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Identification and Characterization of a Negative Regulatory Element Within the Epidermal Growth Factor Receptor Gene First Intron in Hormone-Dependent Breast Cancer Cells

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Abstract The epidermal growth factor receptor (EGFR) exhibits an inverse correlation with estrogen receptor (ER) expression in the majority of breast cancers, predicting a poor response to endocrine therapy and poor survival rate. Inappropriate overexpression of EGFR in breast cancer is associated with a more aggressive phenotype. Transcriptional regulation is the major regulatory mechanism controlling EGFR overexpression in breast cancer cells. We have identified a region within the first intron of the EGFR gene that mediates transcriptional repression of EGFR gene expression in ER +/low EGFR expressing but not in ER−/high EGFR expressing breast cancer cells. Utilizing transient transfections of homologous and heterologous promoter-reporter constructs, we localized optimal repressive activity to a 96 bp intron domain. The 96 bp fragment displayed differential DNA-protein complex formation with nuclear extracts from ER + vs. ER− breast cancer cells. Moreover, factors interacting with this intron negative regulatory element appear to be estrogen-regulated. Consequently, our results suggest that we have identified a potential mechanism by which maintenance of low levels of EGFR expression and subsequent EGFR upregulation may be attributed to the loss of transcriptional repression of EGFR gene expression in hormone-dependent breast cancer cells. J. Cell. Biochem. 85: 601–614, 2002. © 2002 Wiley-Liss, Inc.

Key words: gene expression; transcriptional repression; EGFR overexpression; estrogen-receptor positive; estrogen regulated

Clinically, breast cancer can be characterized by its estrogen receptor (ER) status and is generally believed to progress from an ER +, hormone-dependent, anti-estrogen sensitive phenotype to an ER−, hormone-independent, anti-estrogen insensitive phenotype [Fitzpatrick et al., 1984; Sainsbury et al., 1985]. Initially, the majority of breast tumors are under the control of estrogen, which acts through the ER, and elicits multiple responses including cellular proliferation and inhibition of apoptosis [el-Ashry and Lippman, 1994; Dong et al., 1999]. Subsequent progression of breast tumors to a hormone-independent phenotype involves the disruption of normal control mechanisms, as well as the loss of estrogen regulation of cellular processes. One mechanism by which cells overcome the estrogen-dependence of growth is through upregulation of growth factor receptors and/or their ligands [van Agthoven et al., 1992]. The epidermal growth factor receptor (EGFR) is one particular receptor that has been demonstrated to be overexpressed in breast cancer [van Agthoven et al., 1992].

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There is a noted inverse correlation between the levels of ER and EGFR with the majority of breast tumors being either ER+/EGFR− or ER−/EGFR+ [Harris, 1988; Koenders et al., 1991; Klijn et al., 1992; van Agthoven et al., 1992]. While a significant number of tumors co-express both receptors [Koenders et al., 1991; Klijn et al., 1992], immunohistochemical analysis of cells from ER+/EGFR+ tumors demonstrate that individual cells within this population overexpress only one receptor (i.e., ER or EGFR), but not both. These data emphasize the inverse relationship between these two receptors. The expression of EGFR, independent of ER status, is indicative of a more aggressive phenotype and predicts for poor response to endocrine therapy [Nicholson et al., 1988] and poor survival rate [Toi et al., 1991], suggesting that upregulation of EGFR is involved in the progression to a more aggressive hormone-independent phenotype.

EGFR is a 170 kDa N-glycosylated transmembrane protein with intrinsic tyrosine kinase activity [Xu et al., 1984; Harris, 1988]. Upon ligand binding, including EGF and TGF-α, the receptor dimerizes and undergoes autophosphorylation, initiating a cascade of intracellular signaling events leading to growth stimulation [Carpenter and Zendegui, 1986; Harris, 1988]. The EGFR has been demonstrated to be overexpressed in a wide variety of tumors including lung, bladder, gastric, head, neck, and breast, with overexpression correlating with advanced disease or predicting for poor survival [Gullick, 1991]. Whereas increased EGFR expression has been attributed to gene amplification in some types of tumors, this is rare in breast cancer [Davidson et al., 1987; Gullick, 1991]. Through nuclear run-on experiments and correlation between protein and mRNA levels, it has been established that transcriptional control is the predominant mechanism responsible for EGFR overexpression in breast cancer [Davidson et al., 1987; Haley et al., 1987; Kageyama et al., 1988a,b].

The EGFR gene, located on chromosome 7p12–14, is 110 kb and consists of 26 exons, with the first intron alone being 18 kb [Merlino et al., 1985; Haley et al., 1987; Kondo et al., 1992]. The EGFR gene promoter, which does not contain either TATA or CAAT elements [Ishii et al., 1985], contains GC rich elements [Ishii et al., 1985; Haley et al., 1987] and multiple transcription start sites [Ishii et al., 1985]. Factor binding sites in the promoter have been identified using HeLa (human cervical carcinoma cells) and A431 (human epidermoid carcinoma cells, which contain an amplified EGFR gene) cells that are responsible for both enhancing and repressing EGFR gene transcription, including: Sp1 [Ishii et al., 1985; Haley et al., 1987]; TC factor (TCF) [Haley et al., 1987; Merlino et al., 1989]; ETF1 and ETF2 [Kageyama et al., 1988b; Merlino et al., 1989]; GCF1 and GCF2 [Kageyama and Pastan, 1989; Reed et al., 1998]; and ETR [Hou et al., 1994]. Both positive and negative regulatory factors appear to play an important role in the regulation of EGFR gene expression in other cell lines [Kageyama et al., 1988a; Kageyama and Pastan, 1989] and are suspected to do so in breast cancer cell lines.

We have investigated the differential regulation of EGFR gene expression in ER+ vs. ER− breast cancer cells, with particular emphasis on the transcriptional mechanisms responsible for this inverse relationship [Chrysogelos, 1993; McInerney et al., 2001; Yarden et al., 2001]. We have previously demonstrated that regions of the EGFR gene first intron are involved in both the enhancement and repression of EGFR gene expression in breast cancer cells [Chrysogelos, 1993; McInerney et al., 2001]. Here we demonstrate that a 96 bp element within the first intron of the EGFR gene is involved in the transcriptional repression of EGFR gene expression in ER+/low EGFR expressing breast cancer cells, but not in ER−/high EGFR expressing breast cancer cells. This negative regulatory element interacts with a complex of factors that are required for the observed repressive transcriptional activity. Furthermore, the factors interacting with this intron negative regulatory element appear to be estrogen-regulated. Our observations propose a mechanism by which EGFR gene expression is regulated through an intron repressor element, further contributing to the accumulated information regarding the overall mechanism by which EGFR is transcriptionally regulated.

MATERIALS AND METHODS

Tissue Culture

Breast cancer cell lines were obtained from the Lombardi Cancer Center Tissue Culture Core Facility. The BT549 breast cancer cell line was originally obtained from the American Type Culture Collection (ATCC, Rockville, MD).

