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A Strong Intronic Enhancer Element of the EGFR Gene is Preferentially Active in High EGFR Expressing Breast Cancer Cells

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Abstract Hormone-independent human breast cancer is characterized by estrogen receptor (ER) loss and the acquisition of high epidermal growth factor receptor (EGFR) levels. Despite the tendency for an inverse correlation between EGFR and ER, EGFR is a strong prognostic indicator for poor survival rate independent of ER status suggesting that EGFR overexpression is an important step in the progression to estrogen independence. We have previously shown that several DNase I hypersensitive sites which correspond to potential regulatory regions reside within the EGFR gene first intron exclusively in hormone-independent breast cancer cells. CAT assays investigating the transcriptional activity of the first intron of EGFR indicate that a 140 bp region has an enhancer ability specifically in these hormone-independent breast cancer cells. The DNA–protein interaction that occurs in this enhancer was localized to a 35 bp region and displayed enhancer-like activity in the same hormone-independent breast cancer cells. Furthermore, the protein that binds to this 35 bp region seems to be ubiquitous in the cell lines tested but in higher abundance in high EGFR expressing cells. Identifying the specific regulatory elements involved in EGFR up–regulation could lead to the development of therapies for preventing and treating estrogen-independent breast cancer. J. Cell. Biochem. 80:538–549, 2001.

Key words: epidermal growth factor receptor; intron; *cis*-acting element; gene expression; breast cancer

Breast cancer often progresses from an estrogen receptor (ER) positive, hormone-dependent phenotype that is sensitive to anti-estrogens, to a more aggressive, ER negative, hormone independent phenotype that does not respond to anti-estrogens [Fitzpatrick et al., 1984; Sainsbury et al., 1987]. The loss of ER in the hormone independent state implies that the cell utilizes other pathways to bypass the requirement of estrogen for proliferation. One theory is that the up–regulation of growth factors and/or their receptors in these cells provides a mechanism for escaping estrogen dependence [Derynck et al., 1987; DiMarco et al., 1989]. The epidermal growth factor receptor (EGFR) is one such receptor that has been well documented to be overexpressed in several types of tumors [Gullick, 1991]. In human breast cancer, the estrogen-independent state is associated with the loss of ER and the acquisition of high levels of EGFR [Davidson et al., 1987; Fitzpatrick et al., 1984; Sainsbury et al., 1985]. The EGFR status of a tumor has been shown to predict a more aggressive clinical course, a poor survival rate and the failure of endocrine therapy, independent of ER status [Bolufer et al., 1990; Nicholson et al., 1989, 1991]. Additionally, tumors that do not show an inverse relationship between ER and EGFR almost exclusively co-express the two

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receptors [Koenders et al., 1991]. These results strongly support the hypothesis that overexpression of the EGFR is a critical step in the progression to estrogen independence.

The EGFR gene is 110 kb in size, has 26 exons and is located on chromosome 7p12 [Habets et al., 1992]. The first intron, which is the focus of our studies, is 18 kb in size. The GC-rich promoter of EGFR does not have a TATA or CAAT box [Ishii et al., 1985]. In breast cancer, EGFR is often overexpressed but rarely amplified at the gene level [King et al., 1985], and total EGFR message levels correlate with receptor-binding sites per cell [Davidson et al., 1987]. Nuclear run-off data have shown that transcriptional control is an important regulatory mechanism for EGFR expression in breast cancer cell lines [Davidson et al., 1987]. This implies that the EGFR overexpression characteristic of the hormone independent state in breast cancer is under the control of specific regulatory factors. Previous studies in our laboratory have utilized DNase I hypersensitivity assays to identify potential *cis*-acting elements involved in the differential regulation of the EGFR gene in ER positive and ER negative breast cancer cell lines [Chrysogelos, 1993]. Numerous investigations have shown that DNase I hypersensitive sites are correlated with the binding of regulatory factors [reviewed by Gross and Garrard, 1988]. While hypersensitive regions were found in the promoter and first exon in all the cell lines tested, only the ER negative cell lines that express high levels of EGFR were found to have hypersensitive sites in the first intron [Chrysogelos, 1993]. We therefore hypothesized that there are specific elements within the first intron of EGFR that are involved in its upregulation in ER negative breast cancer cells.

In an attempt to better understand the molecular mechanisms involved in the overexpression of EGFR in hormone-independent breast cancer, we have assessed the transcriptional activity of the 5′ 2.1 kb of the gene's first intron. This region contains the DNase I hypersensitive sites we have previously mapped [Chrysogelos, 1993]. Through reporter assays, gel mobility shift assays and southwestern analysis, we have identified a 35 bp region that acts as an enhancer in the ER negative BT549 cell line and binds a ubiquitous nuclear factor that is in higher abundance in ER negative breast cancer cell lines. Localizing and characterizing such *cis*-acting elements in the first intron of EGFR that are involved in its transcriptional regulation in ER negative breast cancer cells could, in time, lead to new therapeutic targets or molecular markers for hormone-independent breast cancer.

MATERIALS AND METHODS

Cell Culture

All breast cancer cell lines were obtained from the Lombardi Cancer Center Tissue Culture Shared Resource. The BT549 breast cancer cell line was originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). The MCF-7 cell line was originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation). All cell lines were maintained in Richter's modified minimal essential medium (IMEM; Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Biofluids). The cells were maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2, and the media was changed approximately every 3 days. The cells were harvested by trypsinization and split 1:15 approximately every 7 days.

Constructs

The series of CAT reporter constructs shown in Figure 1 were created with the pCAT-promoter plasmid (Promega Co., Madison, WI) which contains the SV40 promoter proximal to the CAT gene and an enhancer cloning site distal to the CAT gene into which regions of the EGFR first intron were subcloned. For our purposes we have renamed the pCAT-promoter plasmid pSV-CAT to distinguish it from our reporter constructs containing the EGFR promoter. Portions of the EGFR first intron were subcloned into this parental vector from two clones that were generously donated by Dr. Glen Merlino (National Cancer Institute, NIH, Bethesda, MD): a CAT construct, pERCAT2DE [Maekawa, 1989] and a genomic clone, pEP1 [Ishii et al., 1985]. First, a 2.1 kb *Bam*HI restriction fragment from the pERCAT2DE construct that contained a region of the first intron of EGFR delineated by *Pvu*II restriction sites was subcloned into the *Bam*HI site of pGEM-4Z (Promega Co., Madison, WI) and named EGFR-3′*Bam*. The pSVI-CAT construct was then made by subcloning the 2.1 kb *Bam*HI fragment of the first intron of EGFR from the

