Transcriptional Repression of the neu Protooncogene by Estrogen Stimulated Estrogen Receptor

Kerry Strong Russell and Mien-Chie Hung

Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Both neu gene overexpression and loss of estrogen receptor (ER) expression have been found to correlate with a poor prognosis in human breast cancer. Studies of breast tumor specimens have suggested that these two factors are not independent, leading us to hypothesize that expression of the neu gene protein product, p185<sub>neu</sub>, in two ER positive but not an ER negative breast cancer cell line(s). We have produced sublines which stably express human ER from a previously ER negative human breast cancer cell line. We demonstrate that the expression of neu in these cell lines is sufficient to confer the ability to respond to estradiol by down-regulating neu expression at both the protein and RNA levels. Utilizing neu promoter-chloramphenicol acetyltransferase constructs in transient cotransfection assays, we have also shown that this regulation occurs at the transcriptional level and requires the presence of both ER and estradiol. Furthermore, utilizing promoter deletion constructs, we provide evidence that a 140-base pair region of the neu promoter is required for this transcriptional regulation. When placed in a heterologous promoter, this 140-base pair region allows transcriptional repression by estradiol stimulated ER; thus, it represents an estrogen responsive region within the neu promoter. Finally, we have used gel mobility shift analysis to demonstrate an alteration in the nuclear factor(s) binding to this promoter region in estradiol stimulated versus estradiol deprived breast cancer cells. This study provides the first evidence that the inverse clinical correlation between neu and ER expression may be due to transcriptional repression of neu by estradiol stimulated ER.

INTRODUCTION

Amplification and overexpression of the neu (HER-2 or c-erb-B-2) protooncogene occurs in as many as 30% of all breast cancers and has been found to be correlated with poor patient prognosis (1–5). There are several lines of evidence which imply that neu overexpression either in the absence of gene amplification or at levels which are disproportionate to that of gene amplification may play an important role in these cancers. In several cell lines established from human breast tumor specimens, the level of neu mRNA expressed is in excess relative to the neu gene copy number present (6). neu gene overexpression can lead to transformation of NIH-3T3 cells (7) and is able to induce mammary tumors in transgenic mice (8, 9).

Deregulation of neu gene expression could occur through several mechanisms. At the posttranscriptional level, prolonged neu mRNA or protein half-life could both lead to excessive levels of p185<sub>neu</sub>. At the transcriptional level, alterations either in the neu promoter which interfere with negative gene regulation (cis-acting) or in factors which interact with neu promoter regulatory elements (trans-acting) could lead to neu overexpression. For example, loss of a protein factor which normally down-regulates neu gene expression could lead to increased neu transcription with subsequent overexpression of p185<sub>neu</sub> and cellular transformation. Breast cancers in which this mechanism of transformation is important would be expected to have a decreased level (or loss of function) of this negative regulatory factor in conjunction with increased levels of neu expression. Several lines of evidence suggest that the ER<sup>+</sup> might be such a negative regulatory factor. Loss of ER expression or function, like neu overexpression, is also strongly correlated with poor prognosis in breast cancer patients (10). Some studies of human breast cancer specimens have shown a correlation between overexpression of neu and loss of functional ER (11–13). Finally, two studies using ER<sup>+</sup> human breast cancer cell lines, MCF-7 and T47D, found an inverse relationship between estrogen stimulation and neu expression (14, 15). These facts led us to investigate the possibility that estrogen stimulation of ER<sup>+</sup> but not ER<sup>-</sup> cells leads to decreased neu expression through transcriptional repression of the neu gene by ER. The specific questions to be addressed in this work are as follows. Does estrogen stimulation of ER<sup>+</sup> breast cells decrease their level of neu expression? Is the presence of ER necessary and sufficient for this repression? Can repression of neu by ER be seen at the transcriptional level? Which neu promoter region is responsible for this repression? Is there an estrogen inducible alteration in a factor(s) which binds to this promoter region to mediate repression?