The MCF-7 breast cancer cell line was originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation). MCF-7 and BT549 cells were maintained in modified IMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD) (growth medium). Cells were maintained at 37°C in a 95% air–5% CO_2 humidified incubator. Media was changed every 3–4 days, and cells were passaged approximately every week.

MCF-7 cells were also treated with the antiestrogen ICI 182,780 (ICI) (Zeneca Pharmaceuticals, UK); growth medium was supplemented with 1×10^{-7} M ICI, dissolved in 100% ethanol. Cells were treated with ICI for 5 days prior to harvest for nuclear extract preparation, and media was changed after 3 days.

DNA Constructs

Constructs used throughout this work were initially subcloned from two clones we received from Dr. Glenn Merlino (National Cancer Institute, NIH, Bethesda, MD), pERCAT2DE [Maekawa et al., 1989] and pEP1 [Ishii et al., 1985]. All restriction enzymes used throughout this work, with the exception of *Sst* I, were from New England Biolabs, Inc. (Beverly, MA); *Sst* I was from Gibco/BRL (Life Technologies, Inc., Rockville, MD). The positions of all EGFR elements are relative to the translational start site.

The SV40 promoter CAT constructs, designated promCAT (or pC), were constructed from Promega's pCAT-promoter plasmid (Promega Co., Madison, WI), which contains the SV40 promoter directly upstream of the CAT gene and a downstream multiple cloning site. Initially, the 730 bp EGFR intron 1 *Pst* I fragment (+ 232 to + 962) from the pEP1 genomic clone [Ishii et al., 1985] was subcloned into the *Pst* I site of the promCAT vector [McInerney et al., 2001], and designated pC-730. A 305 bp *Sau3A* I fragment (+ 313 to + 617) was isolated from the 730-bp fragment and subcloned into the *BamH* I site of the promCAT vector, and designated pC-305F and pC-305R, where F and R correspond to the forward and reverse orientation, respectively.

The EGFR promoter CAT constructs, designated pJFEC (or pJ), originate from the pJFCAT vector obtained from Dr. Judith Fridovich-Keil (Emory University, Atlanta, GA), which contains a triple polyadenylation cassette upstream of the promoter cloning region [Fridovich-Keil et al., 1991]. 840 bp of the EGFR gene proximal promoter from pERCAT2DE (which extends from −855 to −15 relative to the translational start site and contains all of the mapped in vivo and in vitro transcriptional start sites [Ishii et al., 1985; Haley and Waterfield, 1991]) was subcloned into the *Bgl* II-*Xho* I sites of the pJFCAT vector, upstream of the CAT gene [McInerney et al., 2001], and designated pJFEC. All EGFR intron fragments were subcloned into the *Kpn* I/*Sst* I restriction sites in pJFEC.

The 305 bp *Sau3A* I intron 1 fragment (+ 313 to + 617) was subcloned into the *BamH* I site of the pGEM-7Zf vector (Promega), before being subcloned into pJFEC; these constructs were designated pJ-305F and pJ-305R, where F and R correspond to the forward and reverse orientation, respectively. The 80-bp *Pst* I-*Sau3A* I intron 1 fragment (+ 232 to + 312) from the 730-bp region was subcloned into the *Pst* I and *BamH* I sites of pBluescript II KS (Stratagene, La Jolla, CA), and then into pJFEC; this construct was designated pJ-80. The 309-bp *Sau3A* I-*Pst* I intron 1 fragment (+ 618 to + 926) from the 730-bp region was subcloned into the *Pst* I and *Bgl* II sites of pJFCAT, digested with *Pst* I and *Xho* I, subcloned into the *Pst* I and *Ava* I sites of pBluescript II KS, and subcloned into pJFEC; this construct was designated pJ-309.

Digestion of the 305-bp intron region with *Alu* I and *Pml* I resulted in three fragments, 56-, 96-, and 150-bp fragments. The 56-bp *Kpn* I-*Pml* I intron fragment (+ 313 to + 371) was subcloned into the *Kpn* I and *Sma* I sites in pBluescript II KS, before being subcloned into pJFEC; this construct was designated pJ-56. The 150-bp *Alu* I-*Sst* I intron fragment (+ 468 to + 617) was subcloned into the *Sma* I and *Sst* I sites in pBluescript II KS, and then subcloned into pJFEC; this construct was named pJ-150. The plasmid designated pJ-96 was constructed by PCR amplification of the 96-bp fragment (+ 372 to + 467) within the 305-bp intron region. The primers used for amplification, as well as the addition of *Hind* III and *BamH* I restriction sites, were: forward-96, 5′ ATTAATAAGCTT-GTGCGCCCCGCGCTG 3′, and reverse-96, 5′ CAGACTGGATCCCTTCCCTAAACAGTG 3′. The amplified 96-bp fragment was subcloned into the *Hind* III and *BamH* I sites in pBluescript II KS and then subcloned into pJFEC.

Oligonucleotides representing the 59-bp and 37-bp intron fragments (+372 to +431 and +432 to +467, respectively) were utilized to construct the plasmids pJ-59 and pJ-37. The oligos were synthesized with *Hind* III and *BamH* I ends and annealed in annealing buffer (100 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM EDTA pH 8.0) with equivalent amounts of sense and anti-sense strands followed by incubation at 90°C for 1 min, incubation at 65°C for 10 min, and then cooled to room temperature. The annealed oligos were subcloned into pBluescript II KS at the *Hind* III and *BamH* I sites, cut out with *Kpn* I and *Sst* I, and subcloned into these sites in pJFEC.

Transient Transfections and Chloramphenicol Acetyltransferase (CAT) Assays

DNA constructs were prepared by a modified alkaline lysis method [Sambrook et al., 1989] as described [McInerney et al., 2001]. The purified DNA was quantitated by spectrophotometry and checked by electrophoresis in a 1% agarose gel. Transient transfections were performed with at least three different DNA preparations. Breast cancer cells were plated for transfection in 100 mm dishes at a density of approximately $1–5 \times 10^6$ cells. Transient transfections with promoter-EGFR intron CAT constructs, carried out in duplicate, were performed the day after plating when cells were 75–85% confluent by the lipofectamine method [Hawley-Nelson et al., 1993]. DNA (10 μg) was mixed with 0.5 ml serum-free media and 20 μl (40 μg) of lipofectAMINE™ Reagent (Gibco/BRL Life Technologies; Rockville, MD) was mixed with 0.5 ml of serum-free media. These two components were then mixed together and incubated at room temperature for 30 min. This transfection mixture was diluted with 3 ml of modified IMEM supplemented with 5% FBS and incubated on cells for 16–18 h. After incubation, the transfection mixture was removed from the cells and replaced with growth media plus 1% penicillin/streptomycin (Biofluids, Rockville, MD), and any additional treatments, for 48 h. Cells were washed once with cold phosphate buffered saline (PBS; Life Technologies, Rockville, MD) and harvested in TEN Buffer (0.04 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.15 M NaCl). Cell pellets were stored at −70°C.