EGFR-3′*Bam* construct into the *Bam*HI enhancer polylinker site of pSV-CAT. The $pSVI_1$-CAT construct contains the 730 bp 5′ intron 1 *Pst*I fragment from the genomic clone pEP1 subcloned into the *Pst*I site in the enhancer polylinker of pSV-CAT. The $pSVI_2$-CAT and $pSVI_3$-CAT constructs contain the more distal 780 and 660 bp *Pst*I fragments, respectively, from the EGFR-3′*Bam* construct subcloned into the *Pst*I site in the enhancer polylinker of pSV-CAT. The $pSVI_{2+3}$-CAT construct was made by a partial *Pst*I digestion of EGFR-3′*Bam* and the resulting 1440 bp *Pst*I fragment was subcloned into the *Pst*I site in the enhancer polylinker of pSV-CAT. The constructs $pSVI_{3a}$-CAT and $pSVI_{3b}$-CAT were made by subcloning the 500 and the 140 bp *Xba*I fragments, respectively, from $pSVI_3$-CAT into the *Xba*I site of the enhancer polylinker of pSV-CAT. The pSVIΔ3b-CAT construct was made from the pSVI-CAT construct by removing the 140 bp *Xba*I fragment (3b) and religating.

The CAT reporter constructs containing the EGFR promoter (shown in Fig. 6) were generated from the pJFCAT vector we received from Judith Fridovich-Keil [Fridovich-Keil et al., 1991]. First, a 2.1 kb *Xho*I restriction fragment from the pERCAT2DE construct that contained the EGFR promoter region and a portion of exon 1 was subcloned into the *Xho*I site of pGEM-7Zf (Promega) and named EGFR-5′*Xho*. An 840 bp *Bgl*II–*Sst*I fragment of the EGFR promoter from EGFR-5′*Xho* was then subcloned into the promoter polylinker of pJFCAT to generate the construct pJFEC. The reporter construct containing the 35mer region of the EGFR first intron was produced by first subcloning the 35mer oligonucleotide (described below) into the *Sma*I site of pBluescript (Promega) and sequencing to determine orientation. This plasmid was then digested with *Kpn*I and *Sst*I and the resultant fragment was subcloned into the enhancer polylinker site of pJFEC to generate the construct pJFEC-35. All restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA).

Transient Transfection

Plasmid DNA was prepared for transfection by a modified alkaline lysis procedure [Sambrook et al., 1989]. In this procedure the DNA was extracted by conventional alkaline lysis, passed through a 0.2 μm syringe filter, purified by a 5 M LiCl precipitation, incubated with 10 μg/ml RNase, and then precipitated with 1.6 M NaCl and 13% polyethylene glycol. This precipitate was then extracted with 1:1 phenol/chloroform twice and chloroform once and ethanol precipitated. All DNA that was prepared for transfection was quantitated by spectrophotometry and by electrophoresis on a 1% agarose gel. Cells were plated for transfection in 100 mm dishes at a density of $\approx 5 \times 10^6$ cells per dish and were transfected by the lipofectamine method [Hawley-Nelson et al., 1993] when the cells reached 80–85% confluency.

Briefly, this method involved diluting 10 μg of DNA in 0.5 ml serum-free media, and 20 μl of lipofectamine (Gibco/BRL, Gaithersburg, MD) in 0.5 ml serum-free media, and then mixing these two components together. The mixture was incubated for 30 min at room temperature, diluted with 3 ml of IMEM supplemented with 5% FBS and then placed on the cells for an incubation period of 18–24 h. After the transfection incubation, the media was replaced with IMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Biofluids, Rockville, MD) for another 24 h. The cells were then washed with phosphate-buffered saline (PBS; Gibco/BRL, Gaithersburg, MD) and harvested in TNE (0.04 M Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA). The cell pellets were stored at −70°C.

Chloramphenicol Acetyltransferase Assay

The chloramphenicol acetyltransferase (CAT) assay was performed by the thin layer chromatography method (TLC) [Gorman et al., 1982; Prost and Moore, 1986]. Whole cell lysates were prepared in 0.25 M Tris–HCl, pH 7.8 by freeze/thaw and their protein concentration was determined by the Bradford assay (BioRad, Melville, NY). Lysates were normalized for protein concentration and added to a reaction mixture that contained 0.5 M Tris–HCl, pH 7.8, 0.125 μCi ^{14}C-Chloramphenicol and 0.5 mM acetyl CoA in a final volume of 150 μl and incubated at 37°C for 2 h. The reaction mixture was then extracted with ethyl acetate (Fisher, Pittsburgh, PA) and dried. The extracted organic compounds were resuspended in ethyl acetate, spotted on a thin layer chromatography plate (Fisher) and incubated in a 95:5 chloroform/methanol tank. After the solvent had run ≈3 cm from the top, the TLC was dried and exposed to film. The substrate and product spots were then cut out of the

plates and scintillation counted. A percent substrate conversion was calculated and this value was normalized to protein levels. The data are shown as a fold induction with the activity of the parental vector defined as one. The CAT assay data for each figure were calculated from at least four different CAT assays that were transfected with at least three different preparations of DNA. Transfection efficiency of the cell lines was determined by a previously published method [Murphey and Chrysogelos, 1995] and did not effect the trends in the CAT assay data. The SIGMAPLOT software program was utilized to calculate the standard error of the data and to calculate the *P*-value of selected data via the Student's *t*-test.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays or gel shift assays [Fried and Crothers, 1981; Garner and Revzin, 1981] were performed using breast cancer cell line nuclear extracts and short radiolabeled DNA fragments from the EGFR first intron. Nuclear extracts from the breast cancer cell lines were made as described by Dignam et al. [1983]. HeLa nuclear extract was a kind gift from Dr. Anna Riegel (Lombardi Cancer Center and Department of Pharmacology, Georgetown University, Washington, DC). Binding reactions contained 1–10 μg of nuclear extract, 1 μg *E. coli* DNA (Sigma, St. Louis, MO) as a non-specific competitor, binding buffer (10 mM Tris, pH 7.5, 0.1 M KCl, 1 mM DTT, 1 mM EDTA, 6% glycerol), 160 nM NaCl and the protease inhibitors (Boehringer Mannheim, Indianapolis, IN) leupeptin (10 μg/ml), Pefabloc (3.5 μg/ml), and pepstatin (20 μg/ml). Concentrations of 0.3–1 ng of ^{32}P-labeled probe were added and the binding reactions were electrophoresed in a 6% non-denaturing polyacrylamide gel. DNA–protein complexes were detected by autoradiography as retarded bands relative to the free probe. The gel shift assays were perfomed three or more times with different preparations of nuclear extract and probe.