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell lines ZR-75-1, MCF-7, and BT-474 were obtained through the American Type Culture Collection (Rockville, MD). These lines were routinely maintained in phenol red free RPMI supplemented with 10% fetal bovine serum. NIH-3T3 and CV-1 fibroblast lines were maintained in phenol red free DMEM supplemented with 10% calf serum. In order to remove steroid hormone agonists present in normal serum, the serum used in these experiments was treated by incubation with dextran coated charcoal (16). The final concentration of estrogen in serum thus treated was measured by radioimmunoassay (ICN Biomedicals, Lisle, IL) and found to be $\leq 10^{-12}$ M. Stock solutions of estradiol and tamoxifen (Sigma Chemical Co., St. Louis, MO) were dissolved in ethanol at concentrations of 0.1–1 $\mu$M and stored at −20°C for periods of up to 1 month. The concentration of ethanol in all plates, both steroid treated or deprived, was kept at 0.01%.

Immunoblot Analysis. Cells lysates were prepared at various time points after addition or deprivation of estradiol. Immunoblotting for p185<sub>neu</sub> was performed as previously described (17) utilizing the antineu antibody, MAb3 (Oncogene Science, Manhasset, NY), and the enhanced chemiluminescent detection system (Amersham, Arlington Heights, IL).

Northern Blot Analysis. Total cellular RNA was harvested using the RNAzolB method (Cinnatex Biochemicals). Equivalent amounts of RNA (as measured by absorbance at 260 nm) from each sample were loaded for Northern analysis. Filters were subsequently probed with 32P-labeled 3-kilobase 3′ HindIII-KpnI fragment of the human neu gene

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2 To whom requests for reprints should be addressed.
complementary DNA, pCER204 (18), or with 32P-labeled 2.1-kilobase human ER probe cut with EcoRI from the ΔER expression vector (19). Filters were then stripped and reprobed with a 32P-labeled γ-actin probe (20) as a loading control.

Transient Transfection Assays. NIH-3T3 or CV-1 cells were plated at low density in estrogen deprived medium (DMEM without phenol red plus 10% dextran coated charcoal treated fetal bovine serum) 24 h prior to transfection. Four h prior to transfection, the medium was changed to either estrogen deprived DMEM or estrogen deprived DMEM plus 10 nm estradiol. Transfections and enzymatic assays for CAT were carried out essentially as described previously (21). To correct for differences in transfection efficiencies, equivalent amounts of β-galactosidase reporter gene were cotransfected in all experiments and the level of β-galactosidase gene activity was used to normalize the amounts of cell extract from each transfection utilized in subsequent CAT assays. The rat neu promoter-CAT reporter genes were constructed in our laboratory (21). The human neu promoter-CAT constructs, HER2-CAT (22) and Hu neu 2-CAT, were generously provided by Dr. H. Saya (University of Texas M. D. Anderson Cancer Center, Houston, TX). The ΔER estrogen receptor expression vector was provided by Dr. M. J. Tsai (Baylor College of Medicine, Houston, TX) (19).

Formation of ER+ Sublines. The pXThER viral vector containing 2.1 kilobases of human ER complementary DNA along with the neomycin resistance gene (neo8) and PXTI vector containing neo8 alone were generously provided by Dr. D. Steffen (23). This vector was linearized and electroporated into the GP+enAM-12 (PAM12) amphotropic retroviral packaging line. The generation of GP+enAM-12 cells was done as in Ref. 24, courtesy of Dr. A. Bank (Columbia University, New York, NY). Sublines of virus producing cells were selected by their ability to grow in G418 (Sigma). Medium containing virus from these cells was subsequently harvested, passed through a 0.45-μm filter, and added to the BT-474 host line in the presence of 8 μm polybrene (Sigma).

Gel Mobility Shift Analysis. ZR-75-1 cells were plated at low density in estradiol deprived medium with or without 10 nm estradiol and grown for a period of 1 week. Nuclear extracts from these cells were made according to the protocol of Dignam et al. (25). Protein concentrations of these nuclear extracts was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Equivalent amounts of protein from each nuclear extract preparation were then incubated with the 32P-labeled Stul-XhoI region of the neu promoter under conditions previously described (26) with some modifications. Specifically, 4–6 μg of total protein from each nuclear extract were incubated with binding buffer (final concentrations of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 60 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.3 mg/ml bovine serum albumin), 8 μg of poly-(deoxyinosinate-deoxycytidylate)-poly(deoxyinosinate-deoxyctydylate), and 10,000 cpm (0.5–1 ng) of [32P]dCTP-end-labeled neu/Stul-XhoI promoter fragment on ice for 10 min. These samples were then electrophoresed through a 5% acrylamide gel at 4°C. Gels were then dried and autoradiographs were obtained.