Whole cell lysates were prepared from harvested cell pellets in 0.25 M Tris-HCl, pH 7.8, by the freeze/thaw method. Cells were incubated in an ethanol/dry ice bath for 5 min, followed by incubation at 37°C for 5 min. After three rounds of freezing and thawing, cell debris was pelleted at 4°C at 12,000 rpm for 5 min. Soluble proteins were transferred to new tubes, and the protein concentration of the cell lysates was determined using the Bradford Assay (Bio-Rad, Melville, NY). Lysates were normalized for protein concentration and were used in the CAT assay reaction.

Chloramphenicol acetyltransferase (CAT) assays were performed by the thin layer chromatography (TLC) method [Gorman et al., 1982; Prost and Moore, 1986]. Between 50–200 μg protein were used in CAT assay reactions. Products were resolved on TLC plates run in a chloroform:methanol (95:5) mixture. Spots from TLC plates corresponding to substrate (unacetylated chloramphenicol) and products (acetylated chloramphenicol) were cut out of the TLC plates and counted by scintillation and percent conversion of substrate was calculated. Results from CAT assays were normalized to protein concentration, and percent activity of individual intron elements was determined relative to the activity of the parental vector (i.e., promoter alone). Results are presented as percent activity ± the standard error. Statistical analysis of CAT data was performed using SigmaPlot's Student's *t*-test (Jandel Scientific).

Electrophoretic Mobility Shift Assay

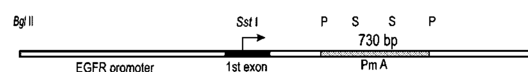
Electrophoretic mobility shift or gel shift assays were performed as described [Fried and Crothers, 1981; Garner and Revzin, 1981]. Nuclear extracts from various breast cancer cell lines, as well as HeLa cells, were prepared according to the method of Dignam et al. [1983]. Reaction mixtures were incubated at room temperature for 20 min, 1 μl (0.4 ng) (between 10,000 and 30,000 dpm/μl) of the DNA probe was added, and incubated for another 10 min at room temperature, before being placed on ice. Reaction mixtures were separated in 6% non-denaturing polyacrylamide gels, and DNA-protein complexes were visualized by exposure of dried gels to Kodak XAR-5 film at −70°C. Probes were labeled by using T4 polynucleotide kinase (PNK; NEB, Beverly, MA) and 32P-ATP (Amersham Life Science, Inc., Arlington heights, IL).

RESULTS

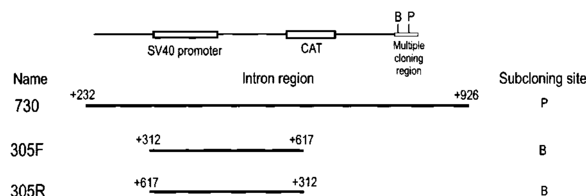
Identification of an Intron Negative Regulatory Region

Both in vivo and in vitro experiments performed in our laboratory have demonstrated the importance of regions within the EGFR gene first intron in the differential regulation of EGFR gene expression in ER+ vs. ER− breast cancer cell lines [Chrysogelos, 1993; McInerney et al., 2001]. Initial investigations with EGFR intron 1 elements suggested that there were regions demonstrating differential transcriptional activity [McInerney et al., 2001]. As a result, we were interested in identifying and characterizing intron elements involved in the negative regulation of EGFR transcriptional activity in breast cancer cells. Transient transfections were performed with heterologous and homologous promoter/CAT constructs in ER+/low EGFR expressing MCF-7 cells vs. ER−/high EGFR expressing BT549 cells. The SV40 promoter was used to address the ability of intron fragments to regulate a heterologous promoter (designated promCAT), while the homologous EGFR promoter was used to investigate the ability of intron elements to regulate the transcriptional activity of its own promoter (designated pJFEC). EGFR gene first intron elements were subcloned into the multiple cloning sites within these promoter/CAT constructs (Fig. 1).

Based on indications from previous experiments, we further investigated the transcriptional activity of a 730-bp region within the EGFR gene first intron (Fig. 1A) that demonstrated differential transcriptional activity with an SV40 promoter CAT construct in breast cancer cell lines [McInerney et al., 2001]. In MCF-7 breast cancer cells, which are ER+/low EGFR expressors, results from transient transfections of SV40 promoter-EGFR intron constructs demonstrated that the 730-bp region of the EGFR gene first intron exhibited a decrease in transcriptional activity, repressing transcription $50\% \pm 12.3$ ($P < 0.001$) ($n = 7$) compared to the parental SV40 promoter (Fig. 2A). However, in BT549 breast cancer cells, which are ER−/high EGFR expressors, results from transient transfections of SV40 promoter-EGFR intron constructs demonstrated that the 730-bp region did not exhibit decreased transcriptional activity (Fig. 2B). In fact, the 730-bp region increased SV40 transcriptional activity in the BT549 cells. Using convenient



A. SV40 promoter constructs



B. EGFR promoter constructs

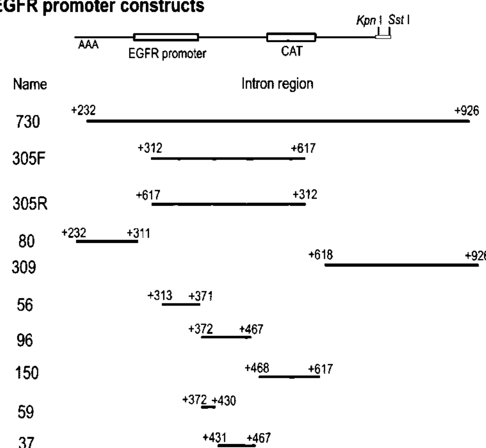


Fig. 1. Depiction of EGFR gene first intron fragments and promoter/CAT constructs. Map of ~2.5 Kb of the EGFR gene including the proximal promoter, first exon, and ~1.5 Kb of the first intron is shown. Schematic representation of EGFR gene first intron elements subcloned into reporter constructs used in transient transfections to investigate transcriptional activity. The indicated positions of the EGFR intron fragments are relative to the translational start site. **A**: SV40 promoter-EGFR intron constructs. EGFR intron elements were subcloned into the parental SV40-promoter-CAT construct, designated promCAT (or pC) as described in Materials and Methods. Sites of subcloning are indicated. **B**: EGFR promoter-intron constructs. EGFR intron elements were subcloned into the parental EGFR promoter-CAT construct, designated pJFEC (or pJ) as described in Materials and Methods. Intron elements were subcloned into the *Kpn* I and *Sst* I restriction sites. F and R refer to the forward and reverse orientation, respectively, of subcloned intron fragments. A, *Alu* I; B, *BamH* I; P, *Pst* I; Pm, *Pml* I; and S, *Sau3A* I.