Competition experiments were performed [Singh et al., 1986] with unlabeled fragments added to the preliminary binding reaction. The 35mer oligonucleotide was manufactured by the Lombardi Cancer Center Synthesis and Sequencing Shared Resource, and consists of the sequence 5′-CTTAGAGGTTATGACTGCCAAGACACCATTTCATG-3′. The other oligonucleotides were purchased from Stratagene (La Jolla, CA) and have the following sequences: NF-1, 5′-ATTTGGCTTGAAGCCAATATG-3′; Sp1, 5′-GATCGATCGGGGCGGGGCGATC-3′; AP-2, 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′. The underlined portions of these oligonucleotides denote the consensus transcription factor binding sequences.

Southwestern Analysis

Southwestern analysis was performed according to Yan and Hung [Yan and Hung, 1991], with minor variations. Nuclear extract concentrations of 100 μg from a panel of cell lines were separated in a 12% SDS-polyacrylamide gel, proteins were transferred to a nitrocellulose Bioblot-NC membrane (Costar, Cambridge, MA), and blots were hybridized with a probe corresponding to the EGFR intron enhancer element. Pre-stained standard molecular weight protein markers (Amersham) were run alongside the nuclear extracts to determine the molecular weight of proteins interacting with DNA probes. Blots were blocked in blocking buffer (5% non-fat dry milk in 10 mM Hepes, pH 7.9) for 1 h at room temperature. The blots were hybridized with ^{32}P-labeled 35mer probe in hybridization buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.25% non-fat dry milk) along with 22 μg/ml single-stranded and 22 μg/ml double-stranded non-specific *E. coli* DNA for 1–3 h at room temperature, and blots were washed with washing buffer (10 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 0.1 mM DTT, 0.25% non-fat dry milk, 200 mM NaCl) for 2–3 h at room temperature with three buffer changes. Blots were exposed to Kodak XAR-5 film at −70°C to visualize DNA–protein interactions.

RESULTS

Transcriptional Activity of EGFR Intron 1

The proximal 2.1 kb region of the EGFR gene first intron contains general DNase I hypersensitive sites which are formed in breast cancer cells expressing high levels of EGFR. To characterize the transcriptional activity of this region, a series of CAT reporter gene constructs (Fig. 1) were made consisting of portions of the EGFR first intron cloned into the enhancer site of the parental pSV-CAT vector which contains the SV40 promoter.

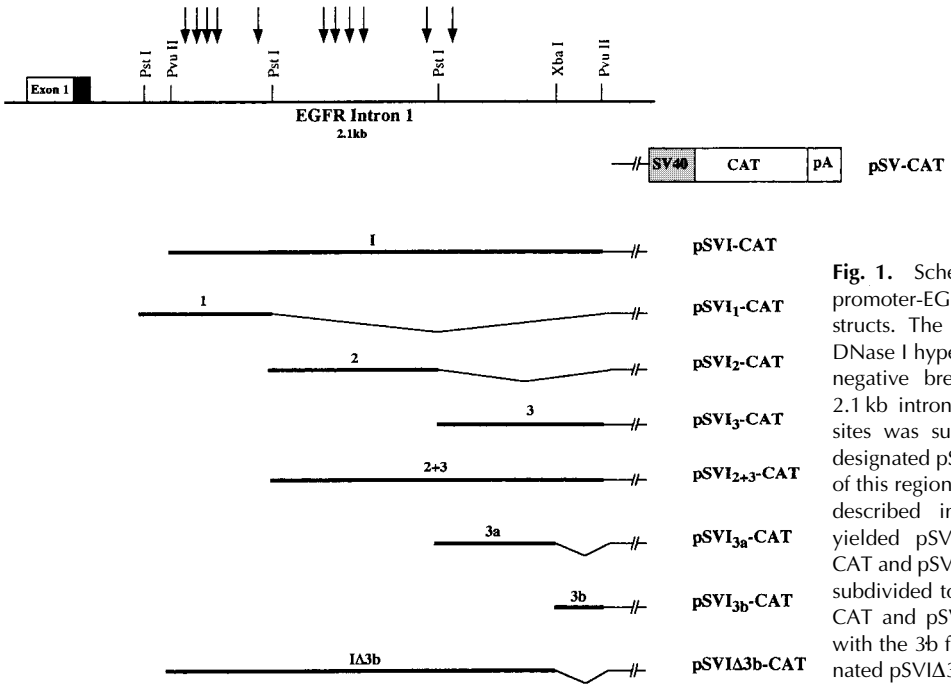

Fig. 1. Schematic drawing of the SV40 promoter-EGFR intron CAT reporter constructs. The arrows denote the intronic DNase I hypersensitive sites present in ER negative breast cancer cell lines. The 2.1 kb intron region delineated by *Pvu*II sites was subcloned into pSV-CAT and designated pSVI-CAT. The further division of this region subcloned into pSV-CAT (as described in Materials and Methods) yielded $pSVI_1$-CAT, $pSVI_2$-CAT, $pSVI_3$-CAT and $pSVI_{2+3}$-CAT. Region 3 was then subdivided to produce constructs $pSVI_{3a}$-CAT and $pSVI_{3b}$-CAT. The 2.1 kb region with the 3b fragment removed was designated pSVIΔ3b-CAT.

These constructs were transiently transfected into ER positive/low EGFR expressing MCF-7 breast cancer cells and ER negative/high EGFR expressing BT549 breast cancer cells, and the results are shown in Figure 2. The 2.1 kb first intron region of EGFR was capable of enhancing expression of the heterologous promoter in both cell lines, though to a greater extent in the ER negative/high EGFR expressing BT549 cells. This experiment indicated the presence of an intronic enhancer whose activity paralleled the EGFR expression observed in these cell lines. Truncation experiments showed that most of this activity was contained in the 660 bp region 3 fragment (Fig. 2A).

