ breast cancer cell nuclear matrix proteins), but not in the preparations of nuclear matrix proteins from 'normal' breast epithelial cells. We refer to these proteins as nuclear matrix proteins in breast cancer (NMBCs), using the nomenclature proposed by Pienta and colleagues (114). Five NMBCs exclusive to $ER^+$ cell lines and one NMBC exclusive to the $ER^-$ cell lines were identified (113). None of these proteins were found in MCF-10A1. As we are interested in the diagnostic and prognostic potential of these nuclear matrix proteins in human breast cancer, we looked at tumour tissue samples. Using $ER^+$ and $ER^-$ human breast tumours, we were able to confirm the presence of NMBCs 1-5 in $ER^+$ human breast tumours and NMBC 6 in $ER^-$ tumours (113).

Interestingly, we found that most, but not all, abundant nuclear matrix bound proteins are also bound to DNA *in situ* (44,115). This was found by *in situ* protein-DNA cross-linking by *cis*-diamminedichloroplatinum, also called cisplatin, which preferentially cross-links nuclear matrix proteins to DNA (58). Cisplatin is an antitumour drug used in the treatment of several types of cancer.

## 5. Transcription factors and the nuclear matrix

Transcription factors are associated with the nuclear matrix (19,49). It has been proposed that the nuclear matrix recruits transcription factors, facilitating their interaction with regulatory DNA elements (116). We modified the cisplatin cross-linking procedure to investigate whether nuclear matrix associated transcription factors were bound to nuclear matrix attached DNA *in situ* in MCF-7 cells (44). The nuclear matrix associated transcription factors and cofactors studied were: ER, a transcription factor essential to the proliferation of hormone-dependent breast cancer cells; hnRNP K, a single-strand DNA-binding transcription factor (117); HET/SAF-B, a MAR-binding protein that acts as a repressor of hsp27 gene expression (118); and HDAC1, a corepressor (44). We found that all of these nuclear matrix bound transcription factors and corepressors were cross-linked to MAR DNA *in situ* (44). In contrast, a nuclear matrix protein SRm160 involved in RNA splicing (119) was not cross-linked to DNA by cisplatin. These observations provide the first direct evidence that nuclear matrix bound transcription factors are also bound to MARs *in situ*. Further, the results provide evidence that the nuclear matrix is not simply a storage site for inactive transcription factors/cofactors. Clearly, the nuclear matrix associated transcription factors/cofactors are functional in the sense that they are bound to nuclear DNA sequences.

These results also suggest a new mechanism for the antitumour activity of cisplatin. Several mechanisms appear to be involved in cisplatin's inhibition of transcription. First, architectural factors, such as high mobility group proteins, may be 'highjacked' to cisplatin-adducted DNA (120). Second, cisplatin adducts may block transcription factor access to a regulatory DNA element (121). We suggest a third mechanism, in which the cross-linking of functionally important transcription factors/cofactors to DNA by cisplatin compromises the transcription factors/cofactors function (44). Cross-linking between the transcription factor/cofactor and MARs may interrupt their dynamic interactions and interfere with the interaction between the transcription factor or cofactor and components of the transcription machinery. The cross-linking of the corepressor, HDAC1, is of particular interest as cross-linking of this enzyme to DNA by cisplatin would effectively reduce the population of HDAC that could be recruited by transcription factors to repress specific genes [e.g., the recruitment of the Rb-HDAC1 complex by E2F (24)]. Further, HDAC is also involved in the maturation of newly synthesized chromatin (122,123). Thus, the sequestering of HDAC1 may affect transcription, cell cycle progression and replication processes.

Our finding that HDAC1 is bound to MARs *in situ* is particularly intriguing and unexpected. Previous studies showed that HDAC1 in complex with several other proteins, including the co-repressors mSin3A and N-CoR, was recruited to regulatory elements by DNA bound repressors (e.g., Mad-Max) (reviewed in ref. 20). Our results suggest that HDAC1 is associated with MARs at the base of loops. This has intriguing possibilities in chromatin architecture. Crane-Robinson and colleagues reported that at the boundaries of the DNAse I sensitive, transcriptionally competent ß-globin chromatin domain of chicken erythrocytes there is a marked change in the acetylation state of the histones (124). The ß-globin chromatin domain is associated with highly acetylated histones, while histones at the boundaries are poorly acetylated. Several studies have shown that the boundaries of a DNAse I sensitive domain co-map with MARs (10). The association of nuclear matrix bound HDAC1 with MARs would provide a mechanism by which the histones at the boundaries of the domain are deacetylated. Further support for this idea comes from two recent reports showing that MeCP2, a transcriptional repressor, recruits the HDAC complex (125,126). Strätling and colleagues found that attachment region binding protein (ARBP), a nuclear matrix associated MAR-binding protein, was MeCP2 (127). Thus, ARBP/MeCP2 anchoring MARs to the nuclear matrix could recruit the HDAC1 complex (Fig. 2). The retinoblastoma protein, a tumour suppressor, is another example of a nuclear matrix associated repressor that recruits HDAC1 (128-131). It will be interesting to know whether the recruitment of HDAC1 is a general property of nuclear matrix associated MAR-binding repressor proteins.

## 6. Histone modifications and transformation

There is evidence that oncogene-transformed cells have an altered chromatin structure (1,132,133). Such an alteration in chromatin structure could be a result of alterations in levels of histone subtypes or in the post-translational modification of the histones, including acetylation and phosphorylation. It has been reported that some transformed cells have alterations in the amounts of H1 variants and that this may be significant in cell transformation (134). In addition, NIH 3T3 cells transformed by the c-Ha-*ras* oncogene have a decreased level of histone H1° subtype and an increase in nucleosome repeat length (135). The authors suggest that the decrease in H1° may play a role in chromatin changes in transformed cells by weakening internucleosomal interactions and destabilizing the chromatin structure.

The analysis of the involvement of histone modifications in cellular transformation has been limited. The main exception being histone H1 phosphorylation, which has been the focus of a number of studies involving oncogene-transformed cell lines and tumour tissues of mouse. H1 C-terminal and N-terminal domains can be phosphorylated (Fig. 3). Phosphorylation of H1 has been extensively studied, and it has been proposed that H1 phosphorylation may lead to decondensation of chromatin which could facilitate access of the chromatin to factors involved in transcription and replication (136). Lennox *et al* (137) have analyzed the phosphorylated forms of H1 in cell lines derived from murine teratocarcinomas. They identified H1b as the subtype with the most highly phosphorylated isoforms. Increased phosphorylation of H1b has also been observed in tumour tissues of mouse (Lewis lung carcinoma) in comparison to normal lung tissues (137). We found an increased level of phosphorylated H1b in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein (MAP) kinase (133,138). It was hypothesized that the persistent activation of the MAP kinase pathway in these cells may have altered the cyclin E associated H1 kinase activity resulting in the observed increase in phosphorylation of H1b (133) (Fig. 4). Fibroblasts lacking the tumour suppressor Rb also exhibit an increased

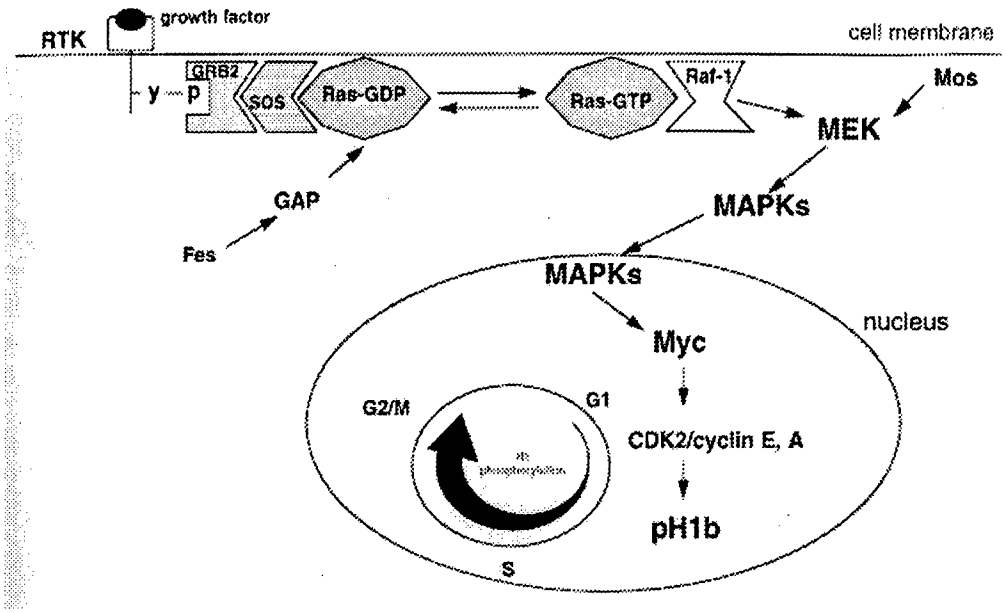


Figure 4. Histone H1b phosphorylation via the Ras-MAP kinase signal transduction pathway. In this model activated MAP kinase phosphorylates Myc, which in turn activates the cyclin E gene transcription. Cyclin E/CDK2 kinase is thought to be the H1b kinase. Increased phosphorylation of H1 throughout the cell cycle is indicated.

level of phosphorylated H1 and relaxed chromatin structure and deregulation of CDK2 may be directly involved (139). We showed that phosphorylation of H1b was dependent upon transcription and replication processes; it is the only histone modification known to be dependent upon both of these nuclear processes (140). We proposed the exposure of H1b to the cyclin E-CDK2 kinase by either the transcription or replication process results in the phosphorylation of H1b (140,141). Once modified, H1b would contribute to the relaxation of the transcribed chromatin fiber. Decondensation of the chromatin fiber would facilitate subsequent rounds of transcription.

Acetylated histones are associated with transcriptionally active and poised genes, and histone deacetylation is implicated in transcriptional repression. Until recently there had been no clear link between histone acetylation and cancer. Deregulation of the recruitment of HAT or HDAC complexes, amplification of coactivators with HAT activity, and synthesis of fusion proteins with HAT activity have been found in acute myeloid leukemia, acute promyelocytic leukemia, and breast cancer (142-149). Several groups have demonstrated that the retinoblastoma protein exists in a complex with HDAC1 and the transcriptional activator E2F (24,130,131). Thus transcriptional repression mediated by the tumour suppressor Rb, a nuclear matrix associated protein, may occur through recruitment of HDAC1. Phosphorylation of Rb by CDK4 or CDK6 results in release of Rb from E2F and the nuclear matrix (128), thus allowing transcription of E2F regulated genes, such as cyclin E. As previously mentioned, cyclin E complexed with CDK2 is thought to be involved in phosphorylation of H1 in transformed cells. Possibly, an altered chromatin structure acquired by deregulation of processes controlling histone modifications such as phosphorylation of H1 and acetylation of core histones may facilitate aberrant gene expression in the process of cellular transformation.

## 7. ER: a nuclear matrix associated transcription factor that affects chromatin structure

In recent years major advances have been made in our understanding of the mechanisms by which the ligand bound ER activates transcription. ER has two domains involved in transcriptional activation functions (AFs). AF-1 is located in ER's amino-terminal region, while AF-2 is located in ER's carboxyl-terminal, ligand-binding region (150). These domains interact with components of the general transcription machinery and with coactivators that have the ability to modify chromatin structure. Coactivators interacting with the estrogen bound ER ($E_2$-ER) include CBP/p300, SRC-1, and ACTR (also called AIB1, amplified in breast cancer 1) (for review see ref. 20). All of these coactivators are HATs. The recruited HATs would presumably acetylate neighbouring core histones (Fig. 5). Acetylation of core histones results in the relaxation of chromatin structure, probably by interfering with fiber-fiber interactions (20,42). Thus, the $E_2$-ER can interact with a host of proteins that may be components of the general transcription machinery (151) or remodel the chromatin template (20,150, 152), facilitating transcription and the loading of transcription factors onto regulatory DNA elements. It should be noted, however, that there are situations where $E_2$-ER inhibits gene expression (153).