restriction sites, this 730-bp region was divided into smaller fragments in order to identify the negative regulatory element responsible for this repressive activity. In MCF-7 breast cancer cells, results from transient transfections, demonstrated that a 305-bp portion of this intron region (Fig. 1A) retained repressive activity and repressed transcriptional activity of the SV40

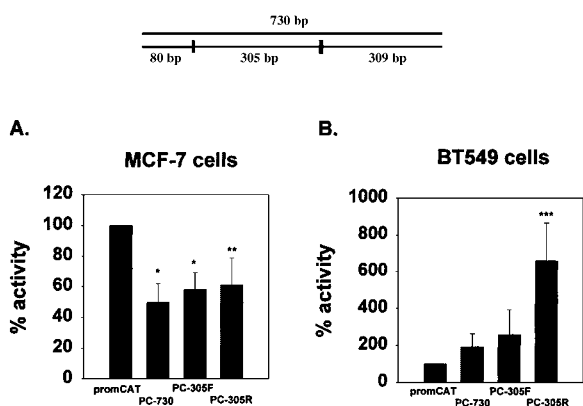


Fig. 2. Identification of an intron element that represses transcriptional activity in ER+/low EGFR expressing MCF-7 cells. Transient transfections of heterologous SV40 promoter-EGFR intron constructs were performed as described in Materials and Methods. A schematic of the 730-bp intron region along with its subfragments is depicted. **A**: Activity in ER+/low EGFR expressing MCF-7 cells. **B**: Activity in ER−/high EGFR expressing BT549 cells. Activity of intron fragments is expressed relative to parental vector (promoter alone/prom CAT) activity ± SE. F and R refer to forward and reverse fragment orientation, respectively. *P* values: $*P < 0.001$; $**P = 0.002$; and $***P = 0.04$.

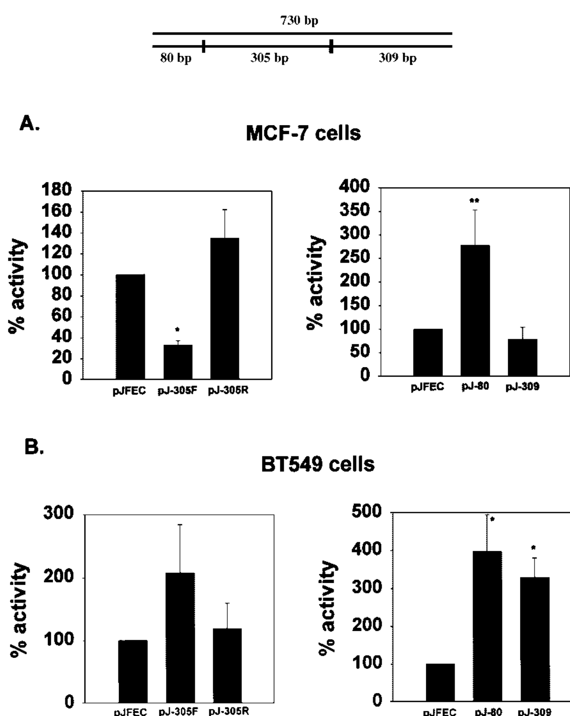


Fig. 3. Intron regions within the EGFR gene first intron demonstrate differential transcriptional activity in breast cancer cells. As described in Figure 2, except transient transfections were performed with EGFR promoter-intron constructs. **A**: Activity in ER+/low EGFR expressing MCF-7 cells. **B**: Activity in ER−/high EGFR expressing BT549 cells. *P* value: $*P < 0.001$ and $**P = 0.04$.

promoter 44% ± 11 ($P < 0.001$) (n = 9) in the forward orientation and 39% ± 17.9 ($P = 0.02$) (n = 6) in the reverse orientation (Fig. 2A). In BT549 breast cancer cells, results from transient transfections of SV40 promoter-EGFR intron constructs demonstrated that the 305-bp intron fragment did not decrease SV40 transcriptional activity either in the forward or reverse orientation (Fig. 2B). Moreover, in the reverse orientation, the 305-bp fragment increased transcriptional activity of the SV40 promoter 6.6-fold ± 2.1 ($P = 0.04$) (n = 5) (Fig. 2B). These results indicated we had identified a region within the EGFR gene first intron that exhibits differential activity in MCF-7 vs. BT549 breast cancer cells with the heterologous SV40 promoter.

We next investigated the ability of these EGFR intron fragments to regulate the transcriptional activity of the EGFR promoter in ER+ vs. ER− breast cancer cells. Results from transfections of EGFR promoter-intron constructs (Fig. 1B) demonstrated that the 305-bp fragment repressed EGFR transcriptional activity 67% ± 4.3 ($P < 0.001$) (n = 12) in the forward orientation in MCF-7 cells, but had no significant effect in the reverse orientation (Fig. 3A). In BT549 cells, similar to results obtained with SV40 promoter-EGFR intron constructs, the 305-bp fragment did not decrease transcriptional activity of the EGFR promoter in either orientation (Fig. 3B). Transient transfections of EGFR promoter-intron constructs containing the remaining fragments comprising the 730-bp intron region (Fig. 1B) demonstrated that, in MCF-7 cells, the 80-bp fragment increased transcriptional activity 2.8-fold ± 0.76 ($P = 0.04$) (n = 7) while the 309-bp fragment had no significant effect on EGFR transcriptional activity (Fig. 3A). The 80- and 309-bp fragments enhanced transcriptional activity of the EGFR promoter in BT549 cells fourfold ± 0.97 ($P < 0.001$) (n = 4) and 3.3-fold ± 0.5 ($P < 0.001$) (n = 3), respectively (Fig. 3B). These results indicated that we had identified a 305-bp region within the EGFR gene first intron demonstrating differential repressive activity with both heterologous and homologous promoter constructs in ER+/low EGFR expressing MCF-7 cells, but not in ER−/high EGFR expressing BT549 cells. The variability of the repressive activity with respect to orientation, as well as promoter specificity, is consistent with literature reports, particularly with

regards to repressors found within introns [Frenkel et al., 1993; Takimoto and Kuramoto, 1993; Bossu et al., 1994; Stewart et al., 1994; Wang et al., 1994].

Identification of the Minimal Intron Negative Regulatory Element

To identify the minimal intron element within the 305-bp region responsible for transcriptional repression of EGFR gene expression in ER+ vs. ER− breast cancer cell lines, the 305 bp fragment was divided into smaller subfragments using convenient restriction sites (Fig. 1B). To evaluate the transcriptional activity of these smaller intron elements, fragments were subcloned into the EGFR promoter construct, pJFEC, and transient transfections were performed. Electrophoretic mobility shift assays were also performed to investigate factor binding within these fragments and to define the minimal protein-binding element.