RESULTS

Effect of Estrogen on p185neo Levels in Human Breast Cancer Cell Lines. In order to test whether estrogen stimulation results in modulation of neu-encoded p185neo, two well characterized ER+ human breast cancer cell lines were selected: ZR-75-1 and MCF-7. Steroid agonists, present in normal tissue culture media, such as phenol red (27), and serum (28), as well as endogenous steroids within the cells themselves, may mask effects of additional steroid under tissue culture conditions (29, 30). Therefore, cells from each line were plated at a low density in estrogen deprived medium for either 0, 4, or 8 days to allow complete deprivation from estrogen stimulation. The level of p185neo expression in these cells was then measured by immunoblotting. The addition of 10 nm estradiol to these estrogen deprived cells leads to a dramatic decrease in their expression of p185neo as early as 1 day and reaching a low level by 4 days of estrogen stimulation. Consistent with the concept that estrogen stimulation decreases the expression of p185neo, during a period of estrogen deprivation, both ZR-75-1 (Fig. 1A) and MCF-7 (Fig. 1B) cells show a very slight increase in expression of p185neo. By contrast, the human breast cancer cell line BT-474 which is ER- (31) shows no effect of either estradiol deprivation or stimulation on the expression of p185neo (Fig. 1C).

Addition of ER to an ER Negative Cell Line Results in Repression of neu Expression by Estradiol. The previous results suggest that ER is necessary for the repression of neu expression by estradiol; however, since the cell lines tested are likely to have multiple differences other than their ER status, these data cannot be used to determine whether ER is sufficient to mediate this repression in these breast cell lines. In order to test this, the ER- BT-474 cell line was used to make ER+ sublines of BT-474. This was accomplished by placing a human ER expression vector, pXThER, in these cells utilizing a viral infection scheme. As a control, cells were also infected with virus containing the parental vector for pXThER, PXTI, which is identical to pXThER but does not contain the ER gene. Several BT-474 clones were then selected as described in "Materials and Methods" to test for responsiveness to estradiol. A Western
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blot analysis of p185<sub>neu</sub> from several clones after 4 days of treatment by estradiol deprivation, with estradiol, or with the estrogen antagonist, tamoxifen, is shown in Fig. 2A. The level of p185<sub>neu</sub> in the ER<sup>+</sup> (i/ER) clones is significantly decreased in the presence of estradiol when compared to the level under either estradiol deprived conditions or in the presence of tamoxifen. There is no difference in the level of p185<sub>neu</sub> in the control (i/neo) clones. This repression of neu expression can also be seen at the mRNA level as seen in a sample Northern blot for the BT-474(i/ER) clone, B10(i/ER) (Fig. 2B). Again, there is no effect of estradiol treatment on the level of neu mRNA seen in control, ER<sup>+</sup> clone B3 (i/neo). A parallel Northern analysis shows that only the (i/ER) clones but not the (i/neo) clone express ER mRNA (Fig. 2C). These experiments demonstrate that the addition of ER alone to the ER<sup>+</sup> BT-474 cell line is sufficient to allow estradiol to repress the expression of neu in these cells.

Repression of neu Expression Occurs at the Transcriptional Level. Transcriptional regulation of a number of genes by ER has been well described (32–34). We therefore chose to investigate whether the down-regulation of neu expression seen at the RNA and protein levels occurs at the transcriptional level. To address this question, a transient cotransfection system was utilized. Two cell lines were chosen for their relative ease of transfectability and lack of ER expression: (a) NIH-3T3 mouse fibroblasts; and (b) CV-1 monkey fibroblasts. In these experiments, a human estrogen receptor expression vector (ΔHER) pXTHIER vector described above was cotransfected with a series of human and rat neu promoter sequences each linked to a CAT reporter gene. Fig. 3A shows the effect of ER and estradiol on transcription from a 537-base pair portion of the human neu promoter. There is a significant decrease in transcriptional activity from this promoter when ER is cotransfected in the presence of estradiol when compared to the activity of this promoter in the absence of estradiol and ER. In cotransfections with ER in the absence of estradiol (Fig. 3A, Lanes 6 and 8) or without ER in the presence of estradiol (Fig. 3A, Lanes 1 and 3), no significant difference from the case where neither ER nor estradiol is present (Fig. 3A, Lanes 5 and 7) is seen. When a larger 2.2-kilobase portion of the rat neu promoter-CAT reporter gene is used in this system, similar E2/ER dependent repression is seen (data not shown). Thus, for both the human and rat neu genes, repression by E2/ER is indeed seen at the transcriptional level.