Antiestrogens, such as hydroxytamoxifen (OH-TAM) or ICI 182,780, induce a different conformation in ER's ligand binding domain than does estradiol, resulting in the binding of a different set of cofactors (149,150,154,155). OH-TAM-ER binds to a corepressor complex, consisting of mSin3A,

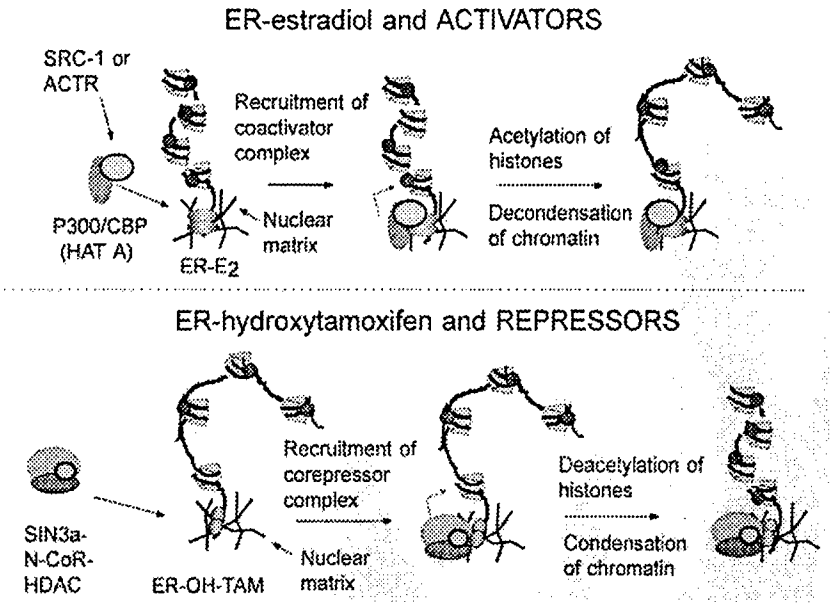


Figure 5. Model for ligand switching of ER activity. $E_2$-ER is shown in the top panel recruiting coactivators with HAT activity, resulting in the acetylation of neighbouring core histones and decondensation of chromatin. Below, OH-TAM is shown recruiting the HDAC complex, resulting in the deacetylation of the core histones and condensation of the chromatin fiber.

N-CoR, SAP30, HDAC1, and HDAC2 (149,156). The HDACs presumably deacetylate neighbouring core histones, resulting in the condensation of chromatin (Fig. 5). Thus, the ligands binding to ER act as molecular switches to alter the structure and function of the ER through its interaction with a battery of proteins.

To initiate these ligand-induced ER activities, the ER must be recruited to the gene's regulatory element, an estrogen response element (ERE). The classic mode of recruitment is by ER binding to a palindromic sequence through its DNA binding domain. However, ER may be recruited indirectly to regulatory DNA elements by binding to another transcription factor (e.g., Sp1) (157). Much of our knowledge of the binding of $E_2$-ER to a consensus ERE is from *in vitro* studies using naked DNA. From these studies, we have learned that both the ERE DNA and ER undergo a conformation change upon binding to each other (158,159). The conformational change that ER undergoes appears to depend upon the ERE sequence, and the resulting ER conformation determines which cofactors it will bind. Liganded ER can bind to the ERE as a dimer or monomer (159,160). Although we have learned much from these studies, it is important to remember that *in vivo* the regulatory DNA sequences of an estrogen responsive gene may be associated with nucleosomes. A recent study shows that $E_2$-ER can bind to the ERE in a nucleosome. An example is the pS2 gene, an estrogen responsive gene in breast cancer cells. Here, nucleosome positioning is key in placing the ERE in the correct position on the nucleosome such that $E_2$-ER can gain access to it (161).

Several studies show that ligand enhances ER binding to an ERE in chromatin (162). However, *in situ* cross-linking studies with formaldehyde suggest that unliganded ER binds to its target sites in chromatin (163). Because formaldehyde causes protein-DNA and protein-protein cross-links, it is not possible to decide whether ER was binding directly or indirectly to DNA through an intermediate DNA-binding protein.

In a perusal of characterized estrogen responsive genes expressed in human breast cancer cells, it is evident that for many of these genes ER acts through the transcription factor Sp1 (157). The Sp1$(N)_x$ERE half-site DNA-binding motif is involved in the estrogen-regulated expression of several genes, including cathepsin D (164), c-*fos* (165), retinoic receptor α (166), and hsp27 (167). The estrogen dependent expression of the c-*myc* gene in hormone dependent breast cancer cells may involve a similar Sp1 mediated recruitment of ER (168,169).

As mentioned above, we recently reported that the ER binds DNA while associated with the nuclear matrix (44). Possibly, ER brings hormone-responsive genes to nuclear matrix sites. Depending on the mode of interaction of the ER with the hormone responsive element and the ligand it is bound to, ER will recruit coactivator complexes with HAT activity or corepressor complexes with HDAC activity. These histone modifying enzymes, which are associated with the nuclear matrix, will induce a remodeling of the three-dimensional structure of chromatin. Thus, while much remains to be uncovered, the ER is an example of the interconnection between transcription factors, chromatin, nuclear structures and gene regulation.

## 8. Summary

In this review we have presented evidence for alterations in the composition and structure of the cell at the levels of chromatin, nuclear matrix and the cytoskeleton in cancer evolution. These different levels of structural organization are all interconnected. Change in the expression of any gene is both a cause and result of a change in its three-dimensional chromatin organization. While changes in the expression of any given gene are not

likely to cause gross morphological changes in the nuclear or cellular structure, changes in cell and nuclear shape are likely to cause alterations in the three-dimensional chromatin structure of genes and therefore affect their regulation. This view is supported by the use of aberrant nuclear shape as a diagnostic marker for cancer.

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# APPENDIX B

*Mol. Biol. of the Cell* (submitted)
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# Direct Visualization of the Human Estrogen Receptor α Reveals a Role for Ligand in the Nuclear Distribution of the Receptor

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## ABSTRACT

The human estrogen receptor α has been tagged at its amino terminus with the S65T variant of the green fluorescent protein, allowing the subcellular trafficking and localization to be observed in living cells by fluorescence microscopy. The tagged receptor, GFP-ER, is functional as a ligand-dependent transcription factor, responds to both agonist and antagonist ligands, and can associate with the nuclear matrix. Its cellular localization was analyzed in four human breast cancer epithelial cell lines, two ER+ (MCF7 and T47D) and two ER- (MDA-MB-231 and MDA-MB-435A), under a variety of ligand conditions. In all cell lines, GFP-ER is observed only in the nucleus in the absence of ligand. Upon the addition of agonist or antagonist ligand, a dramatic redistribution of GFP-ER from a reticular to punctate pattern occurs within the nucleus. In addition, the full antagonist ICI 182780 alters the nucleocytoplasmic compartmentalization of the receptor and causes partial accumulation in the cytoplasm in a process requiring continued protein synthesis. GFP-ER localization varies between cells, despite being cultured and treated in a similar manner. Analysis of the nuclear fluorescence intensity for variation in its frequency distribution helped to establish localization patterns characteristic of cell line and ligand. During the course of this study, localization of GFP-ER to the nucleolar region is observed for ER- but not ER+ human breast cancer epithelial cell lines. Finally, our work provides a visual description of the "unoccupied" and ligand-bound receptor and is discussed in the context of the role of ligand in modulating receptor activity.

## INTRODUCTION

Steroid hormones elicit diverse biological responses, important during growth, differentiation, inflammation, pregnancy, and homeostasis among many other processes. The genomic actions of steroid hormones are mediated by steroid receptors, members of the nuclear receptor superfamily of ligand-dependent transcription factors. In the absence of hormone, steroid receptors exist in a complex with chaperone proteins capable of high affinity binding to steroid hormones. Hormone binding leads to a conformational change in the receptor that results in its dissociation from chaperone proteins and ultimately in the binding of the receptor as a homodimer to cognate sites in steroid-responsive genes (reviewed in Tsai and O'Malley, 1994; Mangelsdorf et al., 1995; Beato et al., 1996).

Immunohistochemistry and biochemical fractionation show the unoccupied steroid receptors to reside predominantly in the cytoplasm, the nucleus, or both compartments, depending on the receptor, in a complex with chaperone proteins (Jensen, 1991; DeFranco et al., 1995; Beato et al., 1996; Pratt and Toft, 1997). For the predominantly nuclear receptors, such as the estrogen receptor, the unoccupied receptor exists in the nucleus either bound or not bound to its cognate site in target genes. Hormone binding leads to activation of the receptor and transcriptional regulation of the responsive genes (Press et al., 1989; Picard et al., 1990; Parker, 1992; Tsai and O'Malley, 1994).

We and others have previously shown that the subcellular localization and trafficking of the glucocorticoid receptor (GR) can be followed with a green fluorescent protein (GFP) fusion (Ogawa et al., 1995; Rizzuto et al., 1996; Carey et al., 1996; Htun et al., 1996). The chimeric receptor GFP-GR can be fully functional as a ligand-dependent transcription factor and shows the ligand specificity of glucocorticoid receptor. The ligand-dependent translocation of GFP-GR from the cytoplasm to nucleus can be observed in real time in a single cell. Most interestingly, we observed an intranuclear pattern and distribution of GFP-GR that reflects the type of ligand, either agonist or antagonist, used to activate the receptor (Htun et al., 1996).

In this report, we have chosen a similar strategy with the human estrogen receptor α, referred to throughout the paper as ER, to see if ligand affects the nuclear distribution of this receptor. Previous biochemical studies described the existence of two biochemically distinct forms of ER (Jensen et al., 1968; Gorski et al., 1968). In the absence of ligand, the "unoccupied" ER with a sedimentation coefficent of 9S is "loosely" associated with the nucleus; ligand causes a biochemical transformation to a complex with a sedimentation of 5S that associates more "tightly" with the nucleus (Greene and Press, 1986; Press et al., 1989; Jensen, 1991). To determine if the biochemical difference is reflected by a change in the intranuclear distribution of

ER, we have directly visualized ER in living cells by tagging the receptor with the S65T variant of the naturally fluorescent protein, GFP.

## MATERIALS AND METHODS

**Cell Lines and Plasmids.** Human breast cancer epithelial cell lines, MCF-7, T47D, and MDA-MB-231, were obtained from ATCC (ATCC, Rockville, MD). MDA-MB-435A is a derivative of MDA-MB-435 (Yee et al., 1996). Unless otherwise noted, cells were maintained in a 225 $cm^2$ cell culture flask (Costar Corp., Cambridge, MA) in 35 ml of Dulbecco's Modified Eagle's Medium (DMEM) with phenol red as pH indicator (Life Technologies, Grand Island, NY; cat. # 11960-044), supplemented with 10% fetal bovine serum (FBS; Life Technologies; cat. # 10437-028), 2 mM L-glutamine, 4.8 ug/ml insulin, and 100 units penicillin G/100 ug streptomycin sulfate per ml at 37°C in a 5% $CO_2$-water jacketed incubator. Medium was changed every 2 days. At confluence, cells were harvested by first washing with Dulbecco's phosphate-buffered saline (D-PBS) without calcium or magnesium and then treating with 0.05% trypsin-0.02% EDTA without phenol red. Cells were reseeded in a fresh flask at about a one-to-four dilution. Four days before transfection, cells were placed in DMEM medium lacking phenol red (Life Technologies; cat. # 31053-028) and supplemented as described above except dextran/charcoal-treated FBS (Hyclone, Logan, UT; cat. # SH30068.03) was used in place of FBS. The reporter gene, pERE-tk-CAT, contains two copies of a perfect estrogen response element in tandem, upstream of the thymidine kinase minimal promoter hooked up to the chloramphenicol acetyltransferase reporter gene (Seiler-Tuyns et al., 1986). The human ER expression vector, pSG5-HEGO (Tora et al., 1989), contains a wild-type human ER under the control of the SV40 early promoter. The GFP-ER expression plasmid, pCI-nGL1-HEGO (GenBank data base; accession no. AF061181), was prepared by first replacing the S65T GFP coding region in the plasmid pCI-nGFP-C656G (Htun et al., 1996) with an S65T GFP coding region optimized for expression in mammalian cells from pGreenLantern-1 (Life Technologies) and then replacing the rat GR with the human ER coding region in pSG5-HEGO, previously mutated with the Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, CA) to introduce a MluI site in the first three amino acids of the ER coding region with the oligonucleotide:

5' TGGTGTGGAGGGTCAACGCGTTGGTCCGTGGCCGCG 3'. To enrich for cells transfected with the expression plasmids, the plasmid, pCMV-IL2R, that expresses the human interleukin-2 receptor (IL-2R), was used in the transfection experiments, as previously described (Htun et al., 1996).