Digestion of the 305-bp intron negative regulatory element with the enzymes *Pml* I and *Alu* I resulted in three fragments, 56, 96, and 150-bp in size (Fig. 1B). The results from transient transfections with the 56-bp fragment and the 150-bp fragment in MCF-7 cells demonstrated that these fragments exhibited decreased transcriptional activity, repressing EGFR transcriptional activity 51% ± 10 ($P < 0.001$) (n = 6) and 60% ± 10 ($P < 0.001$) (n = 5), respectively (Fig. 4A). In contrast, results from transient transfections demonstrated that the 96-bp fragment dramatically reduced EGFR transcriptional activity 93% ± 3.7 ($P < 0.001$) (n = 4) in MCF-7 cells (Fig. 4A). In BT549 cells, results from transient transfections demonstrated that the 56-bp (n = 7) and the 150-bp (n = 5) fragments did not significantly affect transcriptional activity of the EGFR promoter (Fig. 4B). However, the 96-bp fragment appeared to enhance transcriptional activity 2.7-fold ($P = 0.05$) (n = 4)(Fig. 4B). Like the 305-bp intron fragment, these sub-fragments repressed EGFR transcriptional activity in the ER+/low EGFR expressing MCF-7 cells, but not in the ER−/high EGFR expressing BT549 cells. More importantly, the 96-bp sub-fragment

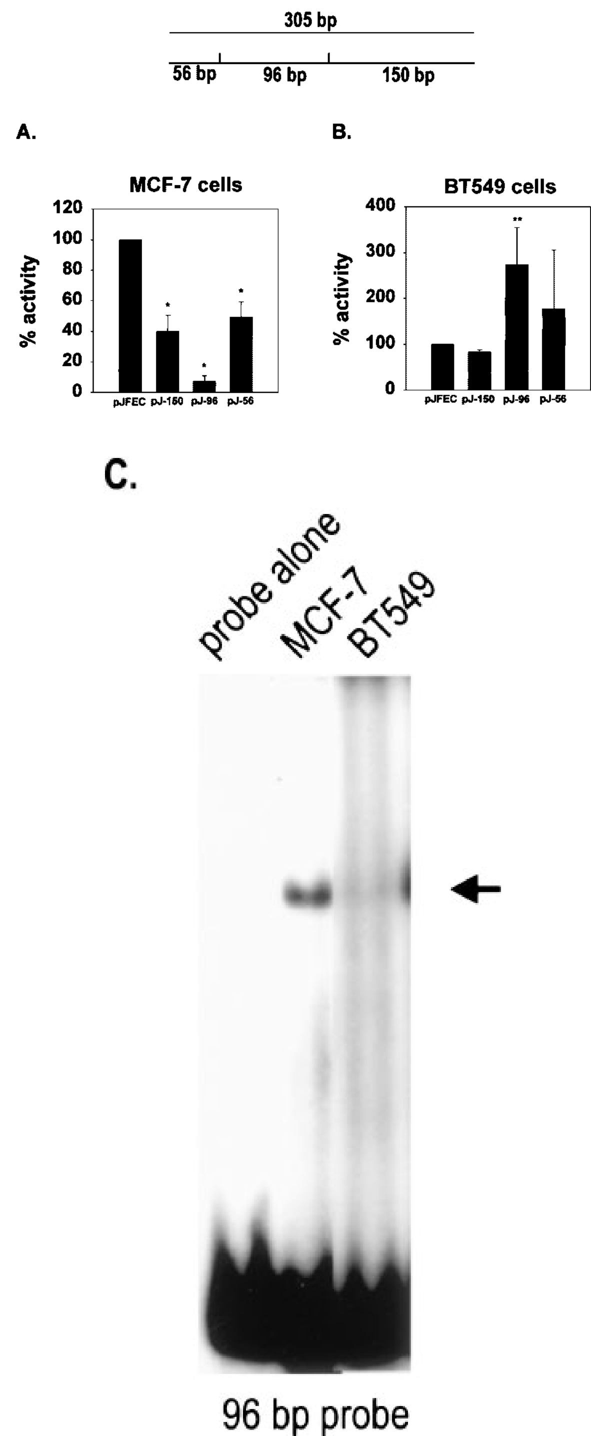


Fig. 4. Localization of the minimal intron negative regulatory domain. Schematic of the 305-bp intron negative regulatory element and its subfragments. Transient transfections with EGFR promoter-intron constructs containing smaller intron fragments were performed as described in Materials and Methods. **A**: Transcriptional activity in ER+/low EGFR expressing MCF-7 breast cancer cells. **B**: Transcriptional activity in ER−/high EGFR expressing BT549 breast cancer cells. *P* values: $^*P < 0.001$ and $^{**}P = 0.05$. **C**: Gels shift assay with the 96-bp intron fragment. Five micrograms of MCF-7 and BT549 nuclear extracts were incubated with a probe corresponding to the 96-bp minimal intron negative regulatory element. Shifted DNA-protein complexes demonstrate a slower migration compared to the probe alone.

demonstrated the greatest extent of repressive activity, which suggested that the 96-bp element contains the major negative regulatory element responsible for exhibiting differential repressive activity.

Gel shift assays were performed with the 56-, 96-, and 150-bp fragments to investigate the shift patterns of DNA-protein complexes between ER+ vs. ER− nuclear extracts. Incubation of the 56-bp or the 150-bp fragments with nuclear extracts from MCF-7 and BT549 breast cancer cell lines resulted in DNA-protein complexes that were similar in both cell lines (data not shown). In contrast, gel shift assays with the 96-bp fragment demonstrated a differential shift pattern in ER + vs. ER− nuclear extracts. Incubation with MCF-7 nuclear extract resulted in one distinct DNA-protein complex, as compared to probe alone, that was greatly diminished in BT549 nuclear extracts (Fig. 4C). These results demonstrated that the 96-bp intron fragment had differential binding activity in ER+ vs. ER− breast cancer cells that directly correlated with functional data. These results further substantiated the localization of the major negative regulatory element within the 96-bp intron fragment.

Dissection of the 96-bp Intron Negative Regulatory Domain

To further delineate the minimal negative regulatory element responsible for repressive activity, EGFR promoter-intron constructs were generated which contained smaller regions within the 96-bp fragment. The 96-bp negative regulatory element was divided into two smaller fragments, 37 and 59-bp in size (Fig. 1B). Results from transient transfection assays demonstrated that the 59-bp fragment repressed activity 50% ± 11 ($P < 0.001$) in MCF-7 cells (n = 4) (Fig. 5A) and, unexpectedly, 62% ± 11 ($P < 0.001$) in BT549 cells (n = 6) (Fig. 5B). The 37-bp fragment increased EGFR transcriptional activity 3.1-fold ± 1.1 ($P = 0.04$) (n = 4) in MCF-7 cells (Fig. 5A) but demonstrated no significant effect in BT549 cells (n = 6) (Fig. 5B). While the 59-bp intron fragment repressed EGFR transcriptional activity, the extent of repression was lower than the repression observed with the entire 96-bp fragment. Furthermore, differential transcriptional repression between MCF-7 and BT549 cells was lost.