Localisation of the Promoter Region Responsible for E2/ER Repression of neu. Cotransfection experiments similar to those described previously were carried out using neu promoter-CAT constructs with successive 5′ deletions to determine which regions of the neu promoter are necessary for repression by E2/ER. Deletion of the human neu promoter to 250 base pairs does not abolish the ability of E2/ER to repress transcription from this promoter (Fig. 3B). Therefore, this 250-base pair region from the human neu promoter must contain one of the elements which contribute repression of this promoter by E2/ER.

Our laboratory has cloned a 2.2-kilobase portion of the rat neu promoter and characterized several cis-acting elements (21, 26) as well as some trans-acting factors which regulate transcription within this promoter region (17, 21, 35). In order to analyze the neu promoter for both short- and long-range elements which might be regulated by E2/ER and to allow comparison of such elements with the elements already defined in our laboratory, this 2.2-kilobase neu promoter region was analyzed using 5′ deletions as described above. Fig. 4 shows that all of the rat neu promoter constructs tested are repressed by
E2/ER except for the shortest, least transcriptionally active, Xhol-CAT construct. Since the Xhol-CAT construct has very weak promoter activity (21) as compared to the other neu-CAT constructs, a longer exposure of data from this construct was required to evaluate its nonresponsiveness to E2/ER. These data are summarized in Table 1 in order to emphasize the point that the transcriptional activity of the Xhol-CAT construct is not significantly affected by the presence of E2/ER. These results indicate that the promoter region located within the Stul and Xhol restriction sites contains at least one of the elements which is responsible for E2/ER repression of neu gene transcription. Sequence comparison between this rat Stul-Xhol region and the homologous regions in the human and mouse promoters reveals a high degree of homology (21, 22, 36). To test whether the Stul-Xhol region alone is sufficient to confer the ability of E2/ER to repress transcription on a heterologous promoter, this region was subcloned in front of the tk gene minimal promoter linked to a CAT reporter gene (tk-CAT or PBLCAT2) (37). This construct was then tested in transient cotransfection assays with the ER as described above. Fig. 5 shows that E2/ER can repress transcription from this (Stul-Xhol)tk-CAT construct. In contrast, no effect is seen when E2/ER is cotransfected with a control construct containing the tk promoter alone (tk-CAT). Therefore, the neu promoter fragment contained within the Stul and Xhol restriction sites contains at least one element which can respond to E2/ER by repressing transcription from the promoter in which it is contained. Comparison of the activity of (Stul-Xhol)tk-CAT and the tk-CAT constructs in the absence of E2/ER confirms that the Stul-Xhol region indeed contains an enhancer-like activity as previously described by our laboratory (21, 38).

### Table 1. Summary of neu-CAT deletion data

<table>
<thead>
<tr>
<th>CAT construct</th>
<th>n</th>
<th>% of repression by E2/ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>neu EcoRI-CAT</td>
<td>4</td>
<td>76.3 ± 1.3*</td>
</tr>
<tr>
<td>neu Xho-CAT</td>
<td>4</td>
<td>33.5 ± 2.5*</td>
</tr>
<tr>
<td>neu RV-CAT</td>
<td>8</td>
<td>60.1 ± 3.7*</td>
</tr>
<tr>
<td>neu RV'-CAT</td>
<td>4</td>
<td>42.5 ± 8.7*</td>
</tr>
<tr>
<td>neu Stu-CAT</td>
<td>6</td>
<td>61.8 ± 7.2*</td>
</tr>
<tr>
<td>neu Xho-CAT</td>
<td>8</td>
<td>10.6 ± 6.9</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to no (0%) repression.