**Transfections.** Plasmid DNA was transiently introduced into cells either by calcium phosphate coprecipitation, electroporation, or liposome-mediated gene transfer. For ERE reporter assays, MDA-MB-435 cells were plated in 100-mm dishes in DMEM media lacking phenol red with 5% twice charcoal-stripped FBS two days prior to transfection and were given fresh media 24 h prior to transfection. Cells were maintained at 37°C in a 5% $CO_2$ incubator prior to transfection. Cells were transfected by the $CaPO_4$ /BES precipitation method (Kingston et al., 1995). One ml of precipitate contained 4 μg of pCH110 (β-galactosidase reporter plasmid) as an internal control, 5 μg of ERE-tk-CAT reporter plasmid, pCI-nGL1-HEGO in amount indicated, and pCEP4 as carrier DNA to a total of 15 μg of DNA. Cells were in contact with precipitate for 14 h, then washed twice with D-PBS and treated with fresh phenol red-free media plus 5% twice charcoal-stripped FBS containing 17β-estradiol, ICI 182780 or 4-hydroxytamoxifen as indicated. Cells were harvested 30 h after the removal of the calcium phosphate precipitates. All transfections were done in triplicate. Chloramphenicol acetyltransferase (CAT) assays (Kingston et al., 1995) and β-galactosidase assays (Sambrook et al., 1989) were done using the method, essentially as described. CAT activity was normalized to β-galactosidase activity, and the mean and standard error of the mean for three sets of data were plotted. For electroporation, cells were electroporated with the indicated amount of pCI-nGL1-HEGO DNA with or without 5 μg pCMV-IL2R DNA for 2 $x10^7$ cells in 0.2 ml of cold phenol red-free DMEM at 250 V and 1100 μF in a 0.4-cm electrode gap electroporation chamber supplied with the Cell-Porator Electroporation System (Life Technologies; catalog number 71600-019), left to recover on ice for 5 min, and then diluted in phenol red-free DMEM supplemented with 10% dextran/charcoal-treated FBS before plating. Cells were then grown for 12-16 h in 37°C, 5% $CO_2$ incubators before imaging. Liposome-mediated gene transfer was used as directed by the manufacturer of DOSPER Liposomal Transfection Reagent (Boehringer Mannheim; catalog number 1 811 169) for nuclear matrix isolation experiments.

**Enrichment of Transfected Cells and Analysis of Total Cell Lysates with Human ER Monoclonal Antibody, H226.** Approximately 18 h after electroporation with 5 μg of pCMV-IL2R and various amounts of pCI-nGL1-HEGO, cells were washed twice with PBS, then sorted with magnetic beads coated with antibody to the IL2 receptor, as previously described (Htun et al., 1996). IL2R positive cells were washed several times and then divided into two

equal aliquots. One aliquot was lysed with a NP40 lysis solution (0.5% NP40, 50 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5 and a cocktail of protease inhibitors) after incubating on ice for 5 min and then centrifuged for 5 min in a microfuge to remove the insoluble debris. The protein concentration in the soluble extract was determined by the Bradford method (Bradford, 1976) using a Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). The other half of the cells was used to prepare total cellular lysate with 1x SDS gel-loading buffer (50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue, 10% glycerol; Sambrook et al., 1989) in the same volume as the NP40 lysis solution. Based on the protein concentration, a volume equivalent to 30-40 μg was removed from the total cellular lysate, heated to 90°C for 5 min, subjected to denaturing polyacrylamide gel electroporesis in 8% polyacrylamide (30 acrylamide:1 bisacrylamide) containing 0.1% SDS, and analyzed by the Western blotting method, essentially as described previously (Sambrook et al., 1989), using a rat monoclonal antibody, H226, raised against the human estrogen receptor α (a gift from Geoffrey Greene) as a primary antibody and donkey anti-rat antibody conjugated to horseradish peroxidase as a secondary antibody with the Pierce Super Signal chemiluminescent substrate (Pierce, Rockford, IL).

**Extraction of Nuclear Matrix.** A modified procedure was used for the extraction of nuclear matrix (Belgrader et al., 1991; Berezney, 1991; Jackson and Cook, 1988). Nuclei from frozen MCF-7 cell pellets, that had been transiently transfected with 15 ug pCI-nGL1-HEGO HEGO in 90 ul DOSPER liposomal transfection reagent (Boehringer Mannheim) for $1x10^6$ cells and grown with DMEM media in CSFBS without phenol red, were resuspended in TNM buffer (10 mM Tris pH 8.0, 300 mM sucrose, 100 mM NaCl, 2 mM $MgCl_2$, 1% v/v thiodiglycol, 1 mM PMSF) and homogenized three times in a Dounce homogenizer with a teflon pestle. Triton-X 100 was added to a final concentration of 0.5% v/v, and nuclei were collected by centrifugation at 500 x g for 10 min at 4° C. Nuclei were then resuspended in TNM buffer, homogenized and pelleted as described above. The isolated nuclei were then resuspended to a concentration of 20 $A_{260}$/ml in DIG buffer (10 mM Tris, pH 8.0, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1% v/v thiodiglycol, 1mM PMSF), and digested with 168 units/ml of DNase I (Sigma D5025) for 20 min at room temperature. Ammonium sulphate was added to a final concentration of 0.25 M, and nuclear matrix was collected by centrifugation. The pellet NM-IF1 was then resuspended in DIG buffer, followed by the addition of an equal volume of 4 M NaCl. The nuclear matrix was then collected by centrifugation and re-extracted with 2 M NaCl as above. The final pellet NM-IF2 was resuspended in 1x SDS gel-loading buffer, as previously described . Supernatants from the 0.25 M ammonium sulphate and 2 M NaCl extractions were dialysed overnight against

$ddH_2O$, 1 mM PMSF, and then lyophilized. All four samples were subjected to denaturing polyacrylamide gel and Western blot analyses, essentially as described above, using an antibody (BabCo, Berkeley, CA) against the HA tag (YPYDVPDYA) at the amino-terminus of GFP-ER.

**Microscopy**. For differential interference contrast, the cells electroporated with only pCI-nGL1-HEGO were grown on glass coverslips overnight. The coverslips were rinsed with D-PBS and placed inverted on a microscope slide. Cells were illuminated by white light from a tungsten light source and viewed under a 63X/1.4 NA Plan-Apochromat oil-immersion objective in a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) under Nomarski optics. For green fluorescence, the same cells were examined through a 480 to 490-nm excitation, a 510-nm dichroic mirror, and a 515 to 565-nm emission filter using a 100-watt mercury bulb light source. Images were recorded on Kodak Elite 200 color slide film. In the case of confocal laser scanning microscopy, cells were imaged through a 100X/1.4 NA Plan-Apochromat oil-immersion objective by excitation with the 488 nm line from a krypton-argon laser and the emission was viewed through a 506-538 nm band pass filter. Images were collected on a Zeiss Axiovert 135 platform attached to a Bio-Rad MRC 1024 Confocal Imaging System using Bio-Rad LaserSharp software (Bio-Rad, Hercules, CA).

**Image Analysis.** Image analyses and representation in Figure 2 were performed on a Power Macintosh 8600/200 computer using the public domain NIH Image program version 1.61 (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). To generate Table 1, additional image analyses were performed by determining mean pixel value and standard error for each area containing a nucleus but excluding the nucleoli using IPLab Spectrum software (Signal Analytics Corp., Fairfax, VA) operating on a Power Macintosh 8600/200. To obtain the coefficient of variation for fluorescent intensity, the standard deviation of the pixel values for each nucleus was divided by the mean pixel value. The mean of the coefficient of variation for the population and the standard deviation were determined for each set of cells exposed to the same ligand and are provided in Table 1. Statistical significance was determined using the "z test" (Chase and Bown, 1992).

## RESULTS

**Expression and Functional Analysis of the GFP-ER Fusion Protein.** To follow the subcellular localization and trafficking of the ER in living cells, we tagged the amino terminus of the human estrogen receptor alpha (ER) with the S65T variant of GFP. Figure 1A shows the CMV enhancer/promoter-driven, GFP-ER expression vector. Functionality of GFP-ER as a ligand-dependent transcription factor was assayed on an estrogen-response-element-containing reporter gene in transient transfection experiments in a human breast cancer epithelial cell line, MDA-MB-435A, which lacks endogenous ER (Yee et al., 1996). GFP-ER activates the reporter gene in a dose-dependent manner and shows additional activation in the presence of agonist ligand, 17β-estradiol (Figure 1C). Maximal activation of GFP-ER by 17β-estradiol was observed at 10 nM concentration of ligand (Figure 1D; lanes 1-5), consistent with the previously reported subnanomolar $K_d$ for ER (Kuiper et al., 1997). ICI 182780, a pure antagonist for ER (Wakeling et al., 1991), completely inhibited GFP-ER activation of the reporter gene (Figure 1D, compare lanes 1 and 6). When ER antagonists were present in 25- to 50-fold molar excess, the action of 17β-estradiol was inhibited, albeit the pure antagonist, ICI 182780 compound, was more effective than the partial antagonist, 4-hydroxytamoxifen (Figure 1D, compare lanes 4 and 8; lanes 4 and 9). Thus, GFP-ER functions as a transcriptional activator, and it's activity is fully regulated by ER ligands.

Presence of the GFP tag results in a receptor that is approximately 27 kilodaltons larger than the untagged ER. An immunoblot using a rat monoclonal ER antibody, H226, of total cellular lysates prepared from MCF-7 cells transfected with GFP-ER expression vector, pCI-nGL1-HEGO, shows the presence of a protein with a molecular mass of about 94 kilodaltons, the expected molecular mass of the fusion protein (Figure 1B). Furthermore, from the width and intensity of the band, inclusion of a greater amount of GFP-ER expression plasmid in a transfection results in more fusion protein being expressed in the transfected cells.

Previous investigations have shown that unliganded ER associates loosely with the nucleus but that the liganded ER associates more tightly with the nucleus (Greene and Press, 1986; Press et al., 1989; Jensen, 1991). Several studies have also suggested that steroid receptors are associated with the nuclear matrix (Barrack and Coffey, 1980; Alexander et al., 1987; Samuel et al., 1998). In addition, transcribing chromatin has been reported to be selectively associated with the nuclear matrix (Davie, 1995; Davie et al., 1997). These considerations led us to examine the potential association of GFP-ER with the matrix. Nuclear matrix was prepared from MCF-7 cells transfected with the GFP-ER expression plasmid. Figure 1E shows the result of two successive 2 M NaCl extractions of a nuclear matrix preparation from MCF-7 cells whose nuclei have been digested with DNase I and extracted with 0.25 M ammonium sulphate. From

the Western blot analysis using an anti-HA antibody to detect the hemagglutinin (HA)-epitope at the amino-terminus of GFP-ER, the GFP-ER that remains associated with the initial nuclear matrix preparation is tightly bound to the matrix and resistant to 2 M NaCl extraction (compare lanes 1-3 with lane 4). Not shown is the initial cytosolic fraction, which contains soluble nuclear protein and significant amounts of GFP-ER. Comparable results were obtained when cells were grown in the presence of estrogens (data not shown). These findings are consistent with the earlier description of ER as a cytosolic protein based on biochemical fractionation experiments (Jensen et al., 1968; Gorski et al., 1968; Greene and Press, 1986; Press et al., 1989; Jensen, 1991) as well as a nuclear matrix-binding protein (Barrack and Coffey, 1980; Alexander et al., 1987).

**Cellular Localization of GFP-ER.** To determine the localization of the tagged ER, we examined various human breast cancer epithelial cell lines transfected with the GFP-ER expression vector. In particular, we were interested in the effect of ligand as well as cellular structure and milieu on GFP-ER subcellular localization in the ER-positive (ER+) and ER-negative (ER-) cell lines. Four representative human breast cancer epithelial cell lines were examined--two ER+ (MCF-7 and T47D) and two ER- (MDA-MB-231 and MDA-MB-435A). Green fluorescence can be detected by conventional fluorescent microscopy in MCF-7 cells, following electroporation with the GFP-ER expression plasmid, indicating that the GFP chromophore in the fusion protein is functional (Figure 2B). Comparison with the differential interference contrast image shows green fluorescence to be restricted to the nucleus of a few cells successfully transfected with the GFP-ER expression plasmid (indicated by white arrows in Figure 2A; also, compare Figure 2A and 2B). In the cells with a lower level of green fluorescence, nucleolar outlines are observed, which is consistent with GFP-ER being excluded from the nucleolus (Figure 2B; two left most nuclei and three lower nuclei). Thus, GFP-ER localization is consistent with that previously reported for the endogenous ER in MCF-7 cells (King and Greene, 1984; Welshons et al., 1988).