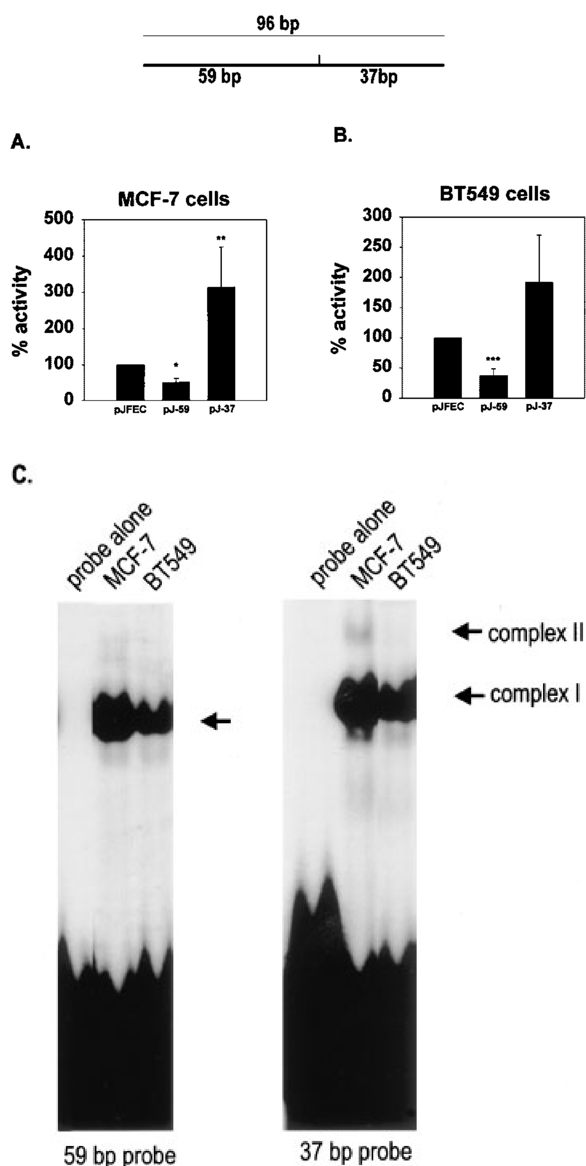


Fig. 5. Disruption of the minimal intron negative regulatory domain results in loss of differential transcriptional activity. Depiction of the 96-bp intron negative regulatory domain and smaller regions contained within. Transcriptional activity of EGFR promoter-intron constructs containing these intron regions in (**A**) ER +/low EGFR expressing MCF-7 breast cancer cells and (**B**) ER−/high EGFR expressing BT549 breast cancer cells. P values: *$P = 0.001$; **$P = 0.004$; and ***$P < 0.001$. **C**: For each probe, 5 μg of nuclear extracts were incubated with probes corresponding to indicated regions within the EGFR gene first intron and analyzed in 6% non-denaturing polyacrylamide gels. Arrows designate protein-DNA complexes.

The 59- and 37-bp fragments were also used as probes in gel shift assays in order to investigate factor binding to these two elements. Incubation of the 59-bp fragment with MCF-7 and BT549 nuclear extracts resulted in similar shifted complexes, relative to probe alone,

although the complex was reduced in BT549 nuclear extracts (Fig. 5C). Incubation of the 37-bp fragment with MCF-7 nuclear extract resulted in two shifted complexes (complexes I & II), relative to probe alone. While complex I was similar in MCF-7 and BT549 nuclear extracts, complex II was greatly reduced in BT549 nuclear extracts (Fig. 5C). The 37-bp demonstrated differential DNA-protein complexes (complex II) in MCF-7 vs. BT549 breast cancer cells. These results were consistent with the recruitment of a specific factor by the 37-bp intron region, represented by complex II, that modulates the activity of the factor(s) interacting with the 59-bp intron element, as well as the entire 96-bp intron negative regulatory domain.

Additional EGFR promoter-intron constructs were generated from oligonucleotides spanning smaller regions of the 96-bp intron negative regulatory domain. Oligonucleotides were chosen based upon their position within the 96-bp fragment, as well as the presence of putative factor binding sites as determined by in vitro DNase I footpriniting (data not shown). Results from transient transfections demonstrated that these individual regions were unable to repress EGFR transcriptional activity in MCF-7 cells (data not shown). Although not shown, multiple gel shift assays and competition experiments with these intron fragments were unable to identify specific transcription factors or individual elements involved in DNA-protein interactions within the 96-bp intron domain. Moreover, the various intron fragments did not compete with identified DNA-protein complexes, demonstrating the specificity of these DNA-protein interactions. Since division of the 96-bp intron negative regulatory domain into smaller fragments resulted in the loss or dramatic reduction of repressive activity in ER+/low EGFR expressing MCF-7 breast cancer cells, these results suggest that the 96-bp intron region is the optimal repressive domain containing binding sites for multiple regulatory factors that interact as complexes. Disruption of this domain results in protein complex disruption, and therefore, loss of transcriptional regulation.

Estrogen-Dependence of Transcriptional Repression

Estrogen directly regulates the expression of EGFR in ER+ breast cancer cell lines resulting in the repression of EGFR [Yarden et al., 1996, 2001]. In addition, treatment of MCF-7 cells with the pure anti-estrogen ICI 182,780 (ICI) results in a two to threefold increase in EGFR levels [Yarden et al., 1996, 2001]. Similar results were observed with respect to the *erbB2* gene; estrogen regulates the activity of an element within the *erbB2* gene first intron [Bates and Hurst, 1997; Newman et al., 2000]. In order to determine if the results observed in ER+MCF-7 vs. ER−BT549 cells were strictly due to the presence or absence of the ER, we investigated the role of the ER in regulating EGFR transcriptional activity. We obtained similar results in our initial experiments in MCF-7 cells that were grown in complimentary conditions with respect to estrogen, including: stripped serum (estrogen-depleted conditions), addition of exogenous estrogen to estrogen-depleted conditions, and blocking estrogen action utilizing the pure anti-estrogen ICI 182,780 (ICI). We chose to utilize the pure anti-estrogen, ICI for our experiments in order to keep experimental conditions similar to those in BT549 cells.

