

Expression of a Constitutively Active Estrogen Receptor Variant in the Estrogen Receptor-negative BT-20 Human Breast Cancer Cell Line¹

Carl G. Castles, Suzanne A. W. Fuqua, Diane M. Klotz, and Steven M. Hill²

Department of Anatomy and Molecular and Cellular Biology Program, Tulane University School of Medicine, New Orleans, Louisiana 70112 [C. G. C., D. M. K., S. M. H.], and Department of Medicine/Oncology, University of Texas Health Science Center, San Antonio, Texas 78284 [S. A. W. F.]

ABSTRACT

Estrogen receptor (ER) expression by breast tumors is an important predictor of disease-free survival in breast cancer patients and, more importantly, is a strong predictor of response to endocrine therapy. Variant forms of the ER may play an important role in the loss of hormone responsiveness and the progression to hormone independence. We have examined a panel of human breast tumor cell lines, both ER-positive and ER-negative, and have identified an ER mRNA variant containing a deletion of exon 5 in the ER-negative BT-20 and ER-positive MCF-7 cell lines. This exon 5 deletion variant has been previously reported to be overexpressed in ER-negative/progesterone receptor-positive breast tumors. Using RNase protection analysis, we have found that the predominant ER transcript in the BT-20 cells is the exon 5 deletion variant, while the principal transcript in MCF-7 cells is the wild-type ER mRNA. The variant ER transcript is translated into a truncated receptor protein of approximately *M*_r 42,000 when expressed in yeast and, more important, in breast tumor cells. This is the first demonstration of an exon 5 deletion variant ER protein. Functional analysis has shown that this variant ER possesses constitutive transcriptional regulatory activity with respect to an estrogen-regulated reporter gene construct in a yeast expression system. The presence of this ER variant in breast tumor cell lines, as well as breast tumor biopsies and uterine tissue, suggests that it is a naturally occurring variant that may arise by alternative splicing, and whose overexpression may be involved in the progression of breast tumors to a hormone-independent state.

INTRODUCTION

Steroid hormones act by binding to their cognate receptors to coordinate complex events involved in development, differentiation, and physiological function. Human breast tumors display a considerable heterogeneity with respect to ER³ expression, and there is ample evidence that ER levels constitute an important predictor of disease-free survival in breast cancer patients and, more importantly, a strong predictor of response to endocrine therapy. Approximately, two-thirds of all primary breast tumors express quantifiable levels of ER (1). Overall, patients with ER-positive tumors are more likely to have a longer disease-free interval than those whose tumors lack ER. Although the presence of ER is a key indicator of estrogen responsiveness, approximately 30% of ER-positive tumors fail to respond to endocrine therapy, whereas 5–10% of ER-negative tumors do respond to endocrine therapy (2).

The presence of abnormal ER proteins in some human breast tumors has been previously suggested from subcellular distribution and nuclear translocation experiments (3, 4). Moreover, studies using immunohistochemical staining techniques have added support to the presence of ER proteins which display functional abnormalities such as inability to bind to the nucleus when ligand is bound, or receptor

proteins which interact with the nucleus in the absence of ligand (5). Although suggestive, these data do not conclusively prove that variant forms of the ER do indeed exist. Recently however, several independent groups have reported variant forms of ER mRNA in human breast tumor biopsies and one breast tumor cell line.

We have previously reported variant ER mRNAs in breast tumors classified as ER-negative/progesterone receptor-positive, or ER-positive/progesterone receptor-negative by ligand-binding analysis (6–8). These ER mRNA variants have either exons 3, 5, or 7 deleted, and were found to be expressed in combination with wild-type ER transcripts. The level of wild-type and variant ER transcripts varied considerably between tumors. Dotzlaw *et al.* (9) have also recently characterized ER mRNA variants in primary breast tumor biopsies which expressed normal ER sequences through exons 1, 2, and 3 but then showed divergent sequences which are unrelated to the normal ER mRNA. In addition to work in primary tumors, 2 groups have isolated variant ER mRNAs from the T47D human breast tumor cell line (10, 11). These alterations include frame-shift mutations that would translate into ERs truncated within either their DNA-binding domain and hormone-binding domain, a large in-frame deletion spanning the hinge region or part of the hormone-binding domain, and deletions of exon 2, 3, or 7. Besides breast tumor biopsies, many of the variants, including the exon 5 deletion ER mRNA variant, have also been identified in normal tissue (12).