Inclusion of ten nanomolar ligand, either agonist 17β-estradiol (Figures 2C and D) or partial antagonist 4-hydroxytamoxifen (Figures 2E and F), during the time of transfection and culturing of the cells for 20 h had no apparent effect on the nucleocytoplasmic compartmentalization of GFP-ER. In contrast, when GFP-ER expressing cells were treated with 10 nM ICI 182780, a pure ER antagonist, green fluorescence was observed not only in the nucleus but also in the cytoplasm (Figures 2G and H). This effect of ICI 182780 on the nucleocytoplasmic compartmentalization of GFP-ER is similar to that previously reported for the untagged ER (Dauvois et al., 1993).

Although a similar effect of ligand on GFP-ER nucleocytoplasmic compartmentalization was observed in T47D, MDA-MB-231, and MDA-MB-435A cells (data-not-shown), we

observed variability in the proportion of cells showing cytoplasmic green fluorescence after overnight treatment with 10 nM ICI 182780. In T47D and MCF-7 cells, cytoplasmic green fluorescence was seen in a minority of the cells within 30 min of 10 nM ICI 182780 treatment and peaked at 6 to 8 h with about ninety percent of the cells showing some degree of cytoplasmic fluorescence (e.g., Figures 3B). In the case of the ER- cell lines, cytoplasmic accumulation was observed in about ten percent of the population despite prolonged treatment (20 h) with 10 nM ICI 182780 (data not shown). However, in no case did total cytoplasmic green fluorescence exceed nuclear fluorescence (e.g., Figures 2H and 3B).

**Effect of Cycloheximide on ICI 182780-Induced Cytoplasmic Accumulation of GFP-ER.** The protein synthesis inhibitor, cycloheximide, was used to block protein synthesis and its effect on cytoplasmic accumulation of GFP-ER was examined following 10 nM ICI 182780 treatment. In MCF-7 cells treated with 200 μg/ml cycloheximide for a period of 20 h immediately following electroporation, no green fluorescence was seen (compare Figures 3C and D with no cycloheximide treated cells in Figures 2A and B), indicating that no GFP-ER was synthesized in the cycloheximide-treated cells. In contrast, allowing protein synthesis to continue for the first 12 h after transfection, and then halting it for 8 h through the addition of cycloheximide to the culture medium showed green fluorescence in the nucleoplasm but not in the nucleolus (Figures 3E and F). Simultaneous inclusion of both cycloheximide and 10 nM ICI 182780 12 h after transfection but 8 h before microscopy failed to reveal any cell with cytoplasmic green fluorescence (Figures 3G and H). Since absence of the protein synthesis inhibitor results in cytoplasmic accumulation of GFP-ER in the presence of ICI 182780 (Figures 3A and B), continued protein synthesis appears to be required for ICI 182780-induced cytoplasmic accumulation of GFP-ER.

**Confocal Laser Scanning Microscopic Examination of GFP-ER Nuclear Distribution.** The ability of ICI 182780 to affect the cytoplasm-nucleus partitioning of GFP-ER suggests that other ER ligands might affect the localization of the receptor. In particular, although no change in the nuclear versus cytoplasmic compartmentalization of GFP-ER was observed for cells treated with either 17β-estradiol or 4-hydroxytamoxifen (compare Figure 2B with Figures 2D and 2F), the localization of GFP-ER might have been altered within the nucleus. To determine whether changes occurred in the nuclear localization of GFP-ER upon the addition of ligand, the GFP-ER-expressing MCF-7 cells were examined by high resolution fluorescent microscopy using a confocal laser scanning microscope.

Figure 4A shows an optical section of four live MCF7 cells grown in the absence of ligand, obtained through a confocal laser scanning microscope. Note the increased resolution of the images (also other images in Figures 4 and 5) over the conventional epifluorescent images in

Figures 2 and 3. Despite differences in the overall level of brightness attributable to cell-to-cell variation in the level of GFP-ER expression (e.g., top left-hand corner compared to the adjacent nucleus or the two right-most pair of nuclei), the tagged receptor is found to be present in a reticular pattern evenly distributed throughout the nucleus. It is, however, excluded from the nucleoli. In contrast, treatment with 10 nM 17β-estradiol, 4-hydroxytamoxifen or ICI 182780 for 1 h leads to the redistribution of the receptor, resulting in the nucleus appearing punctate and highly structured (Figures 4B-D). Note that the ICI 182780-treated cells (1 h) failed to show cytoplasmic GFP-ER, since at this time point, few cells accumulate GFP-ER in the cytoplasm (Figure 4D and also Figures 5D, H, L and data-not-shown). This result is in contrast to the cytoplasmic accumulation seen after an overnight treatment (Figure 2H). Thus, intranuclear localization of the estrogen receptor is altered by treatment with ER ligands (Figures 4B-D).

**Representation of Confocal Laser Scanning Microscopic Images.** Since the confocal laser scanning microscope records images in a digital format, these images can be analyzed by a number of procedures. Figures 4E and F are three-dimensional, colored surfaces in which height and color are used to represent the light intensity in the panel corresponding to the untreated or 17β-estradiol-treated cells (Figures 4A and B). For example, the brightest nucleus in the untreated sample and the two brightest in the 17β-estradiol-treated sample are represented by the tallest nuclei whose surface color either approaches a blue color or is blue in Figures 4E and F, respectively.

The fluctuation in light intensity is readily apparent when a perpendicular slice through the surface plot of the optical section for the cell is made and examined. The left diagram in Figure 4G shows a slice from an untreated nucleus (green line; Figure 4A), while the right diagram shows a slice from a 17β-estradiol-treated nucleus (red line; Figure 4B). In tracing along the green-colored line from left-to-right in Figure 4A and examining the left diagram in the same manner for Figure 4G, it is clear that the fluorescence intensity is zero outside of the nucleus. As the line enters the nucleus, the fluorescence intensity value suddenly rises, remains fairly constant in the nucleus except where it drops dramatically at the nucleolus, which is a little over half way into the nucleus. The fluorescent intensity again recovers outside the nucleolus, and subsequently drops to the baseline upon exiting the nucleus. A similar trend is seen for the slice of the 17β-estradiol-treated nucleus indicated in Figure 4B (right diagram); however, in this case, dramatic changes in the fluorescent intensity values are seen for the nuclear region excluding the nucleolus. Such changes mirror the punctation observed for the 17β-estradiol-treated nuclei (Figure 4B); in contrast, minor fluctuation in fluorescent intensity values in the slice of the untreated nucleus (left diagram) reflects the more even, reticular distribution of GFP-ER observed in Figure 4A.

**Quantitative Analysis of the Effect of Ligand on GFP-ER Nuclear Distribution.** To quantitatively compare the magnitude of fluctuation in fluorescence intensity, the coefficient of variation was determined from the mean and standard deviation of the fluorescence intensity for a segment of each line traversing the nucleus (the portion under the two black bars in Figure 4G). In the case of the untreated sample, the mean fluorescence intensity was 66 with a standard deviation of 12.3; whereas, the 17β-estradiol sample had a mean of 103 with a standard deviation of 35.4. Normalization of the standard deviation by the mean yielded the coefficient of variation, which allows direct comparison of the magnitude in the variation of the fluorescence intensity for samples with different mean values. Indeed, the coefficient of variation was nearly two-fold higher for the 17β-estradiol-treated sample than for the untreated sample (0.344 versus 0.186), indicating a substantial fluctuation in fluorescence intensity for the 17β-estradiol-treated cells than untreated cells.

To determine whether a statistically significant difference exists in the coefficient of variation following ligand treatment, the mean coefficient of variation was determined from a large population of nuclei treated in a similar manner. In the current instance, the coefficient of variation was computed in a similar manner as described in the earlier example, but now encompassing the entire nucleus in the optical section minus the nucleolus. From the coefficient of variation, a mean coefficient of variation along with its standard deviation was calculated for each population treated in a similar manner. As seen in Table 1, the mean coefficient of variation for 164 MCF-7 cells not treated with ligand is 0.225; while that for 82 MCF-7 cells treated with 17β-estradiol is 0.365. Statistical "z test" gives a value greater than 26. This value indicates a chance of less than one in ten million that these two means would show such a difference on the basis of chance alone, and thereby establishes a high degree of statistical significance to these results (Chase and Bown, 1992). Similarly, treatment with 4-hydroxytamoxifen or ICI 182780 resulted in a 1.4-fold increase in the mean coefficient of variation, which is also a high statistical difference from the untreated sample.

Nuclear distribution of GFP-ER was also analyzed in another ER+ cell line, T47D, and two ER- cell lines, MDA-MB-231 and MDA-MB-435A, to determine if the 1 h ligand treatment also caused a statistically significant redistribution of GFP-ER. In each case, ligand treatment results in an increase in the mean coefficient of variation (compare left-most number with others in the row in Table 1) to a value which would occur by chance in less than one out of ten million cases. Thus, for each cell line, a significant redistribution of GFP-ER occurs upon treatment with ligand (compare first panel to other panels in each row for Figure 5).

**Ligand- and Cell Line-Specific Distribution of GFP-ER in the Nucleus.** While it is apparent that no two GFP-ER expressing cells look identical (compare the nuclear appearance of the

group of cells in each panel in Figures 4 and 5), each cell line has a characteristic distribution of GFP-ER and responds in a characteristic manner to ligand. For example, in the absence of ligand, GFP-ER is distributed evenly in a reticular pattern in MCF-7 cells (Figure 3A). However, deviation from this pattern occurs in T47D, MDA-MB-231, and MDA-MB-435A cells, resulting in a small but statistically significant increase in the mean coefficient of variation (compare the value for MCF-7 with others in the first column of Table 1). In the case of T47D cells, GFP-ER can be seen to concentrate at a few nuclear sites over a reticular background (more evident for the brighter nucleus in Figure 5A). The two other cell lines show an uneven distribution of GFP-ER. In the case of the MDA-MB-231 cell line, the unevenness results in the left and lower left half of the nucleus being brighter than the other half (Figure 5E). For MDA-MB-435A, the unevenness is very apparent from the "patchy" GFP-ER patterns within the nucleus (Figure 5I).

Despite the fact that GFP-ER undergoes a dramatic redistribution following ligand treatment, the magnitude of the change differs between different cell lines as each cell line responds in a characteristic manner to ligand. 17β-estradiol treatment leads to a high degree of punctation in the distribution of GFP-ER for MCF-7 and T47D cells, less so in MDA-MB-231 cells, and least in MDA-MB-435A (Table 1). The extent of punctation observed for MCF-7 is greater for 17β-estradiol than for the antagonists, but for T47D, it is not significantly different. While the GFP-ER patterns for the two antagonists are significantly different for MDA-MB-231 cells (probability of less than 1 in 100 that the mean coefficients of variation are those in Table 1), these patterns appear not to be significantly different for MDA-MB-435A cells.

**GFP-ER Presence in the Nucleolar Region.** In the two ER- cell lines, expression of GFP-ER results not only in its accumulation within the nucleus, but also in its presence within the nucleolar region (Figures 5E-G, I, J, and L). This is most evident for the MDA-MB-435A cells. The extent of "nucleolar" accumulation differs between different cells in the population, independent of ligand treatment. Thus, unlike the ER+ cell lines, GFP-ER can accumulate in the nucleolar region of ER- cells.

## DISCUSSION

Early attempts at localization of the estrogen receptor by biochemical fractionation led to the two-step model of steroid hormone action. Binding of steroid by cytosolic steroid hormone receptor leads to its transformation and subsequent translocation to the nucleus where it regulates gene expression (Jensen et al., 1968; Gorski et al., 1968). This view was revised when the human estrogen receptor was shown to be in the nucleus independent of ligand by immunocytology (King and Greene, 1984) and hormone-binding assays of cytoplast and nucleoplast fractions from cytochalasin B-induced enucleation of intact cells (Welshons et al., 1984). Steroid hormone transformed a "loosely" bound nuclear ER to a more "tightly" bound nuclear form, which regulated gene expression (Greene and Press, 1986; Press et al., 1989; Jensen, 1991). However, attempts at defining these two biochemically distinct states of ER by immunocytology failed to reveal any notable difference in the intranuclear localization of the "loosely" and "more tightly" bound forms of ER (Press et al., 1985; Vazquez-Nin et al., 1991; Yamashita, 1995).