Further division of region 3 allowed us to delineate a 140 bp region (region 3b) that accounted for this enhancer activity which was preferentially active in the ER negative/high EGFR expressing BT549 cells as compared to the ER positive/low EGFR expressing MCF-7 cells (Fig. 2B). This difference in the CAT activity detected between the cell lines was significant for both region 3 ($P = 0.013$) and region 3b ($P = 0.019$). In comparison, region 3a did not significantly enhance activity in either cell line. When region 3b was deleted from the 2.1 kb intron region (construct pSVIΔ3b-CAT), the ability of the remaining intron region to induce CAT activity was significantly decreased from 12.6-fold to 1.7-fold ($P = 0.05$) in the BT549 cells (Fig. 2B). In the MCF-7 cells, the deletion of the region 3b from the 2.1 kb intron fragment did not cause a significant change in CAT activity. These results demonstrate the presence of enhancer activity within a 140 bp region (region 3b) of the EGFR gene first intron that is preferentially active in an ER negative/high EGFR expressing breast cell line as compared to an ER positive/low EGFR expressing breast cancer cell line.

Nuclear Factor Binding to the Enhancer Region of the EGFR First Intron

To begin to identify the nuclear factors which mediate the enhancer activity of the EGFR we performed gel shift assays using nuclear extracts from MCF-7 and BT549 cells. In these experiments the 140 bp 3b fragment produced a single distinctive band with nuclear extracts from both MCF-7 and BT549 cells (Fig. 3B). To further localize binding activity, we performed gel shift assays with the smaller 49 bp *Xba*I–*Hinf*I, 91 bp *Hinf*I–*Pvu* II and 91 bp *Dde*I

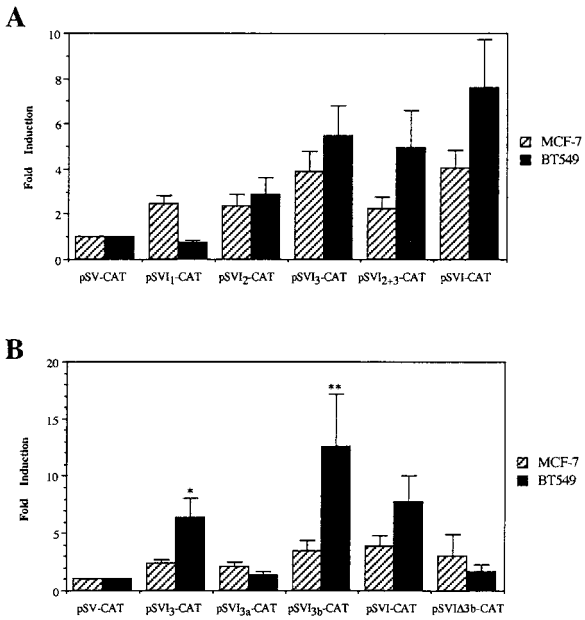

Fig. 2. Comparison of transcriptional activity of the SV40 promoter–EGFR intron CAT reporter constructs in the MCF-7 and BT549 cell lines. **A**: Transcriptional activities of the 2.1 kb intron fragment and its subfragments. **B**: Transcriptional activity of the intron region 3 and its subfragments. $^{*}P = 0.013$, $^{**}P = 0.019$.

fragments that comprise the 140 bp fragment (Fig. 3A). With each of these fragments there was no appreciable difference in the shifted patterns observed with nuclear extracts from MCF-7 and BT549 cells (data not shown), despite the differential levels of EGFR expression in these cells.

Gel shift competitions were then performed with all possible combinations of the 140 bp fragment and its sub-fragments. Based on these competition assays and sequence comparison of these fragments that revealed regions of homology, we synthesized a 35 bp oligonucleotide that was predicted to contain the major factor-binding activity. This fragment, referred to as the "35mer," spans from a 5′ *Dde*I restriction site to a *Hinf* I site, encompassing all but 14 bp of the 49 bp *Xba*I–*Hinf*I fragment (Fig. 3A). When incubated with nuclear extract from MCF-7 or BT549 cells, the 35mer produced a single band shift (Fig. 3C). In competition experiments, a 50-fold excess of the 35mer resulted in the almost complete disappearance of the shifted band observed with the 140 bp *Xba*I–*Pvu*II probe (Fig. 4A). The 35mer was also capable of competing away the shifted band that is seen with the 91 bp *Hinf* I–*Pvu*II probe even though the 35mer does not overlap with the 91 bp *Hinf* I–*Pvu*II fragment (Fig. 4B). Sequence comparison of the 35mer with the 91 bp *Hinf* I–*Pvu*II fragment revealed spans of only 4–6 bp in common. The four base sequences are AAGA, TATG, and TTTC. The five base sequences are TGACT and GAGGT and the six base sequence is AGAGGT. The ability of the 35mer to compete for protein binding sites in the 91 bp *Hinf* I–*Pvu*II fragment suggests that these common sequences could correspond to similar *cis*-acting elements involved in protein binding in multiple sites within the 140 bp fragment.

Competitions were performed with other synthetic oligonucleotides that correspond to known *cis* elements to determine the specificity of binding and to see if the DNA–protein interaction involved any of these known transcription factors (Fig. 5). Utilizing the 140 bp *Xba*I–*Pvu*II probe there was no significant competition with the Sp1 oligonucleotide which has no sequence homology with the 35mer. The AP-2 oligonucleotide was capable of competing with the main band but not as well as the 35mer. Interestingly, there is no homology between the 35mer and the concensus AP-2 binding site; however, two 4 bp sequences match between the oligonucleotides (ACTG and TGAC). The NF-1 oligonucleotide has two 4 and a 5 bp sequence in common with the 35mer, but it was unable to compete with the shifted band that occurs with the 140 bp probe. The lack of appreciable competition with these known *cis*-acting elements and the lack of sequence homology of the 35mer with any *cis*-acting elements in a database sequence analysis (by GCG Wisconsin and TFSEARCH) fails to match the sequences of the 35mer to any currently known transcription factor.