Transient transfections were performed in ER+/low EGFR expressing MCF-7 breast cancer cells in the absence and presence of the anti-estrogen ICI. After transfection, cells were untreated or treated with 10^{-7} M ICI for 48 h. Treatment of MCF-7 cells with ICI did not significantly affect the activity of the EGFR promoter in the absence of intron elements. In untreated MCF-7 cells, the 305-bp element repressed EGFR transcriptional activity 66% ± 4.5 ($P < 0.001$) (Fig. 6A). Treatment with ICI resulted in the reversal of this transcriptional repression exhibited by the 305-bp negative regulatory element in MCF-7 cells. In the presence of the anti-estrogen ICI, the 305-bp intron element induced transcriptional activity of the EGFR promoter sevenfold ± 3 ($P < 0.001$) over the activity of the EGFR promoter alone (Fig. 6A). As indicted by results, treatment with ICI increased the overall activity of the 305-bp intron element, demonstrating a 21-fold increase in activity compared to the activity of the 305-bp intron element in untreated MCF-7 cells (Fig. 6A). These results suggested that the transcriptional repression mediated through the 305-bp negative regulatory element was estrogen-dependent; by blocking estrogen action, transcriptional repression was lost.

We were also interested in the role that estrogen played in DNA-protein complex formation with intron fragments. Nuclear extracts

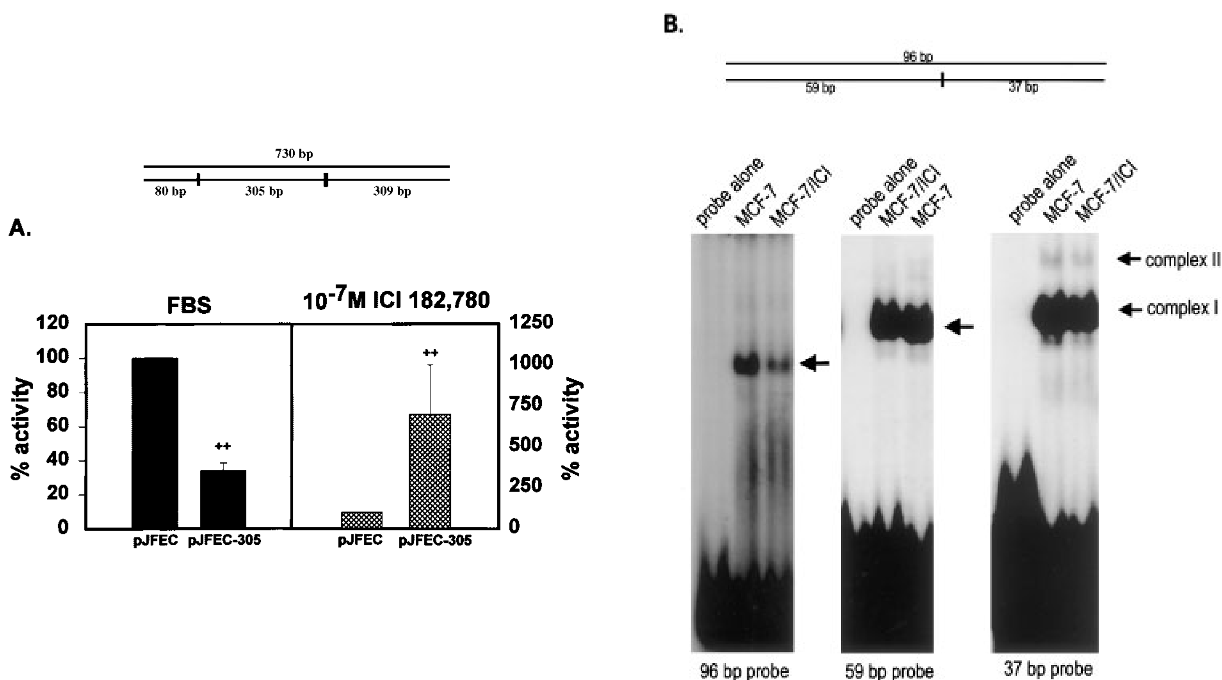


Fig. 6. The anti-estrogen, ICI 182,780 abrogates transcriptional repression mediated by the 305-bp intron negative regulatory element and DNA–protein interactions with intron elements. Map of the EGFR gene first intron demonstrating the relative position of the 305-bp negative regulatory element. **A**: Transient transfections were performed with the EGFR-intron construct containing the 305-bp intron negative regulatory element in MCF-7 cells. Cells were untreated (FBS) or treated with 10^{-7} M ICI (10^{-7} M ICI 182,780) for 48 h post-transfection. Activity is expressed relative to the parental construct (promoter alone) for each treatment condition. *P* values: $^{++}P < 0.001$. **B**: For each probe, 5 µg of nuclear extracts were incubated with probes corresponding to indicated regions within the EGFR gene first intron and analyzed in 6% non- denaturing polyacrylamide gels. Arrows designate protein-DNA complexes. Nuclear extracts were made from MCF-7 cells treated with ICI (MCF-7/ICI) (Materials and Methods).

were made from MCF-7 cells that were treated with 10^{-7} M ICI for 5 days prior to preparation. Yarden et al. [1996, 2001] demonstrated the ICI treatment results in increased EGFR levels. ICI treatment decreased complex formation with the 96-bp intron negative regulatory element (Fig. 6B; compare MCF-7 vs. MCF-7/ICI), similar to the results observed with nuclear extracts from BT549 cells (Fig. 4C). DNA-protein interactions with the 59-bp intron fragment did not appear to be affected by ICI treatment. However, formation of complex II with the 37-bp intron fragment was slightly decreased in MCF-7/ICI nuclear extracts (Fig. 6B). These results suggested that some of the differences in EGFR transcriptional regulation observed between MCF-7 vs. BT549 breast cancer cells were attributable to the ER and estrogen action. Moreover, these results are consistent with the interaction of estrogen-dependent factors within the 96-bp intron negative regulatory domain in ER+/low EGFR expressing MCF-7 breast cancer cells.

DISCUSSION

Inappropriate overexpression of EGFR in breast cancer is associated with a more aggressive phenotype, suggesting that EGFR up-regulation is involved in the progression of breast cancer to a more aggressive, hormone-independent phenotype. Initial investigation by others of transcription factors regulating EGFR gene expression has been performed in HeLa and A431 cells and sites within the EGFR gene promoter were identified [Merlino et al., 1989]. More recently, attention has focused on the involvement of the EGFR gene first intron in the regulation of its expression in human breast cancer [Chrysogelos, 1993; Gebhardt et al., 1999; Buerger et al., 2000; McInerney et al., 2001]. In this study, we identify and characterize a negative regulatory element within the EGFR gene first intron demonstrating differential transcriptional activity and DNA-protein interactions in ER+ vs. ER− breast cancer cell lines that is involved in the repression of EGFR gene expression in human breast cancer cells.