In our current work, we have revisited this issue of the role of ligand in ER localization, using a direct visualization approach in living cells based on GFP-tagging. We have previously shown this approach to be extremely useful for observing the subcellular localization of the glucocorticoid receptor. Using the GFP-tagging approach, we saw for the first time differences in the intranuclear distribution of the receptor that reflected the type of ligand, agonist or antagonist, bound to the receptor. This work represents the first report of the importance of ligand or signal in affecting the distribution of a steroid hormone/nuclear receptor within the nucleus (Htun et al., 1996).

When ER was tagged at its amino terminus with GFP, the tagged receptor is functional by a number of criteria. First, the receptor is capable of transcriptional activation of estrogen response element containing reporter gene. Second, GFP-ER responds to ligands, ER agonist or antagonist, similar to ER. Third, GFP-ER is nuclear in the absence of any added ligand, as has been reported for ER. Fourth, the pure antagonist ICI 182780 causes partial cytoplasmic accumulation of GFP-ER, as has been reported for ER (Dauvois et al., 1993). Finally, GFP-ER can associate with the nuclear matrix, similar to that reported for the wild type ER (Barrack and Coffey, 1980; Alexander et al., 1987; Samuel et al., 1998).

Using this GFP-ER, we find the receptor to be distributed in a reticular pattern in the absence of ligand. This pattern suggests that the majority of the unliganded GFP-ER is not freely diffusing in the nucleus but is rather associated with some nuclear meshwork present throughout the nucleus. Ligand causes a dramatic redistribution of the receptors to numerous nuclear sites, giving a punctate nuclear pattern. These two distinct nuclear localization patterns provide the

first visual evidence of the changes in receptor activity by hormone and may reflect the "loose" and "tighter" binding nuclear forms of ER (Greene and Press, 1986; Press et al., 1989; Jensen, 1991). These findings also complement our earlier work with the glucocorticoid receptor, which demonstrated the importance of ligand on the nucleocytoplasmic compartmentalization and intranuclear distribution of the receptor (Htun et al., 1996). Despite the fact that the current work was performed on human breast cancer epithelial cells, the nuclear redistribution of ER is expected to be a general feature of all ER containing cells and is likely a consequence of hormone-dependent transformation of ER from a "loose" to "tighter" nuclear form.

Closer examination of the various human breast cancer epithelial cell lines shows a cell line-specific intranuclear distribution of GFP-ER. Small but significant differences in GFP-ER localization can be observed in the absence of ligand. In MCF-7 cells, GFP-ER can be seen to localize in a reticular pattern evenly distributed throughout the nucleus, excluding the nucleolus (Figure 4A). This is not so for T47D cells where a composite pattern emerged due to the accumulation of the receptor in a reticular pattern and to a low level of concentration at numerous nuclear sites present throughout the nucleus (Figure 5A). Despite this alteration in the nuclear pattern, comparison of different portions of the nucleus in these ER+ cells show no remarkable difference in the fluorescent intensity and localization patterns, indicative of an even, intranuclear distribution of GFP-ER. In contrast, ER- cell lines lack this even distribution. In MDA-MB-231 cells, the receptor is slightly more abundant on one half over the other half of the nucleus (Figure 5E); whereas, in MDA-MB-435A cells, the unevenness in GFP-ER distribution results in a "patchy" nuclear appearance (Figure 5I). Thus, each cell line shows a characteristic distribution of GFP-ER in the absence of ligand.

In comparing the confocal laser scanning microscopic images of GFP-ER-expressing cells, it is clear that no two nuclei show an identical distribution of GFP-ER despite the cells being treated and handled in a similar manner (compare nuclei within each panel in Figures 4 and 5). Differences in the phase of the cell cycle, physical characteristics, local external environment, and stochastic nature of some biological regulatory processes may contribute to a unique nuclear appearance. To describe the distribution of GFP-ER in a quantitative manner, the coefficient of variation was determined from the frequency distribution of fluorescence intensity. While the coefficient of variation analysis ignores the precise spatial organization of GFP-ER, changes in the GFP-ER patterns are often accompanied by changes in the size and number of GFP-ER clusters and hence the frequency distribution of fluorescence intensity. Under most circumstances, the coefficient of variation will be different, and the significance of this difference can be addressed statistically. However, in cases where the values of the coefficient of variation are similar, direct visual assessment is required to address the issue of similarity or difference in

the GFP-ER patterns. Thus, the coefficient of variation serves as an indirect measure of the spatial distribution of GFP-ER through its effect on the frequency distribution of fluorescence intensity.

When cells not exposed to ER ligand were examined, a reticular pattern can be observed throughout the nuclear volume excluding the nucleolus. However, subtle differences exist (Figures 4A, 5A, 5E, and 5I) that can be evaluated quantitatively and analyzed statistically. When the coefficient of variation was determined from the nuclear fluorescence but excluding the nucleolus, the mean coefficient of variation was smallest for MCF-7 cells, which showed an even and reticular distribution of GFP-ER (Table 1; see Figure 4A). The mean coefficient was largest for the cell line, MDA-MB-435A, which deviated furthest from this distribution as evident from the "patchy" appearance of the nuclei (Table 1; see Figure 5I). Intermediate values were obtained for the untreated T47D and MDA-MB-231 cells, which had a low level of deviation from the MCF-7 nuclear pattern (Table 1; see Figures 5A and E). Heterogeneity in the observed distribution of GFP-ER is partly reflected by the standard deviation, as indicated in Table 1. From the mean and standard deviation, the statistical "z-test" established the statistical significance of the difference in the mean coefficient of variation among the different cell lines, except between T47D and MDA-MB-231, and hence the existence of a unique and characteristic GFP-ER distribution pattern, at least on the average. For T47D and MDA-MB-231, the mean coefficient of variation is similar; however, examination of the confocal sections have shown differences in the nuclear patterns between T47D and MDA-MB-231 cells, as discussed earlier. Thus, to a first approximation, the mean coefficient of variation for the most part adequately summarizes the different GFP-ER localization patterns and helps define a characteristic nuclear distribution of GFP-ER for each cell line.

In the presence of ligand, GFP-ER redistributes within the nucleus. Analysis of the number of cells, indicated within parentheses in Table 1, shows that for all ligands, the greatest redistribution occurs in T47D cells, and the least, in MDA-MB-435A cells. The response in MCF-7 and MDA-MB-231 cells is intermediate and ligand-dependent. In MCF-7 cells, 17β-estradiol caused a greater redistribution than 4-hydroxytamoxifen, which elicited a similar response as ICI 182780. However, in MDA-MB-231 cells, both 17β-estradiol and ICI 182780 caused a similar but greater change than 4-hydroxytamoxifen. Thus, each cell line not only has a characteristic GFP-ER distribution pattern but responds in a characteristic manner to ER ligands.

Responsiveness of human breast cancer to hormonal therapy correlates well with ER status in which up to 60% of ER+ tumors respond to anti-estrogen therapy, in contrast to 10% of ER- tumors (Allegra et al., 1980; Samaan et al., 1981; Williams et al., 1987). Interestingly, treatment of ER- cells, made to express exogenous ER, with 17β-estradiol inhibited cell growth

and proliferation, contrary to what is normally observed in ER+ cells (Garcia et al., 1992; Jiang and Jordan, 1992; Zajchowski et al., 1993; Levenson and Jordan, 1994). This differential response to the activation of ER by 17β–estradiol suggests important differences in cellular and/or nuclear content and/or structure that affects ER function. Comparing GFP-ER localization between the ER+ and ER- cell lines revealed three characteristic differences. First, GFP-ER is distributed more evenly throughout the nuclear volume minus the nucleolus in ER+ than ER- cells. Second, ER- cells had GFP-ER in the nucleolar region unlike ER+ cells, more so in MDA-MB-435A than MDA-MB-231 cells. Third, following 6-8 h treatment with ICI 182780, GFP-ER is seen in the cytoplasm of about 90% of the ER+ cells but only 10% of ER- cells. Thus, the cell line variations in the subcellular localization of GFP-ER demonstrate that ER function is affected by cellular, nuclear or structural differences. The importance of this alteration on the receptor's role in inhibiting the growth and proliferation of ER- cells, transfected with ER expression vector, remains to be elucidated.

Dauvois et al. (1993) proposed ER to constantly shuttle between the nucleus and the cytoplasm in spite of its predominantly nuclear location. They further reported that nuclear uptake was energy dependent and that ICI 182780 disrupted this process resulting in the accumulation of ER in the cytoplasm. We observed that the ability of ICI 182780 to cause cytoplasmic localization of GFP-ER was prevented when breast cancer cells were incubated with both ICI 182780 and cycloheximide, a protein synthesis inhibitor. Possible explanation for this observation is that ICI 182780 prevents the nuclear uptake of newly synthesized GFP-ER. Alternatively, a labile protein factor could be required for ER to be exported from the nucleus to the cytoplasm. Variation in the abundance of this labile factor could account for the observed differences between the rate of cytoplasmic accumulation of GFP-ER in ER+ breast cancer epithelial cells and mouse ER in COS-1 cells (Dauvois et al., 1993), and absence of this labile factor in 90% of the ER- cells might explain why these cells failed to show cytoplasmic green fluorescence following ICI 182780 treatment.

From our current work, it is clear that the effectiveness of ICI 182780 as an antagonist is independent of its ability to induce cytoplasmic accumulation of GFP-ER. In the ER+ human breast cancer epithelial cells, most of the GFP-ER remained in the nucleus. In an ER- human breast cancer epithelial cell, MDA-MB-435A, ICI 182780-treatment effectively suppressed GFP-ER transcriptional activation of a reporter gene; however, only 10% of these cells showed any GFP-ER accumulation in the cytoplasm. Thus, the mechanism of ICI 182780 antagonism does not depend on the nuclear-cytoplasmic re-compartmentalization of the receptor, but rather must occur within the nuclear compartment at a step required for activation of transcription by ER.

Previous studies have shown that in the absence of hormone, steroid receptors are thought not to be bound to hormone-responsive elements in target genes (Wijnholds et al., 1988; Kumar and Chambon, 1988; Pham et al., 1991a; McDonnell et al., 1992; and also reviewed in Tsai and O'Malley, 1994; Shibata et al., 1997). Hormone binding causes a conformational change in the receptor that allows it to bind to its cognate sites and regulate gene transcription. Agonist ligands induce a receptor conformation that can interact with the general transcription factors or transcriptional co-activators to establish a productive transcriptional preinitiation complex. Antagonist ligands, on the other hand, induce a different receptor conformation that interfere with the ability of the receptor to bind to DNA or alternatively prevent formation of a productive transcriptional preinitiation complex by abrogating its interaction with the general transcription factors and/or transcriptional co-activators (Martinez and Wahli, 1989; Sabbah et al., 1991; McDonnell et al., 1991; Pham et al., 1991b; Tsai and O'Malley, 1994; McDonnell et al., 1994; McDonnell et al., 1995; Mymryk and Archer, 1995; Vegeto et al., 1996; Shibata et al., 1997; Brzozowski et al., 1997; Gallo and Kaufman, 1997). Alternatively, antagonist ligands might promote receptor interaction with transcriptional co-repressors to actively maintain a repressed transcriptional state (McDonnell et al., 1992; McDonnell et al., 1994; Smith et al., 1997; Zhang et al., 1998; Lavinsky et al., 1998). In the case of ER antagonists, antagonism appears to occur at a step subsequent to the binding of the receptor to the estrogen response elements of target genes than at the actual step of DNA-binding (Martinez and Wahli, 1989; Sabbah et al., 1991; McDonnell et al., 1991; Pham et al., 1991b; McDonnell et al., 1992; McDonnell et al., 1994; McDonnell et al., 1995; Vegeto et al., 1996; Shibata et al., 1997; Brzozowski et al., 1997; Gallo and Kaufman, 1997). Our visual data favor a model of 4-hydroxytamoxifen and ICI 182780 antagonism in which ER binds to the hormone-response elements of target genes but it's ability to activate transcription is partially or completely abolished, respectively. In support of this assertion, we find that, qualitatively, the punctate pattern observed after 4-hydroxytamoxifen or ICI 182780 treatment is no different from the pattern observed after 17β-estradiol treatment in the four human breast cancer epithelial cell lines. Quantitatively, in the case of T47D cells, no significant difference in the mean coefficient of variation can be established following 4-hydroxytamoxifen, ICI 182780 or 17β-estradiol treatment (Table 1), suggesting a similarity in the number of nuclear sites to which the ligand-bound receptor accumulates. While we favor these sites of GFP-ER concentration to be regions high in the concentration of estrogen response elements, we cannot rule out the possibility that these sites might be ER processing sites, storage sites, or sites of interaction with the nuclear matrix.