Transcriptional Activity of the 35mer Element

We tested the enhancer activity of the 35mer by subcloning it into the parental pJFEC vector in which the expression of CAT is driven by the EGFR promoter (Fig. 6A). The parental construct, pJFEC, has similar levels of activity in both MCF-7 and BT549 cells indicating that cell-specific regulation does not occur through this promoter region. We observed a modest 2.2-fold increase in activity in the MCF-7 cells ($P = 0.16$) while in the BT549 cells the 35mer was able to significantly induce activity 6.5-fold

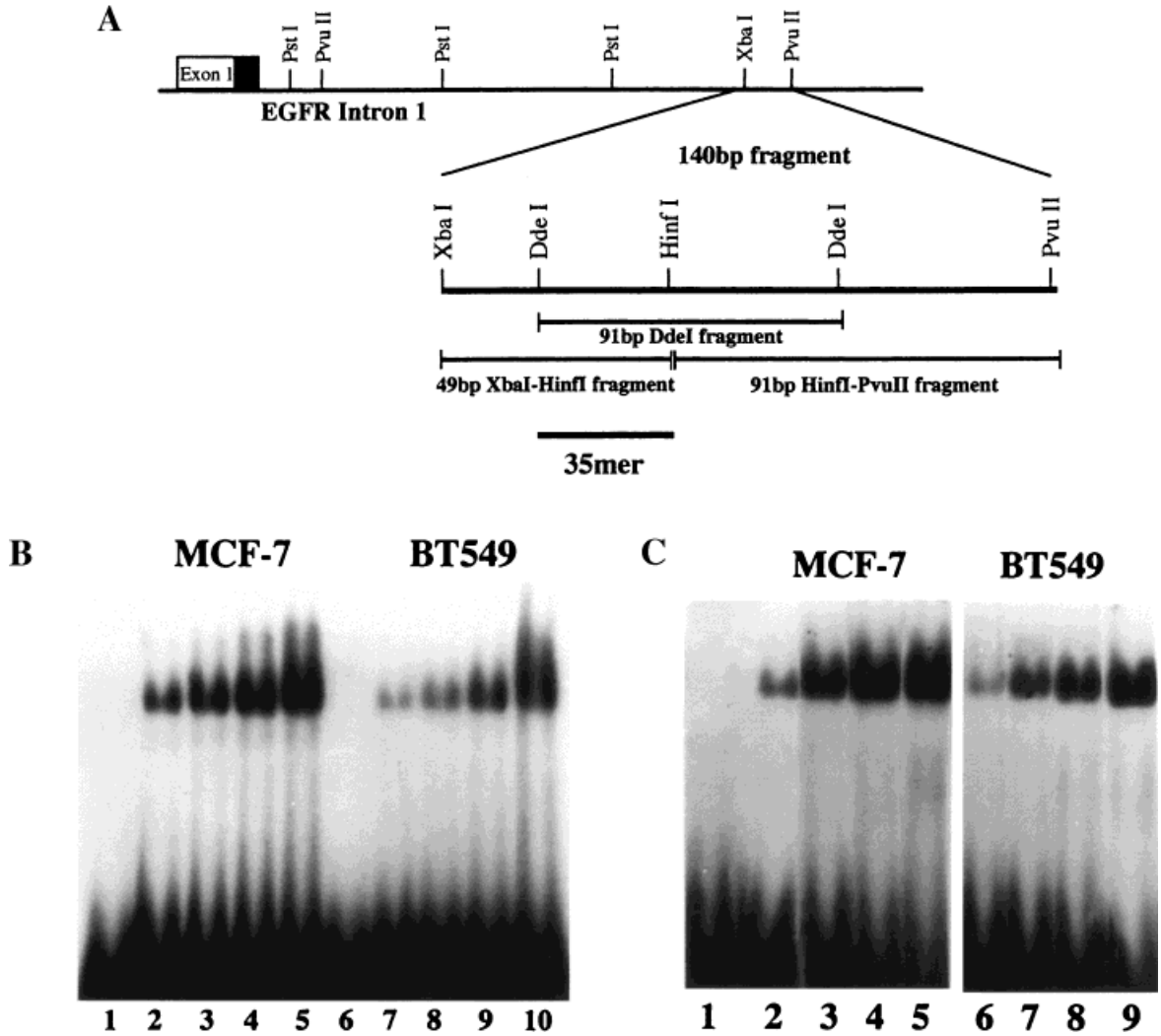

Fig. 3. Protein binding to the EGFR intron 1 enhancer region. **A**: Schematic representation of the fragments used as probes for the gel shift assays. **B**: Gel shift assay using the 140 bp region 3b fragment as a probe. **Lanes 1** and **6** are probe alone; **lanes 2–5** are probe incubated with 1, 2.5, 5, and 10 μg MCF-7 nuclear extract; and **lanes 7–10** are probe incubated with 1, 2.5, 5, and 10 μg BT549 nuclear extract. **C**: Gel shift assay using 35mer as probe. **Lane 1** is probe alone; **lanes 2–5** are probe incubated with 1, 2.5, 5, and 10 μg MCF-7 nuclear extract and **lanes 6–9** are probe incubated with 1, 2.5, 5, and 10 μg BT549 nuclear extract.

($P = 0.002$) over the activity of pJFEC (Fig. 6B). Thus, the 35mer is capable of specific enhancer activity in conjunction with the homologous EGFR promoter in the hormone-independent, high EGFR expressing BT549 breast cancer cell line.

Characterization of the 35mer Binding Protein

As an initial characterization of the protein(s) that binds to the 35mer enhancer element, we performed a southwestern analysis. Nuclear extracts from the ER positive breast cancer cell lines MCF-7 and BT474, the ER negative breast cancer cell lines MDA-MB-231 and BT549, and the non-breast cancer HeLa cell line were electrophoresed in an SDS-PAGE gel, Western blotted and then probed with the 35mer to determine the molecular weight and the abundance of DNA binding proteins. These cell lines express EGFR at a variety of levels: MCF-7 has low levels of EGFR expression, while BT474 and HeLa cells have moderate levels of EGFR, and the MDA-MB-231 and BT549 cell lines overexpress EGFR [Davidson et al., 1987; Xu et al., 1984; Yarden et al., 1996]. Our experiments show that a single protein of