Transient transfections of heterologous and homologous promoter/CAT constructs in ER+/low EGFR expressing MCF-7 breast cancer cells and ER−/high EGFR expressing BT549 breast cancer cells reveal regions within the EGFR gene first intron demonstrating differential repressive activity. In MCF-7 cells, but not in BT549 cells, a 305-bp intron fragment represses transcriptional activity of both the SV40 and EGFR promoters. While the 305-bp intron negative regulatory element does not function as a classical silencer element, repressive activity that is independent of position, orientation, and promoter [Brand et al., 1985], our results are consistent with literature reports regarding intronic negative regulatory elements. Intron negative regulatory elements have been demonstrated to vary in transcriptional activity with respect to orientation dependence, as well as promoter specificity [Frenkel et al., 1993; Takimoto and Kuramoto, 1993; Bossu et al., 1994; Stewart et al., 1994; Wang et al., 1994].

Although multiple fragments within the EGFR gene first intron appear to be involved in the overall transcriptional regulation of EGFR gene expression in breast cancer cells, we focused on the 305-bp intron region since it exhibited differential repressive activity in ER+ vs. ER− cells. Moreover, in vitro DNase I footprinting analysis of the 305-bp intron negative regulatory element (data not shown) identified numerous putative transcription factor binding sites that may be involved in mediating the repressive activity of the 305-bp intron fragment observed in MCF-7 cells. To identify the minimal intron element responsible for mediating repressive transcriptional activity, we dissected the 305-bp intron negative regulatory element into smaller fragments. Results from these experiments identified a 96-bp element that demonstrates maximal repressive activity. Moreover, the 96-bp fragment demonstrates a difference in DNA–protein interaction that corresponds to transcriptional activity. The DNA–protein interactions observed between the 96-bp fragment and MCF-7 nuclear extracts are greatly diminished in BT549 nuclear extract. We concluded from these results that the 96-bp domain contains the multiple transcription factor binding sites which constitute the major negative regulatory element. We constructed a series of DNA fragments representing the 96-bp negative regulatory domain in order to further isolate the minimal *cis*-element within the 96-bp fragment responsible for the observed repressive transcriptional activity. However, disruption of the 96-bp repressor domain results in the loss of differential transcriptional repression most likely due to perturbation of integral DNA–protein and protein–protein interactions.

Our results do not delineate between the two possible reasons for the disruption of repression in ER+/low EGFR expressing MCF-7 cells, which include: 1) the DNA sites disrupted while creating individual intron fragments may contain critical DNA elements responsible for negative transcriptional activity or 2) proteins interacting with DNA sequences contained within each half of the fragments surrounding the disruption site must interact with one another in order to achieve optimal repressive activity and by separating these adjoining sequences, repression is lost. Kondo et al. [1992] demonstrated the latter to be true in the transcriptional repression of the rat epoxide hydrolase gene. Two composite binding sites in the gene promoter interact with distinct factors that, when separated, no longer function as a transcriptional suppressor [Kondo et al., 1992]. Similar to these findings, by disrupting the 96-bp intron negative regulatory domain we identify specific elements that mediate transcriptional repression and dictate the cell-specificity of repression. Furthermore, our results are also consistent with the overall regulation of EGFR transcriptional activity being a balance of positive and negative regulators, as well as subject to limitations due to transcription factor availability and binding site accessibility due to chromatin structure. Our results support the crucial role that protein–protein interactions play in transcriptional repression mediated through the EGFR gene intron negative regulatory element. Overall, results support our conclusion that the 96-bp intron domain contains binding sites for multiple transcription factors and constitutes the major negative regulatory element involved in the transcriptional repression of EGFR gene expression in hormone-dependent breast cancer cell lines.

Sequence analysis of protected regions obtained by in vitro DNase I footprinting identified several putative binding sites for known factors, as well as unidentified sequences, indicating the potential involvement of unique factors. The involvement of a number of these known factors with the negative regulatory

element within the EGFR gene first intron was investigated. Both competition gel shift and antibody supershift assays were performed to look at the potential involvement of Sp1, Sp3, GCF-1, GCF-2, and ETR. As demonstrated previously, binding sites for these factors reside in the EGFR gene promoter and influence EGFR transcriptional activity in a variety of cell lines [Ishii et al., 1985; Johnson et al., 1988; Kageyama et al., 1988a; Kageyama and Pastan, 1989; Hagen et al., 1992; Hagen et al., 1994; Hou et al., 1994; Reed et al., 1998]. Results from these experiments (data not shown) did not suggest the direct involvement of these factors in the transcriptional repression of EGFR activity mediated by the 96-bp intron negative regulatory element.

Previous experiments have demonstrated that estrogen regulates EGFR gene expression in ER + breast cancer cells [Yarden et al., 1996, 2001]. Moreover, the continuous presence of estrogen represses EGFR expression [Yarden et al., 1996, 2001]. Therefore, we investigated the estrogen-dependency of this intronic repressor element. In MCF-7 cells, treatment with the anti-estrogen ICI does not significantly alter the activity of the EGFR promoter alone. However, treatment with ICI increases the overall transcriptional activity of the 305-bp fragment and reverses its repressive activity. Consistent with functional data, DNA-protein complex formation between the 96-bp intron fragment and nuclear extracts from MCF-7 cells treated with ICI (MCF-7/ICI) is decreased. These results strongly suggest that estrogen may play a role in the transcriptional repression mediated through the intron negative regulatory element.

Yarden et al. [1996] previously established the presence of an estrogen-regulated repressor of EGFR transcriptional activity. In ER+ MCF-7 and BT474 breast cancer cells, estrogen transiently upregulates EGFR mRNA levels, with subsequent downregulation, that is dependent upon de novo protein synthesis [Yarden et al., 1996]. Additionally, ICI treatment results in increased EGFR levels while the continuous presence of estrogen reduces EGFR levels [Yarden et al., 1996, 2001]. Indeed, our results are congruent with these observations and suggest that the intron negative regulatory element may be involved in mediating the estrogen-dependent repression of EGFR gene expression. Furthermore, Bates and Hurst [1997] also identified an estrogen-dependent repressor element located within the first intron of the *erbB2* gene. Transcriptional repression mediated through this *erbB2* intron element is dependent upon a functional ER [Bates and Hurst, 1997]. These results also suggest the potential for common intron elements within *erbB* genes that could potentially repress transcriptional activity of *erbB* family members.

Transcriptional regulation of EGFR gene expression is complex and results from the balance between both positive and negative transcription factors. We have identified a negative regulatory element within the EGFR gene first intron which demonstrates repressive activity in the ER +/low EGFR expressing MCF-7 breast cancer cells, but not in the ER−/high EGFR expressing BT549 breast cancer cells. Furthermore, a 96-bp domain within this element demonstrates differential binding to factors in MCF-7 vs. BT549 nuclear extracts that correlates with transcriptional activity. Our results suggest that the underlying mechanism by which transcriptional repression is mediated by the 96-bp intron domain is through the interaction of multiple factors and recruitment of auxiliary factors. Moreover, one or more of the factors interacting with the 96-bp intron domain appears to be estrogen-dependent. These observations provide a mechanism that critically links the progression of breast cancer to a hormone-independent phenotype and the loss of transcriptional repression during this process.

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