Lastly, during mouse embryonic development, ER has been reported to preferentially accumulate within the nucleolar region of a specific subset of cells (Hou et al., 1996). Since this

re-compartmentalization is observed during the ontogeny of ER- from ER+ cells, it has been suggested that this re-compartmentalization might be a consequence of the mechanism involved in ER down regulation. In this regard, we have found ER- human breast cancer epithelial cells to show GFP-ER not only in the nucleus, but also in the nucleolar region (Figures 4 and 5). The presence of GFP-ER in the nucleolar region of ER- human breast cancer epithelial cells further suggests the importance of ER localization for its function and points to parallel regulatory mechanisms governing ER localization during both early mouse embryonic development and human mammary gland development.

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## TABLE.

**Table 1.** Mean coefficient of variation and its standard deviation obtained from the frequency distribution of nuclear fluorescence intensity for a population of cells treated in a similar manner.

| | None | 17β-Estradiol | 4-Hydroxy-tamoxifen | ICI 182780 |
|---|---|---|---|---|
| MCF7 | 0.225±0.042(164) | 0.365±0.068(82) | 0.329±0.059(108) | 0.326±0.057(109) |
| T47D | 0.245±0.050 (76) | 0.365±0.060(60) | 0.370±0.084 (56) | 0.373±0.058 (34) |
| MDA-MB-231 | 0.245±0.040(118) | 0.353±0.057(78) | 0.315±0.078 (54) | 0.342±0.074 (49) |
| MDA-MB-435A | 0.257±0.040 (84) | 0.301±0.040(53) | 0.287±0.042 (44) | 0.294±0.041 (35) |

Coefficient of variation is determined from the frequency distribution of the pixel intensity within each optical section of a nucleus but ignoring the nucleolus, using IPLab Spectrum software (Signal Analytics Corps., Fairfax, VA). From the coefficient of variation, the mean and its standard deviation were determined for the number of nuclei indicated within parenthesis and are separated by the ± sign, respectively.

## FIGURE LEGENDS.

**Figure 1. Construction and characterization of GFP-ER.**

**(A) Map of GFP-ER expression plasmid, pCI-nGL1-HEGO.** The human wild-type ER is fused to the carboxyl terminus of a S65T variant of GFP whose codons have been optimized for translation in mammalian cells. A small linker region containing five glycine-alanine repeats separates the two coding regions. The S65T GFP is tagged with a $(his)_6$ and hemagglutinin epitope (HA) at the amino terminus. Amino acids present in the fusion protein are indicated in the open boxes for GFP and ER.

**(B) Expression of GFP-ER in MCF-7 cells.** MCF-7 cells were electroporated with the indicated amount of GFP-ER expression plasmid pCI-nGFL1-HEGO and 5 μg of interleukin-2 receptor expression plasmid pCMV- IL-2R and allowed to express for 16 h. The population of transiently transfected cells were identified by the presence of the interleukin-2 receptor cell surface marker and isolated by magnetic beads coated with the anti-interleukin-2 receptor monoclonal antibody, as previously described (Htun *et al.*, 1996). The total cellular lysate prepared from these cells was analyzed for the presence of GFP-ER by Western blot analysis using a rat monoclonal antibody, H226, against human ER as primary antibody, donkey anti-rat antibody coupled to horse radish peroxidase as second antibody, and Pierce Super Signal chemiluminescent substrate. Location of GFP-ER is indicated along with that of the immunoglobulin, which is present in the extract due to the cell sorting procedure and is also detected by the secondary antibody. Molecular weight markers are indicated on the left.

**(C) GFP-ER-dependent and 17β-estradiol-dependent transcriptional activation of ERE-containing reporter gene, pERE-tk-CAT, in MDA-MB-435A cells.** Human breast cancer epithelial cells, MDA-MB-435A, are transfected with various amounts of the GFP-ER expression plasmid as indicated along with 5 μg of pERE-tk-CAT reporter gene, and 4 μg β-galactosidase expression plasmid pCH110, by calcium phosphate coprecipitation method. Following replacement with fresh medium the next day, cells were treated (gray bars) or not treated (stippled bars) with 10 nM 17β-estradiol for 30 h. Extracts were prepared subsequently and assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activity. The relative CAT activity was calculated using the β-galactosidase activity to normalize for transfection efficiency.

**(D) Effect of 17β-estradiol concentration and type of ligand on GFP-ER activation of pERE-tk-CAT reporter gene in MDA-MB-435A cells.** Cells were transfected as described in the previous panel using 0.1 μg pCI-nGL1-HEGO expression plasmid, 5 μg pERE-tk-CAT reporter gene, and 4 μg β-galactosidase expression plasmid pCH110. Following transfection,

cells were treated with no additional ligand (lane 1), 0.1 nM 17β-estradiol (lane 2), 1 nM 17β-estradiol (lane 3), 10 nM 17β-estradiol (lane 4), 100 nM 17β-estradiol (lane 5), 10 nM ICI 182780 (lane 6), 10 nM 4-hydroxytamoxifen (lane 7), 500 nM ICI 182780 and 10 nM 17β-estradiol (lane 8), and 250 nM 4-hydroxytamoxifen and 10 nM 17β-estradiol (lane 9) for 30 h before analysis.

**(E) Association of GFP-ER with the nuclear matrix in MCF-7 cells.** MCF-7 cells were grown in charcoal-stripped fetal bovine serum. Lane 1 is the supernatant following extraction of isolated nuclei with 0.25 M ammonium sulphate; lane 2 is the supernatant following the first extraction with 2M NaCl; lane 3 is the supernatant following the second extraction with 2 M NaCl; lane 4 is the nuclear matrix and associated intermediate filaments. Immunodetection of GFP-ER was done using the HA antibody to the HA tag located on the N-terminus of the GFP-ER fusion protein.

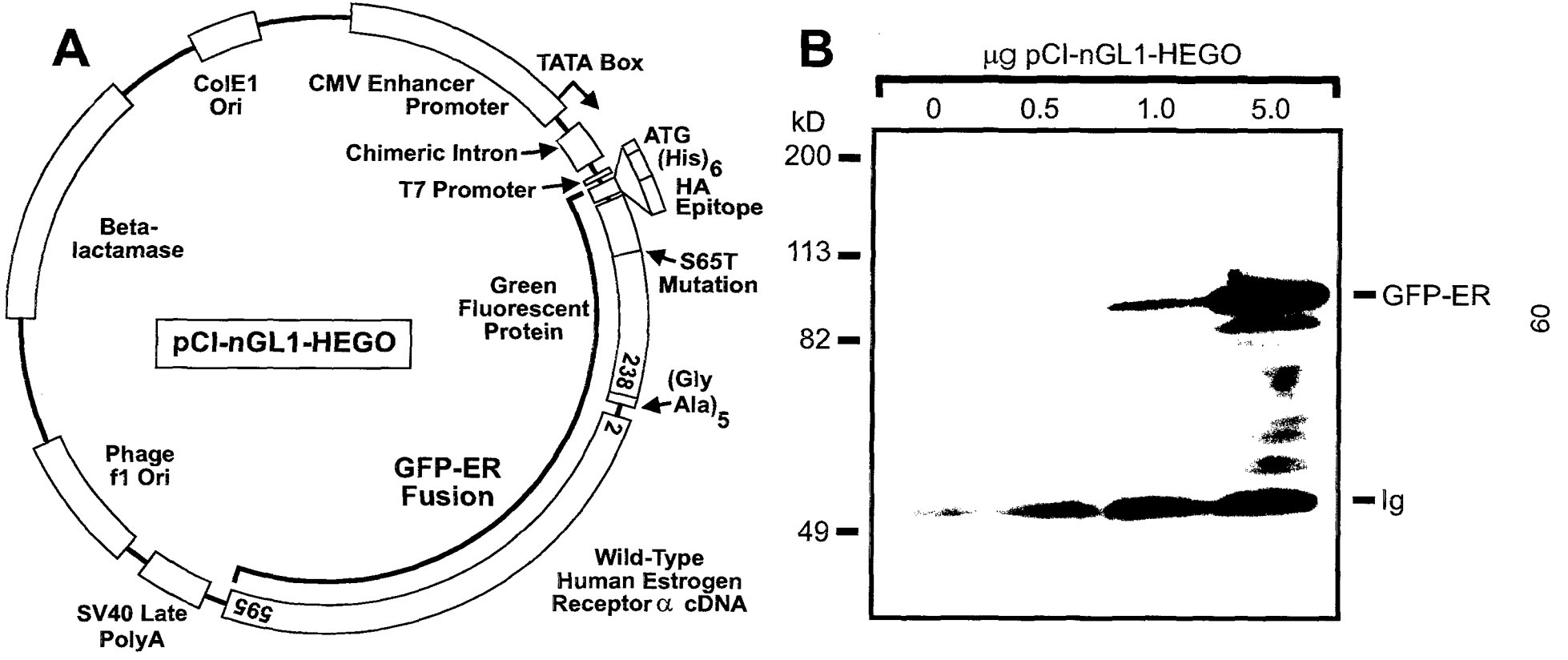
**Figure 2. Effect of ligand treatment on GFP-ER localization in MCF-7 cells.** Cells from a human breast cancer epithelial cell line, MCF-7, were electroporated with 0.2 μg of GFP-ER expression plasmid, pCI-nGL1-HEGO, and cultured on coverslips for 12-16 h before visualization by differential interference contrast (panels A, C, E and G) or epifluorescence with a standard set of FITC filters (panels B, D, F, and H). Cells were treated at the time of culturing with nothing (panels A and B), 10 nM 17β-estradiol (panels C and D), 10 nM 4-hydroxytamoxifen (panels E and F), or 10 nM ICI 182780 (panels G and H). Arrow in the left panel indicates the nucleus of a cell exhibiting green fluorescence, as seen in the right panel.

**Figure 3. Effect of cycloheximide treatment on ICI 182780-induced accumulation of GFP-ER in the cytoplasm.** MCF-7 cells were electroporated with 0.5 μg of pCI-nGL1-HEGO DNAs and left to express for 12 h before 8 h treatment with 10 nM ICI 182780 (panels A and B), 200 μg/ml cycloheximide (panels E and F), or 200 μg/ml cycloheximide and 10 nM ICI 182780 (panels G and H). Alternatively, the cells were treated with 200 μg/ml cycloheximide immediately after electroporation for 20 h (panels C and D). Cells were visualized by differential interference contrast (panels A, C, E, and G) or epifluorescence using a standard FITC filter set (panels B, D, F, and H). Arrows in the left panels point to nuclear fluorescence observed in the right panels.

**Figure 4. Effect of ligand treatment on the intranuclear distribution of GFP-ER in MCF-7 cells.** MCF-7 cells were electroporated with 0.2 μg GFP-ER expression plasmid, pCI-nGL1-HEGO, and cultured on coverslips overnight. Next day, media was changed and the cells were treated with nothing (A), 10nM 17β-estradiol (B), 10 nM 4-hydroxytamoxifen (C), or 10 nM ICI 182780 (D) for 1 h before visualization by confocal laser scanning microscopy on a Bio-Rad MRC 1024 system. Re-presentation of fluorescence intensity in panels A and B as three-

dimensional plots in panels E and F, respectively, with the greater the fluorescence, the higher the peaks and the cooler the colors. White scale bar indicates ten microns. (G) Graph of fluorescence intensity along the green line for the nucleus in panel A (left graph with green curve) and red line for the nucleus in panel B (right graph with red curve). Arrowheads in panels A and B point to the direction of the plot from left to right in panel H. The black bar within each graph in panel H marks the segment of the line for which the mean fluorescence intensity and standard deviations are calculated.

**Figure 5. Effect of ligand treatment on the intranuclear distribution of GFP-ER in three human breast cancer epithelial cell lines.** Three human breast cancer epithelial cell lines, T47D, MDA-MB-231, and MDA-MB-435A, are electroporated with 0.2 μg pCI-nGL1-HEGO and treated and visualized as in Figure 3. The white scale bar in each of the panel for the first column (A, E, and I) is the same scale bar for the other panels in the row and is ten microns long. Note the presence of fluorescence in the nucleolar regions (indicated by white arrows) for the some of the MDA-MDA-231 cells (e.g., panels E-G) which can be quite prominent in MDA-MB-435A cells (e.g., panels I, J, and L).