### Fig. 5. Effect of E2/ER on the neu promoter Stul-Xhol region in a heterologous promoter. NIH-3T3 fibroblasts were transfected with 8 μg of neu (Stul-Xhol)tk-CAT (Lanes 3 and 4) or PBLCAT2 (tk-CAT) (Lanes 1 and 2) in the presence (Lanes 2 and 4) or absence of cotransfected ΔHER plasmid DNA and 10 nm estradiol. The average repression of neu (Stul-Xhol)tk-CAT by E2/ER (compared to activity of neu (Stul-Xhol)tk-CAT in the absence of E2/ER) was found to be 63 ± 11% (n = 6). bp, base pairs.
that proteins involved in these altered complexes may contribute to the modification of neu transcriptional activity induced by E2/ER.

**DISCUSSION**

Steroid hormones play an essential part in regulating the growth of both normal and neoplastic breast cells (39). Specifically, estrogen has a marked effect on the proliferation of breast cells in vivo and in vitro. The mechanisms by which estrogen induces proliferation in ER+ breast cells are incompletely defined. However, modulation in expression of certain growth related cellular protooncogenes by E2/ER has been well described using cell lines established from human breast tumors (40–42). These experiments demonstrate that estrogenic stimulation of two ER+ human breast cancer cell lines results in a reduction in the protein level of neu (43-45). It is interesting to note that in these experiments the presence of tamoxifen does not result in repression of neu either at the RNA and protein levels (Fig. 2, A and B) or at the transcriptional level, as tested by transient transfection assays (data not shown). This suggests that tamoxifen does not act as an estrogen agonist in the down-regulation of neu.

These studies have shown that the presence of E2/ER results in a significant decrease in transcription from both the human and rat neu promoters in transient transfections. This occurs in a manner which is dose dependent on both estradiol and ER. In these transient transfection experiments, the maximal repression seen (55–80%) is limited by several parameters. Two of the main limiting factors are the total amount of DNA which may successfully be transfected and the minimum amount of reporter DNA necessary to detect CAT activity. These two parameters limit the ratio of effector to reporter DNA which may be transfected, which, in turn, limits the amount of transcriptional repression observed. Simply stated, the higher the ratio of effector (ER, in this case) to reporter (neu-CAT, in this case) plasmid transfected, the more likely it is that any given cell receiving the reporter will receive the effector. The importance of this phenomenon is that each cell which receives reporter and not effector will contribute “background” CAT activity, the sum of which lessens the degree of repression seen in each experiment. However, it is also interesting to note that the degree of repression of neu expression at the protein level exceeds that at the RNA level in the BT-474 subclones which suggests that posttranscriptional mechanisms may play a role in repression of neu expression by E2/ER.

A number of estrogen response elements within promoter sequences of genes the transcription of which is stimulated by ER have been described (46–49). Our studies utilizing neu promoter deletion constructs have allowed us to define an estrogen responsive region within the rat neu promoter from which transcription is down-regulated. We are pursuing further analysis of this promoter region by utilizing smaller deletions within this region and gel shift analysis. This 140-base pair region can mediate repression by E2/ER when placed in a heterologous promoter. Sequence analysis of this promoter region has been reported by our laboratory (21). A review of this sequence reveals that there is no region identical to any previously described estrogen response elements. Gel shifting of this region reveals an alteration in the pattern of binding seen in nuclear extracts from estradiol stimulated versus estradiol deprived ZR-75–1 cells. Further studies will help to determine whether the complex binding to this region contains ER and is responsible for E2/ER-mediated transcriptional regulation.

In summary, these studies provide evidence that estrogen regulation of neu expression occurs in an ER dependent manner. This regulation occurs at the transcriptional level. An element which can mediate transcriptional repression by E2/ER is located within a 140-base pair region of the neu promoter and demonstrates a different pattern of binding with nuclear extracts from estradiol stimulated versus estradiol deprived breast cancer cells. This functional link between ER stimulation and the addition of ER expression to the ER+ BT-474 line allows these cells to respond to estradiol by down-regulating neu expression suggests that ER is their missing link in this pathway. Since this pathway is potentially important in growth regulation of these cells, we are currently investigating whether the presence of ER in these cells alters their growth characteristics in a manner related to their expression of neu.
control of gene expression may provide important clues to the mechanism of tumor progression in breast tumors which have lost ER expression.

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