MCF-7 and BT-20 cells are well-characterized human breast cancer cell lines widely used as model systems for the study of estrogen-responsive and estrogen-unresponsive breast cancer, respectively. Hall *et al.* (13) have previously shown that BT-20 breast cancer cells, although classified as ER-negative by ligand-binding analysis, do express an ER mRNA. These observations led us to question whether altered forms of the ER, which may not bind hormone but still remain transcriptionally active, are expressed in these cells. Using a variety of approaches including RNase protection analysis and RT-PCR followed by cloning and sequencing, we have isolated from the supposedly ER-negative BT-20 breast tumor cell line our previously reported exon 5 deletion ER variant, in addition to low levels of wild-type ER transcript. We have also previously reported (6) that the ER-positive MCF-7 breast tumor cell line expresses this exon 5 deletion variant. Using RNase protection analysis, we found that the most abundant transcript in the MCF-7 cells is indeed wild-type ER, while BT-20 cells express almost exclusively the exon 5 deletion ER mRNA variant. This variant ER mRNA is translated into a truncated receptor in the BT-20 cell line. This is the first demonstration of the expression of the exon 5 deletion variant protein.

MATERIALS AND METHODS

Breast Cancer Cell Lines. MCF-7, ZR-75-1, BT-20, and MDA-MB-231 cell lines were obtained from the late William L. McGuire, San Antonio, TX, and the American Type Culture Collection, Rockville, MD. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD), β -mercaptoethanol amino acids, minimal essential medium nonessential amino acids, L-glutamine, penicillin-streptomycin, and porcine insulin (10^{-8} M; Sigma Chemical Co., St. Louis, MO). Stock cells were maintained in 75-cm² culture flasks in a humidified atmosphere of 5% CO₂ and 95% air at a constant temperature of 37°C.

Received 7/2/93; accepted 10/5/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by American Cancer Society Grant IN-1331 to S. M. H. and NIH Grants CA52351 and CA30195 to S. A. W. F.

² To whom requests for reprints should be addressed, at Department of Anatomy, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112.

³ The abbreviations used are: ER, estrogen receptor; cDNA, complementary DNA; RT-PCR, reverse transcription-polymerase chain reaction.

Cells for Northern blot analysis and RNase protection analysis were harvested from 150-cm² culture flasks at 75% confluence with 0.25% trypsin, 1 mM EDTA solution, pelleted at 600 × g, snap frozen in liquid nitrogen, and stored at -70°C until RNA could be extracted. Cells to be used for immunoprecipitation and Western blot analyses were grown in phenol red-free media supplemented with 5% fetal bovine serum treated with dextran-coated charcoal to remove endogenous estrogens.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated in a single step procedure (14) from frozen cell pellets by homogenization in Dounce homogenizers using RNazol B as per manufacturer's instructions (Cinna-Biotech Laboratories, Inc., Houston, TX). The concentration and integrity of RNA were determined spectrophotometrically at an absorbance of 260 nm and by agarose gel electrophoresis. Poly A(+) RNA used for Northern blot analysis was isolated as described previously (15). Thirty μg of poly A(+) RNA from each cell line were heat denatured at 55°C for 15 min and separated electrophoretically on a 1% denaturing agarose gel containing 2.2 M formaldehyde. RNA molecular weight standards, ranging in size from 9.5 to 0.2 kilobase, were run in adjacent lanes. RNA was transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by capillary action in 20× standard saline-citrate and fixed to the membrane by UV-cross-linking. The membrane was then hybridized to a 2.1-kilobase cDNA probe to the human ER (16).

RNase Protection Analysis. Thirty μg of MCF-7 and 60 μg of BT-20 and MDA-MB-231 total RNA were hybridized to a ³²P-labeled antisense complementary RNA ER variant probe. This ER probe was generated by transcribing an ER variant cDNA clone containing portions of exons 4 and 6 (nucleotides 1142–1580), but with exon 5 (nucleotides 1389–1527) deleted. Briefly, 1 × 10⁶ cpm of ³²P-labeled ER variant complementary RNA probe were mixed with 30 μl of hybridization solution containing cell line total RNA. The mixture was heated at 85°C for 5 min, then hybridized at 48°C overnight. Following hybridization, the samples were digested with RNase A (50 μg/ml) for 30 min at 37°C, and RNA digestion was terminated by the addition of 20 μl of 10% sodium dodecyl sulfate and 50 μg of proteinase K (10 mg/ml). Ten μg of carrier tRNA were added to the digested samples, which were then extracted with phenol-chloroform and the aqueous phase precipitated with ethanol. The precipitates were dissolved in 5 μl of gel-loading buffer [80% formamide, 10% 10× Tris-borate 50 mM (pH 8), 1 mM EDTA, 0.5% bromophenol blue, and 0.5% xylene cyanol], heated at 85°C for 5 min, loaded on a 6% polyacrylamide 8 M urea gel, and electrophoresed at 1000 V for 3 h. Gels were dried and exposed to Kodak XAR film at -70°C for 18–48 h.