A
ColE1 Ori
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TATA Box
Chimeric Intron
T7 Promoter
ATG
(His)6
HA Epitope
S65T Mutation
Green Fluorescent Protein
pCI-nGL1-HEGO
Beta-lactamase
238
(Gly Ala)5
2
Phage f1 Ori
GFP-ER Fusion
Wild-Type Human Estrogen Receptor α cDNA
595
SV40 Late PolyA
B
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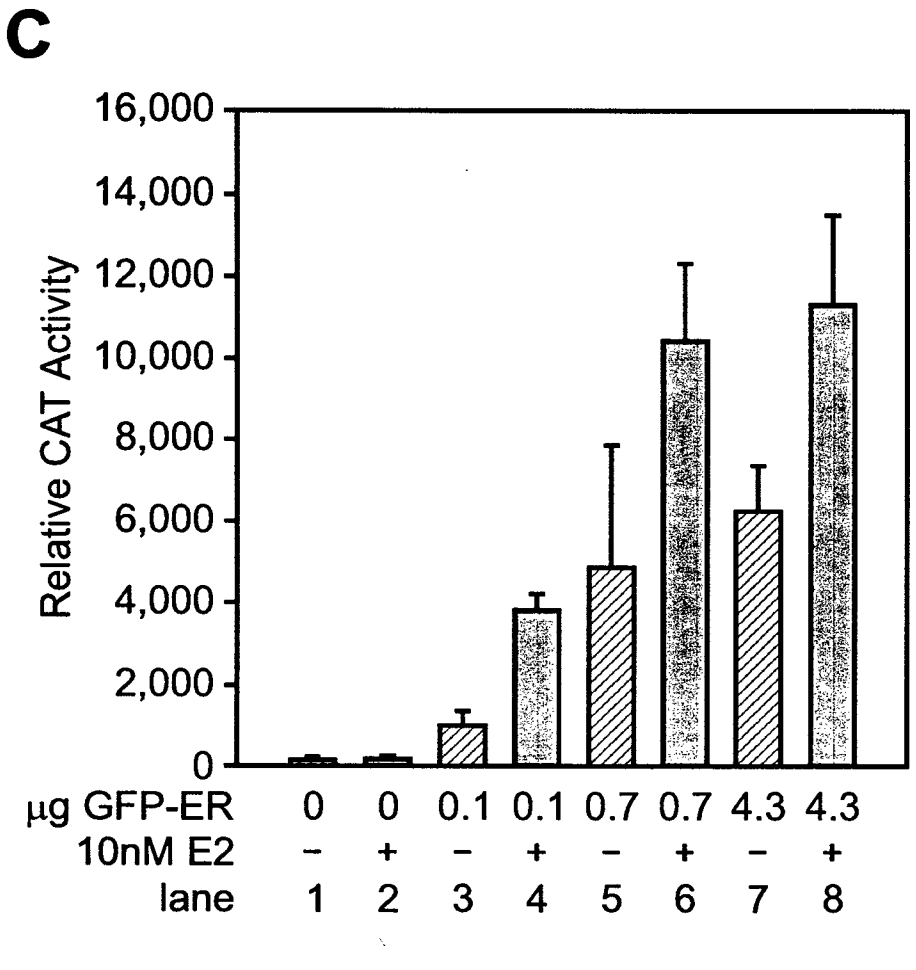
1.0
5.0
kD
200
113
82
49
GFP-ER
Ig

Figure 1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14,000
12,000
10,000
8,000
6,000
4,000
2,000
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10nM E2 – + – + – + – +
lane 1 2 3 4 5 6 7 8

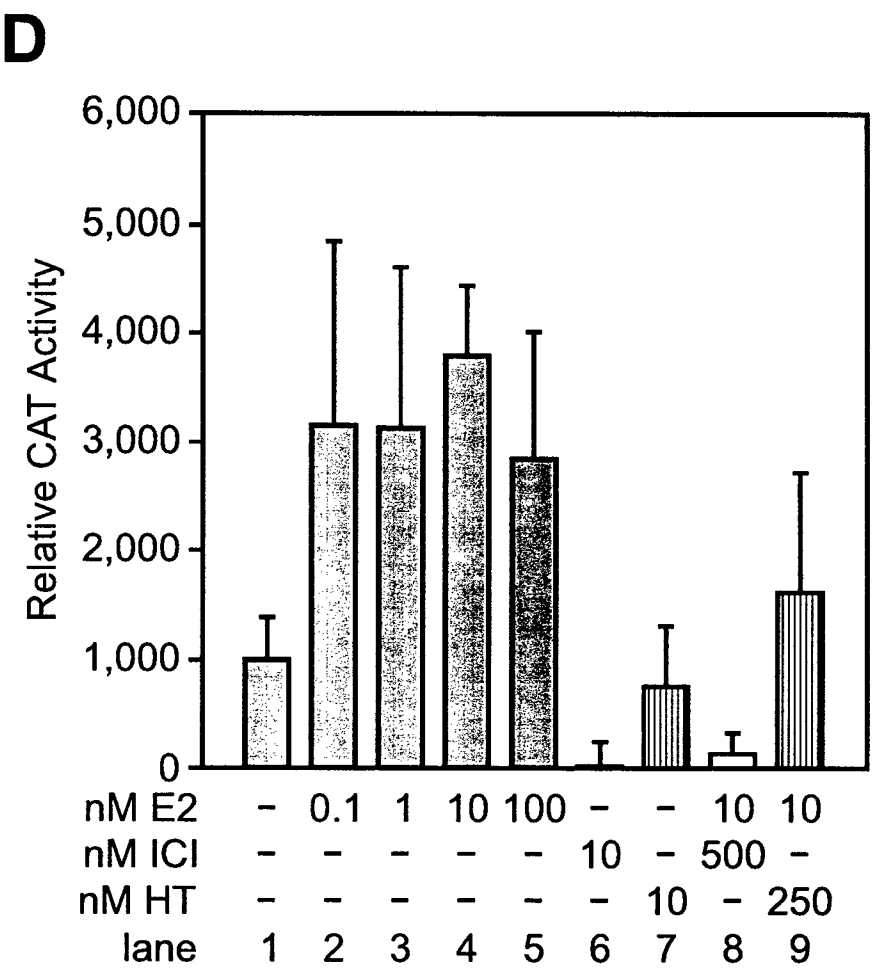

D
Relative CAT Activity
6,000
5,000
4,000
3,000
2,000
1,000
0
nM E2 – 0.1 1 10 100 – – 10 10
nM ICI – – – – – 10 – 500 –
nM HT – – – – – – 10 – 250
lane 1 2 3 4 5 6 7 8 9