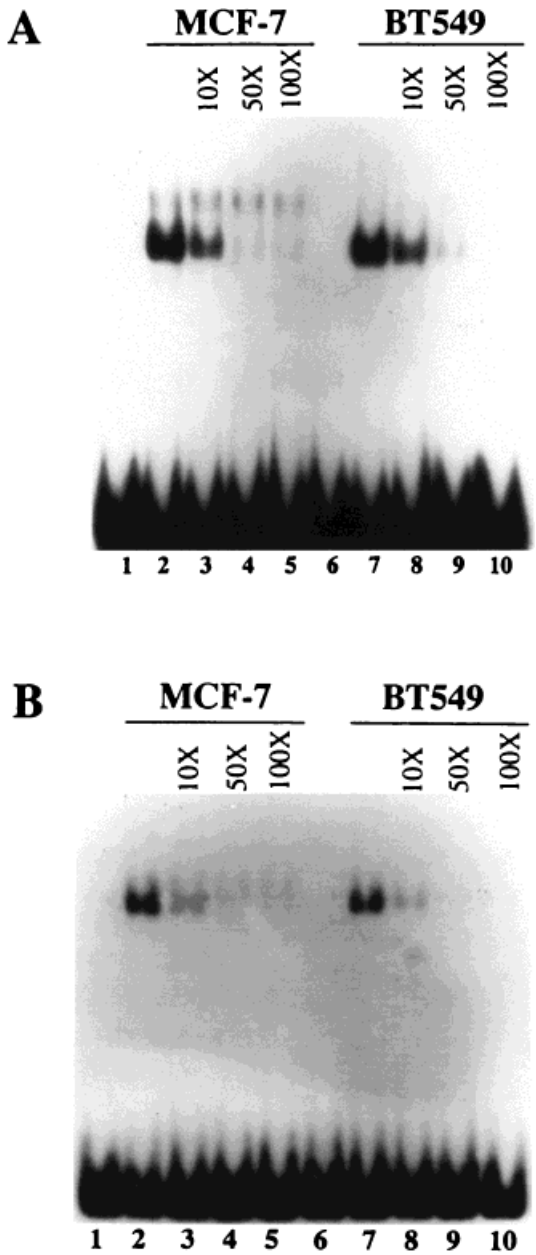

Fig. 4. Gel shift competition for protein binding activity with 35mer. **A**: Competition of 35mer for protein binding to the 140 bp region 3b probe. **Lanes 1** and **6** are probe alone; **lanes 2–5** are probe incubated with 5 μg of MCF-7 nuclear extract and **lanes 7–10** are probe incubated with 5 μg of BT549 nuclear extract; **lanes 3–5** and **lanes 8–10** are competed with increasing amounts of 35mer. **B**: Competition of 35mer for protein binding to the 91 bp *Hinf* I–*Pvu*II probe from region 3b. **Lanes 1** and **6** are probe alone; **lanes 2–5** are probe incubated with 5 μg of MCF-7 nuclear extract and **lanes 7–10** are probe incubated with 5 μg of BT549 nuclear extract; **lanes 3–5** and **lanes 8–10** are competed with increasing amounts of the 35mer.

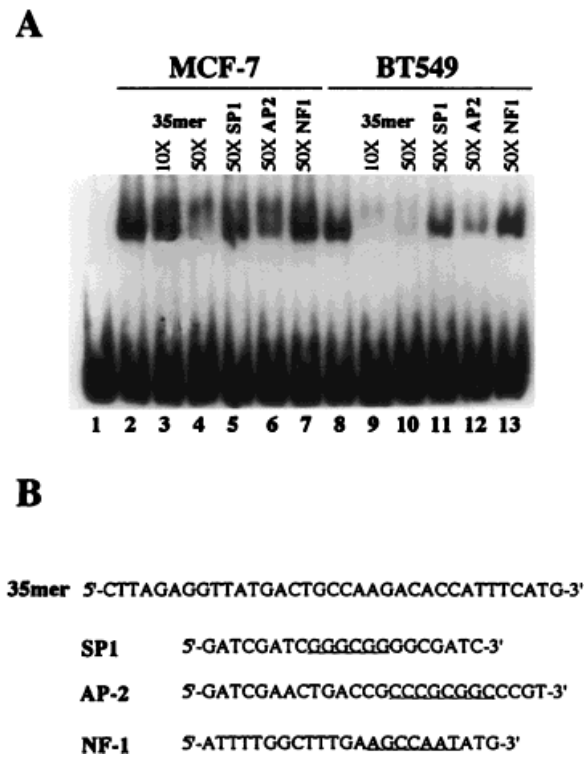

Fig. 5. Gel shift competition analysis of the EGFR intron enhancer region using consensus oligonucleotides. **A**: Competition of consensus oligonucleotides for protein binding to the 140 bp region 3b probe. **Lane 1** is probe alone; **lanes 2–7** are probe incubated with 5 μg of MCF-7 nuclear extract and **lanes 8–13** are probe incubated with 5 μg of BT549 nuclear extract; **lanes 3–7** and **lanes 9–13** are competed with the designated oligonucleotides. **B**: Sequence of the oligonucleotides used for competition. Underlined bases indicate the specific consensus *cis* elements.

≈130 kD exists in these tested cell lines, indicating that this protein might not be ER status or cell type-specific. Notably, the abundance of this protein is dramatically increased in the ER negative breast cancer cell lines as compared to the MCF-7 cell line and corresponds to the relative level of EGFR expression in these five cell lines.

DISCUSSION

We have investigated the regulatory role of the EGFR first intron in the expression of the EGFR gene and have identified a *cis*-acting element that is potentially involved in the overexpression of this gene in ER negative breast cancer cells. A 2.1 kb region of the first intron of EGFR that contains DNase I hyper-

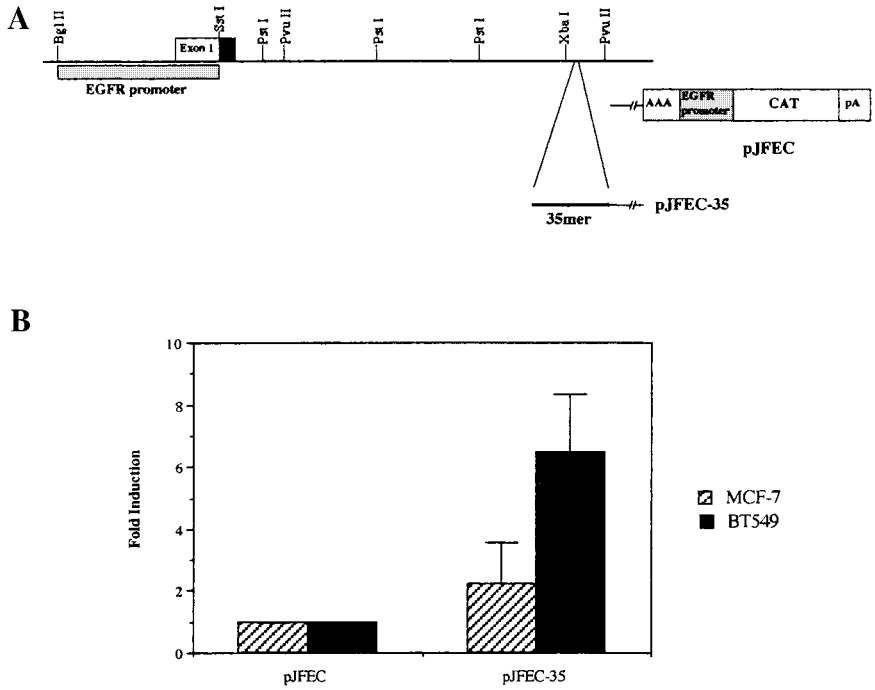

Fig. 6. Transcriptional activity of 35mer in conjunction with the EGFR promoter. **A**: Schematic drawing of the pJFEC and the pJFEC-35 constructs used in the assay. **B**: Activity of the EGFR promoter–35mer CAT reporter construct in MCF-7 vs. BT549 cells ($P = 0.002$). Data are from four separate assays.