Immunoprecipitation of ER and Western Blot Analysis. MCF-7 and BT-20 cells were rinsed in PBS; harvested from semiconfluent 75- and 150-cm² flasks, respectively, by scraping with a rubber policeman; pelleted; resuspended in a high-salt buffer of 10 mM NaMoO₄, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, and 0.6 M KCl, pH 7.5; and disrupted by sonication. The cell preparation was then taken through high velocity centrifugation, and the resulting cytosol fraction was recovered and incubated with the human ER monoclonal antibody H226 (17) (12 μg/ml; Abbott Laboratories, Abbott, IL), followed by the addition of 133 mg/ml protein A-Sepharose (Pharmacia, Piscataway, NJ). Bound ER was extracted from the Sepharose resin with 2 M sodium thiocyanate, and then precipitated with 8% trichloroacetic acid and 0.1% sodium deoxycholate, resuspended in Laemmli's buffer, boiled, and separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel according to the method of Laemmli (18). Proteins were then electrophoretically transferred onto nitrocellulose membranes, incubated with the H226 ER monoclonal antibody (1.0 μg/ml), and tagged with an ¹²⁵I-labeled rabbit anti-rat IgG (ICN, Costa Mesa, CA). Following washes to remove excess radioactivity, the membranes were exposed to Kodak XAR film at -70°C for 3–5 days.

RT-PCR Amplification, Cloning, and Sequencing. RT-PCR was used to amplify regions of the ER mRNA isolated from MCF-7, BT-20, and MDA-MB-231 breast cancer cells, and was performed as described previously (19). To amplify across exon 5, a sense oligonucleotide primer HB10, which corresponds to nucleotides 1142–1162 of the ER cDNA (16), and an antisense primer HB11, which corresponds to nucleotides 1561–1580, were used. To the 5' end of each primer, 2 additional nucleotides were added to facilitate cutting at an introduced *Eco*RI restriction site. RT-PCR products were gel purified and then cloned into pGEM7z(+) vectors (Promega, Madison, WI). Double-stranded plasmid DNA containing the cDNA inserts was alkali denatured (20), and both strands were sequenced (Sequenase version 2.0; United States

Biochemical Corp., Cleveland, OH) using SP6 and T7 promoter primers (21), and then compared to those reported in the Genetic Sequence Data Bank (EMBL/GenBank).

Yeast Expression Assay and Western Blot Analysis. The BCpE2 receptor plasmid and YEPE10 expression vectors were used as described previously (22–24). A *Hind*III/*Bgl*II-digested ER fragment containing the exon 5 deletion variant from the BT-20 cell line was cloned into a similarly cut BCpE2 vector, and sequence analysis was performed to ensure proper insertion into the vector. An *Afl*III/*Kpn*I digest then removed a fragment from this intermediate vector, which was subsequently inserted into a YEPE10 vector, from which a similarly digested fragment had previously been removed, generating the final expression vector YEPEΔ5. Dideoxy-sequence analysis was again performed to ensure proper insertion.

This variant ER construct and a construct containing the wild-type ER (YEPE10) were transformed into a protease-deficient strain of yeast (BJ3505). These cells were also cotransformed with an estrogen-responsive reporter gene construct, YRPE2 (22, 23), which contains 2 copies of the vitellogenin A2 estrogen response element within the proximal promoter elements of the yeast isochromosome C promoter, fused to the β-galactosidase gene. This construct was thus used as an assayable marker. Yeast cells were grown overnight at 30°C in 5 ml minimal media supplemented with 2% glucose. An additional 5 ml of media were added along with 25 μM CuSO₄, and the cells then grown overnight. The absorbance of the cells was determined at 600 nm, and the cells were set up at a density of 0.5 absorbance unit/ml, supplemented with 25 μM CuSO₄ in the presence or absence of 17-β-estradiol (10⁻⁸ M), and allowed to grow for an additional 4 h at 30°C. β-Galactosidase assays were carried out as described previously (22). Protein was isolated from cytosol preparations of yeast cells, and Western blot analysis was performed with the H226 ER monoclonal antibody as described previously (22, 23).