Figure 1

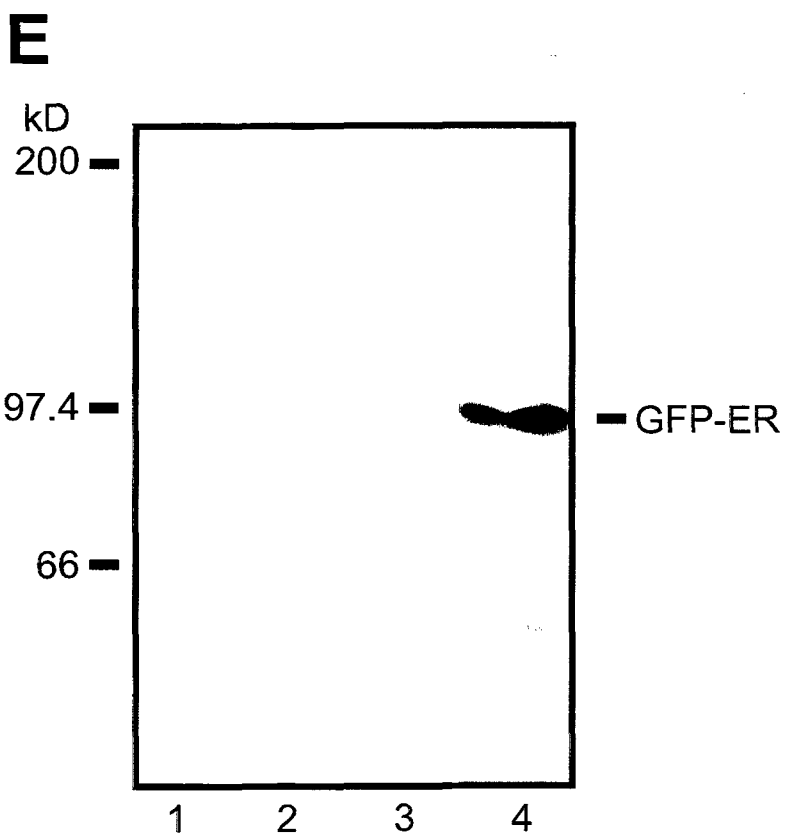



Figure 1

Figure 2

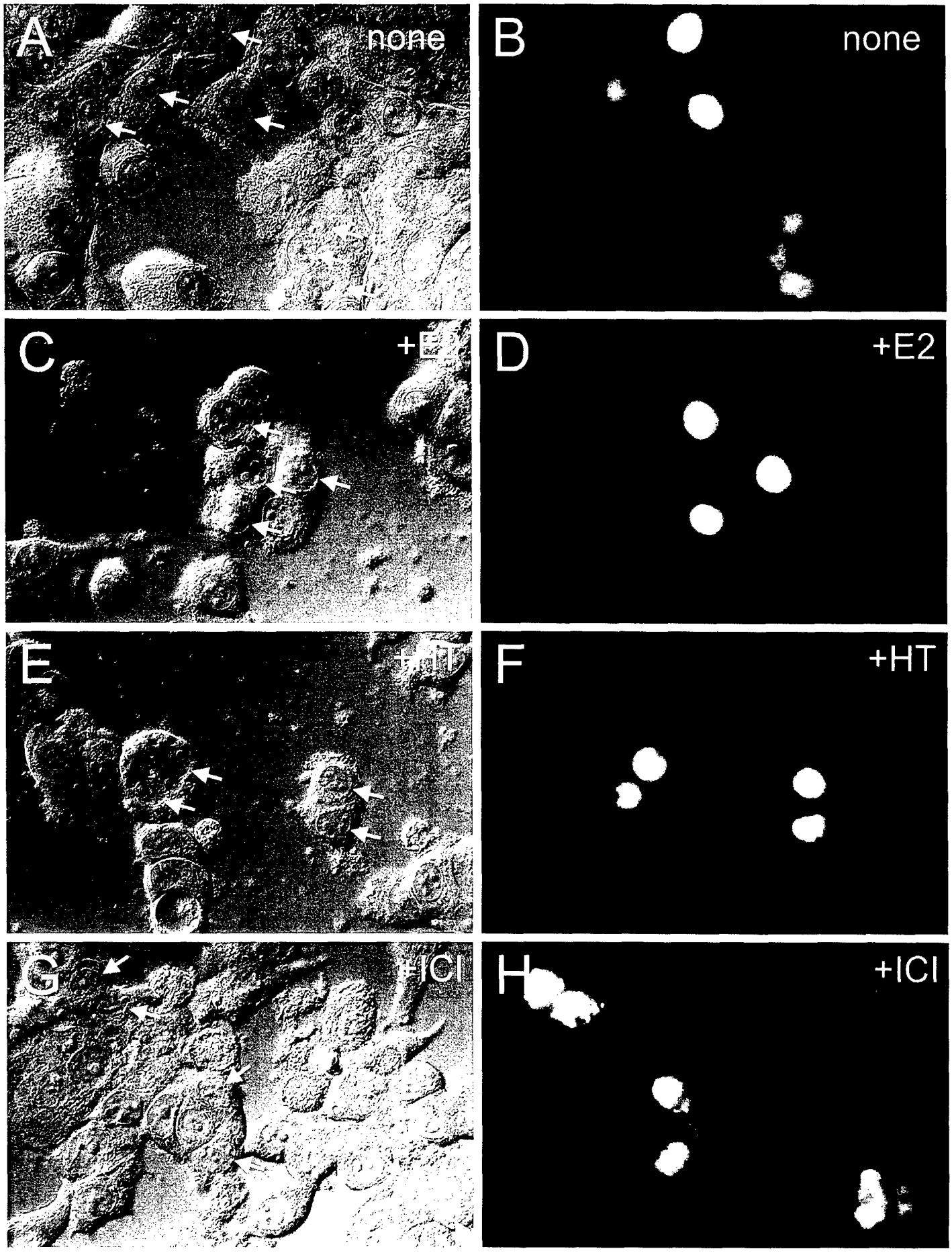
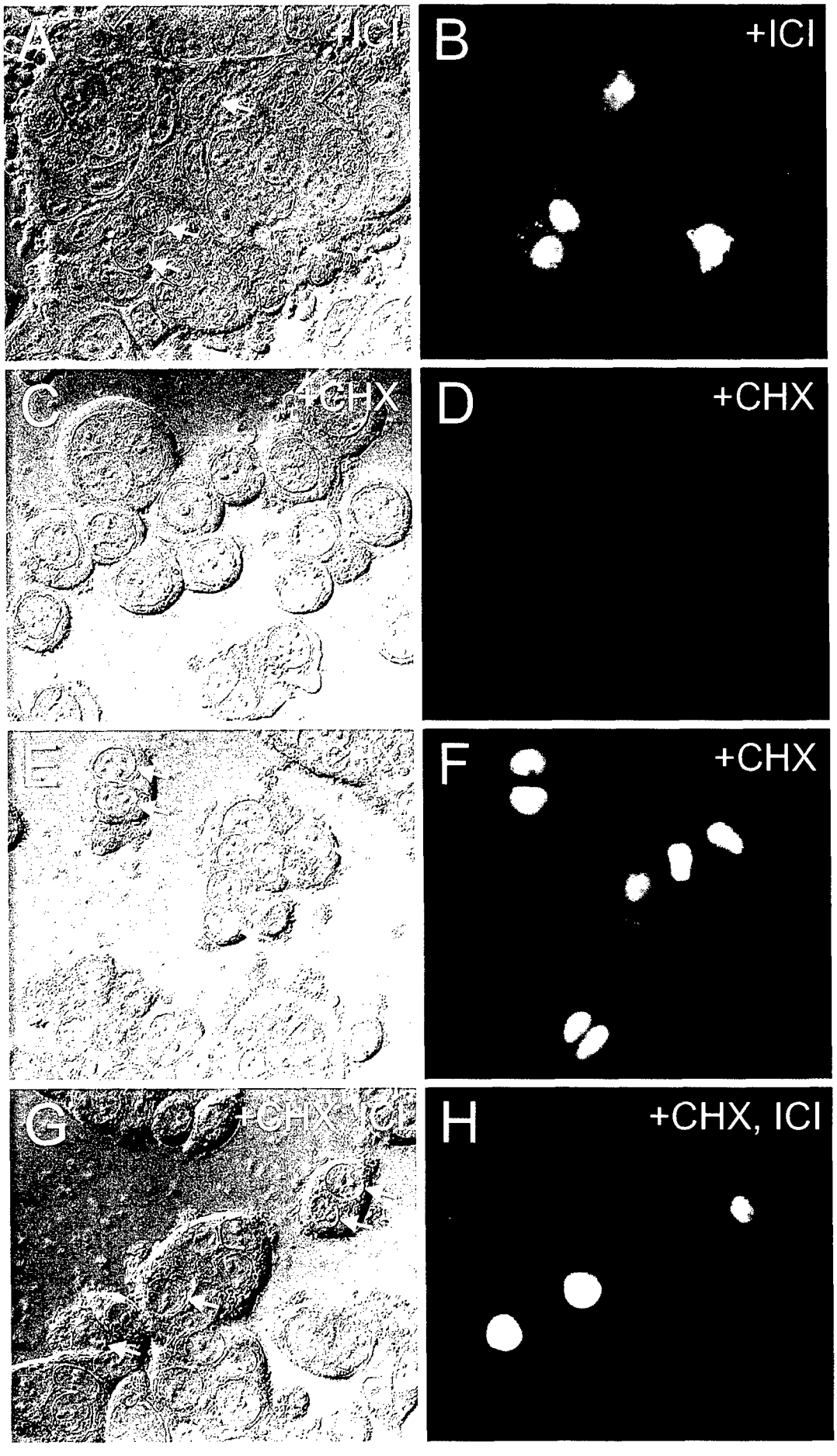
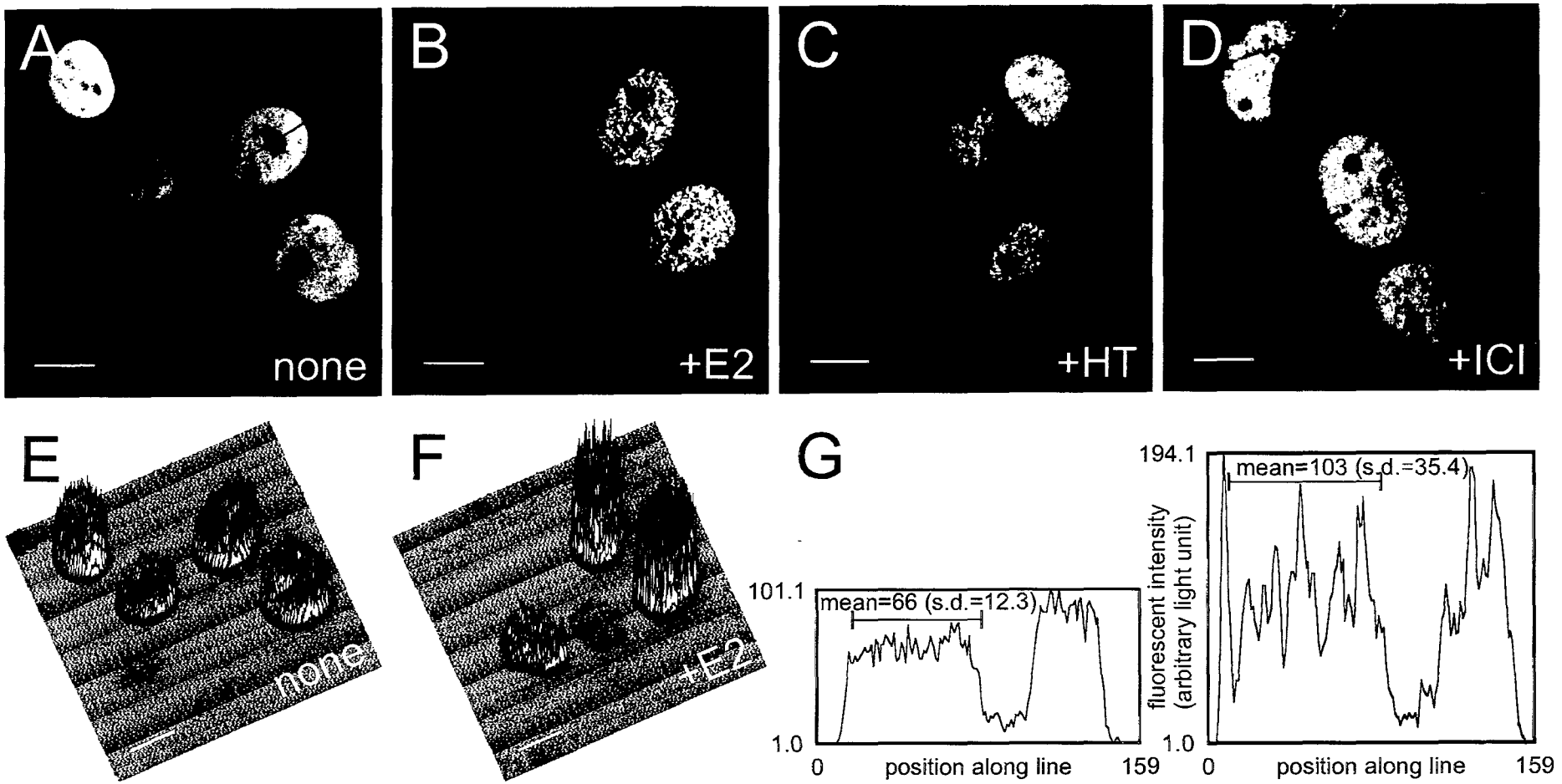


Figure 3

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none
B
+E2
C
+HT
D
+ICI
E
none
F
+E2
G
101.1
1.0
mean=66 (s.d.=12.3)
0
position along line
159
194.1
1.0
mean=103 (s.d.=35.4)
fluorescent intensity
(arbitrary light unit)
0
position along line
159

Figure 4

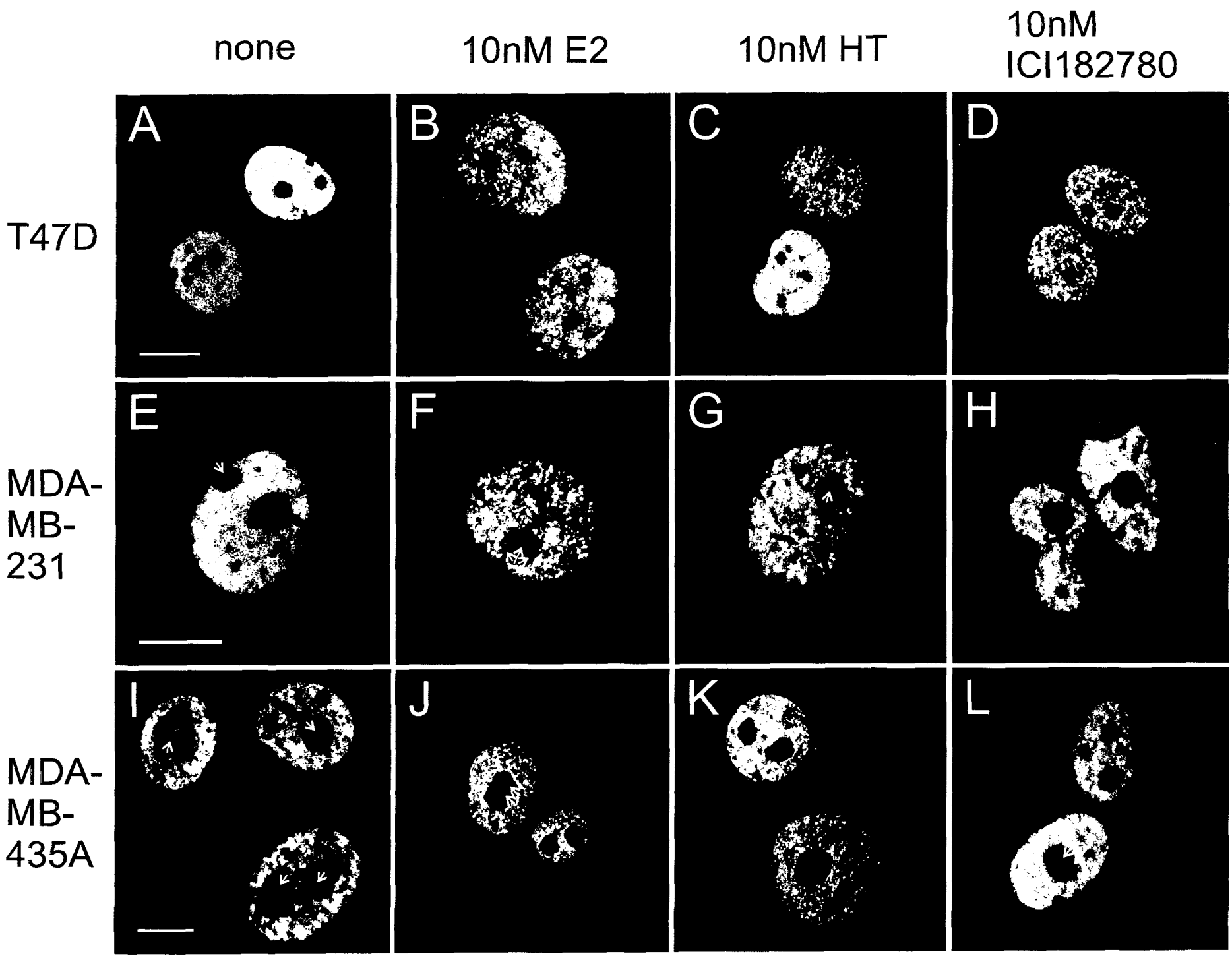
none
10nM E2
10nM HT
10nM ICI182780
T47D
MDA-MB-231
MDA-MB-435A
A
B
C
D
E
F
G
H
I
J
K
L



Figure 5

RECVD 30NOV 00

**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) 15 Nov 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: 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 Grant DAMD17-96-1-6269. Request the limited distribution statement for Accession Document Number ADB239417 be changed to "Approved for public release; distribution unlimited." This report 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 email at Judy.Pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management