sensitive sites specifically in ER negative, high EGFR expressing breast cancer cells [Chrysogelos, 1993] was found to have a positive regulatory effect. Subdivision of this intron region revealed an enhancer (region 3b) that was preferentially active in ER negative, high EGFR expressing breast cancer cells as compared to ER positive, low EGFR expressing breast cancer cells and was found to be essential to the regulatory effect of the 2.1 kb intron region. Further examination of region 3b determined that a 35 bp region contains the major site of DNA–protein interaction. A 130 kD protein that binds to the 35 bp region can be found in a variety of breast cancer cell lines with both low and high levels of EGFR as well as in HeLa cells. The amount of this protein correlates with the level of EGFR expression in these cell lines. Inspection of the sequence of the 35 bp enhancer region indicates no sequence homology with any known *cis*-acting element.

The majority of studies investigating the transcriptional regulation of the EGFR gene to date have utilized HeLa and A431 cells. A431 cells were derived from an epidermoid carcinoma and have an amplified and rearranged EGFR gene [Ullrich et al., 1984]. In vitro transcription assays and binding assays using A431 nuclear extracts demonstrated that numerous factors are capable of interacting with the EGFR promoter [Merlino et al., 1989]. Most of the transcription factors that have been shown to enhance activity through the EGFR promoter are general transcription factors, and their activities cannot account for the characteristic overexpression of EGFR in ER negative breast cancers [Johnson et al., 1988; Kageyama and Pastan, 1989; Kageyama et al., 1988; Merlino et al., 1989].

Further experiments using transient transfection assays looked at the role of the promoter and first intron in EGFR expression in HeLa cells. Two regions, one upstream from the major transcription start site and one downstream in the first intron showed enhancer activity individually [Maekawa et al., 1989]. These enhancers acted synergistically when they were in the same construct [Maekawa et al., 1989], and both of these regions correspond to DNase I hypersensitive sites in A431 cells, implying that regulatory factors bind to these regions in vivo. The downstream intronic enhancer was delineated to a 530 bp region that when transfected into MCF-7 cells repressed

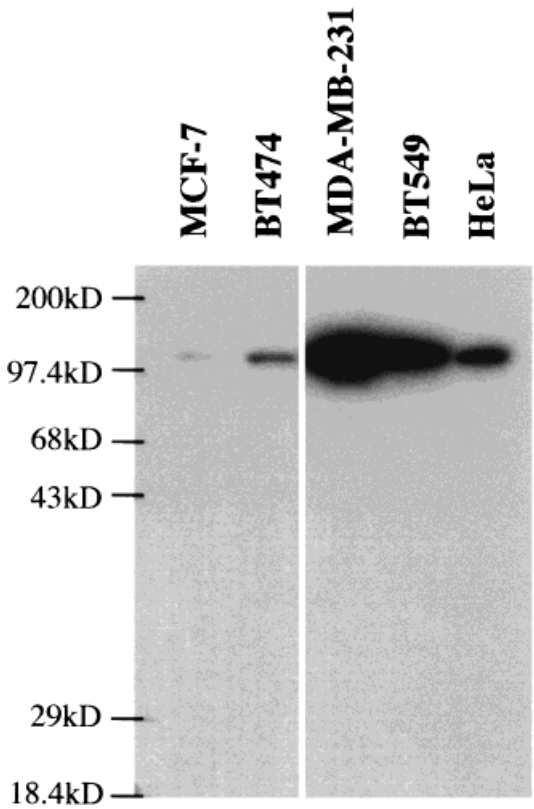

Fig. 7. Southwestern analysis of a factor binding to the 35mer enhancer element. Nuclear extracts (100 μg) from MCF-7, BT474, MDA-MB-231, and BT549 breast cancer cells, and HeLa cells were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the ^{32}P-labeled 35mer fragment.

promoter activity of the EGFR gene [Maekawa et al., 1989]. This prompted our investigation of the regulation of EGFR in breast cancer cells. Additionally, a recent study identified another intronic region that is involved in EGFR expression in breast cancer. This region contains a series of CA repeats whose length correlates to the expression of EGFR. In in vitro run-off assays using A431 nuclear extracts shorter lengths of CA repeats correlated with higher expression of EGFR [Gebhardt et al., 1999]. The lengths of these CA repeats were also found to correlate with EGFR levels in breast cancer cell lines and loss of heterozygosity of these repeats associated with elevated EGFR levels in breast tumors [Buerger et al., 2000]. The 35 bp enhancer we delineated is 113 bp downstream of the intron enhancer defined by Maekawa et al. and is ≈1.2 kb downstream of the CA repeat region.

While it is possible that the level of the factor available for binding to the enhancer region may determine the cell specificity of its function, it is also possible that other regulatory proteins that come in contact with this protein are responsible for the function of this element as an enhancer in high EGFR expressing breast cancer cells and can act to repress its activity in low EGFR expressing breast cancer cells. Additionally, the difference in EGFR expression between high and low EGFR expressing breast cancer cell lines may be affected by the accessibility of *cis*-acting elements in the first intron to their cognate factors. The dramatically different pattern of DNase I hypersensitive sites in the EGFR gene in ER negative, high EGFR expressing breast cancer cell lines versus ER positive, low EGFR expressing breast cancer cell lines [Chrysogelos, 1993] suggests that the chromatin structure of the regulatory regions of the EGFR first intron may play a role in determining the expression level of the gene.

These studies have demonstrated that a region of the EGFR gene first intron has the ability to act as transcriptional enhancer specifically in hormone-independent breast cancer cells that overexpress EGFR. Further work to identify the protein(s) involved and to elucidate the mechanisms by which this element regulates the expression of EGFR in breast cancer will be important in our understanding of the progression of breast cancer to an aggressive, hormone-independent state.

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REFERENCES

Bolufer P, Miralles F, Rodriguez A, Vasquez C, Lluch A, Garcia-Conde J, Olmos T. 1990. Epidermal growth factor receptor in human breast cancer: correlation with cytosolic and nuclear estrogen receptors and with biological and histological tumor characteristics. Eur J Cancer 26:283–290.

Buerger H, Gebhardt F, Schmidt H, Beckmann A, Hutmacher K, Simon R, Lelle R, Boecker W, Brandt B. 2000. Length and loss of heterozygosity of an intron 1 polymorphic sequence of *egfr* is related to cytogenetic alterations and epithelial growth factor receptor expression. Cancer Res 60:854–857.

Chrysogelos SA. 1993. Chromatin structure of the EGFR gene suggests a role for intron 1 sequences in its regulation in breast cancer cells. Nucleic Acids Res 21:5736–5741.

Davidson NE, Gelmann EP, Lippman ME, Dickson RB. 1987. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinol 1:216–223.

Derynck R, Goddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH. 1987. Synthesis of mRNAs for TGF-α and β and epidermal growth factor receptor by human tumors. Cancer Res 47:707–712.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

DiMarco E, Pierce JH, Fleming TP, Krauss MH, Molley CJ, Aaronson SA, DiFiora PP. 1989. Autocrine interaction between TGF-α and EGF-receptor: quantitative requirement for induction of the malignant phenotype. Oncogene 4:831–838.

Fitzpatrick SL, Brightwell J, Wittliff J, Barrows GH, Schultz GS. 1984. Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen and progestin receptor levels. Cancer Res 44:3448–3453.

Fridovich-Keil JL, Gudas JM, Bryan IB, Pardee AB. 1991. Improved expression vectors for eukaryotic promoter/enhancer studies. BioTechniques 11:572–579.

Fried M, Crothers DM. 1981. Equilibria and kinetics of lac repressor–operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res 9:6505–6525.

Garner MM, Revzin A. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res 9:3047–3060.

Gebhardt F, Zanker KS, Brandt B. 1999. Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. J Biol Chem 274:13176–13180.

Gorman CM, Moffat LF, Howard BH. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051.

Gross DS, Garrard WT. 1988. Nuclease hypersensitive sites in chromatin. Annu Rev Biochem 57:159–197.

Gullick WJ. 1991. Prevalence of aberrant expression of the epidermal growth factor in human cancers. Br Med Bull 47:87–98.

Habets GG, van der Kammen RA, Willemsen V, Balemans M, Wiegant J, Collard JG. 1992. Sublocalization of an invasion-inducing locus and other genes on human chromosome 7. Cytogenet Cell Genet 60:200–205.

Hawley-Nelson P, Ciccarone V, Gebeyehu G, Jessee J, Felgner PL. 1993. Lipofectamine reagent: a new, higher efficiency polycationic liposome transfection reagent. Focus 15:73–83.

Ishii S, Xu YH, Stratton RH, Roe BA, Merlino GT, Pastan I. 1985. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc Natl Acad Sci USA 82:4920–4924.

Johnson A, Ishii S, Jinno Y, Pastan I, Merlino GT. 1988. Epidermal growth factor receptor gene promoter: deletion analysis and identification of nuclear protein binding sites. J Biol Chem 263:5693–5699.

Kageyama R, Merlino GT, Pastan I. 1988. Nuclear factor ETF specifically stimulates transcription from promoters without a TATA box. J Biol Chem 264:15508–15514.

Kageyama R, Pastan I. 1989. Molecular cloning and characterization of a human DNA binding factor that represses transcription. Cell 59:815–825.

King CR, Kraus MH, Williams LT, Merlino GT, Pastan IH, Aaronson SA. 1985. Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNA. Nucleic Acids Res 13:8477–8486.

Koenders PG, Beex LV, Geurts-Moespot A, Heuvel JJ, Kienhuis CBM, Benraad TJ. 1991. Epidermal growth factor receptor-negative tumors are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. Cancer Res 51:4544–4548.

Maekawa T, Imamoto F, Merlino GT, Pastan, Ishii S. 1989. Cooperative function of two separate enhancers of the human epidermal growth factor receptor. J Biol Chem 264:5488–5494.

Merlino GT, Johnson AC, Kageyama R, Pastan, I. 1989. Isolation and characterization of DNA binding factors regulating transcription of the EGF receptor proto-oncogene. In: Growth regulation of cancer II: UCLA symposia on molecular and cellular biology. New York: Alan R. Liss, Inc.

Murphey JM, Chrysogelos SA. 1995. Direct measurement of transfection efficiency in transient transfection assays. Biotechniques 18:967–969.

Nicholson S, Sainsbury JR, Halcrow P, Chambers P, Farndon JR, Harris AL. 1989. Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet i: 82–184.

Nicholson S, Richard J, Sainsbury C, Halcrow P, Kelly P, Angus B, Wright C, Henry J, Farndon JR, Harris AL. 1991. Epidermal growth factor receptor; results of a 6 year follow-up study in operable breast cancer with emphasis on the node negative subgroup. Br J Cancer 63:146–150.

Prost E, Moore DD. 1986. CAT vectors for analysis of eukaryotic promoters and enhancers. Gene 45:107–111.

Sainsbury JR, Farndon JR, Sherbert GV, Harris AL. 1985. Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Lancet i:364–366.

Sainsbury JR, Needham GK, Farndon JR, Malcolm AJ, Harris AL. 1987. Epidermal growth factor receptor status as predictor of early recurrence and death from breast cancer. Lancet 1:1398–1402.

Sambrook J, Fritsch EF, Maniatis T, editors. 1989. In: Molecular cloning: A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Singh H, Sen R, Baltimore D, Sharp PA. 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunglobin genes. Nature 319:154–158.

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Liberman TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the

amplified gene in A431 epidermoid carcinoma cells. Nature 309:418–425.

Xu YH, Richert N, Ito S, Merlino GT, Pastan IH. 1984. Characterization of epidermal growth factor receptor gene expression in malignant and normal cell lines. Proc Natl Acad Sci USA. 81:7308–7312.

Yan D-H, Hung M-C. 1991. Identification and characterization of a novel enhancer for the rat *neu* promoter. Mol Cell Biol 11:1875–1882.

Yarden RI, Lauber AH, El-Ashry D, Chrysogelos SA. 1996. Bimodal regulation of EGF receptor by estrogen in breast cancer cells. Endocrinology 137:2739–2747.