RESULTS

Northern Analysis of RNA Isolated from Human Breast Tumor Cell Lines. Thirty μg of poly A(+) RNA from four human breast tumor cell lines (MCF-7, ZR-75-1, BT-20, and MDA-MB-231) were subjected to Northern blot analysis. Hybridization of the resulting blot with the human ER cDNA (16) demonstrated the presence of a major mRNA transcript (Fig. 1) of approximately 6.5 kilobases in the 2 ER-positive breast tumor cell lines (MCF-7 and ZR-75-1). The BT-20 breast tumor cell line, classified as ER-negative by ligand-binding analysis, expressed an ER mRNA species as previously reported (13), but this transcript appeared to be somewhat smaller (6.3 kilobases) than the ER transcript seen in the MCF-7 and ZR-75-1 cell lines. In addition, this smaller transcript is expressed at a significantly lower level in BT-20 cells than the wild-type ER transcripts in the 2 ER-positive cell lines. Due to overloading on this blot, smaller transcripts cannot be conclusively identified in the lane containing the MCF-7 mRNA. No ER transcript was detectable in the MDA-MB-231 cell line.

RT-PCR and Sequence Analysis of Variant ER Transcripts in MCF-7, BT-20, and MDA-MB-231 Breast Tumor Cell Lines. Total cellular RNA from the ER-positive MCF-7 cell line and the ER-negative BT-20 and MDA-MB-231 cell lines were reverse transcribed and the cDNA amplified by PCR using the primers HB10 and HB11. The amplified products were then separated on a 5% polyacrylamide, 8 M urea gel. The ER-negative BT-20 cells expressed 2 amplified products, a larger 438-base pair product which correlated with the expected size of the wild-type ER product, and a smaller 300-base pair variant band (data not shown). Similarly, the ER-positive MCF-7 cells also expressed 2 PCR products, a larger 438-base pair band, and a smaller variant band of approximately 300 base pairs. Polymerase chain reaction analysis of MDA-MB-231 did not identify detectable levels of ER cDNA. Clones containing the larger 438-base pair products, from both BT-20 and MCF-7 cells, were sequenced and shown to correspond to sequences established for the human ER (25), including a G residue at nucleotide 1491 (data not shown). Sequence

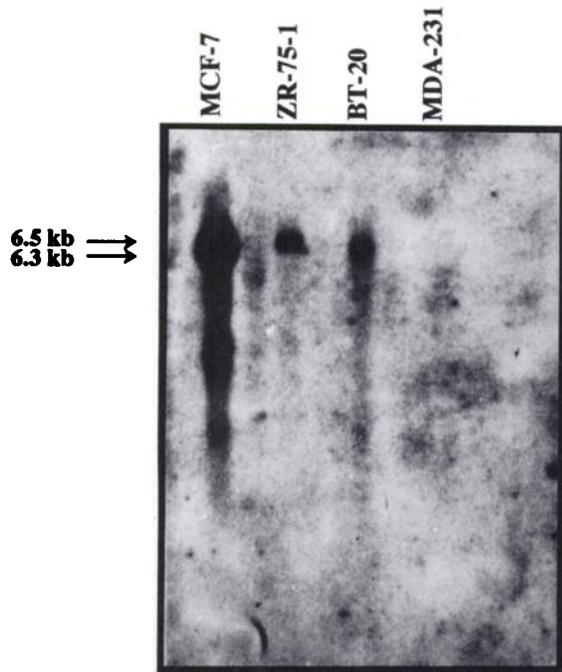


Fig. 1. Northern blot analysis of ER mRNA from human breast cancer cell lines. Thirty μ g of poly-A(+) RNA from each cell line were transferred to nitrocellulose, cross-linked, and hybridized to a 2.1-kilobase 32 P-labeled ER cDNA probe. The BT-20 ER mRNA transcript is smaller (6.3 kilobases) than the normal ER message (6.5 kilobases) observed in the ER-positive MCF-7 and ZR-75-1 cell lines. No ER transcript was detected in the MDA-MB-231 cell line.

analysis of the cloned 300-base pair variant fragment revealed wild-type sequences for exons 4 and 6 with an internal deletion of exon 5 (Fig. 2). Complete sequence analysis of these variants indicates that all of these deletions correspond precisely to known intron/exon boundaries. All other regions of the ER message, excluding the region encoding the carboxy-terminal region of the hormone-binding domain, appear to contain wild-type ER mRNA sequences.

RNase Protection Analysis and Quantitation of ER Transcripts. The relative expression of wild-type and variant ER transcripts was also examined by RNase protection analysis (Fig. 3). In our BT-20

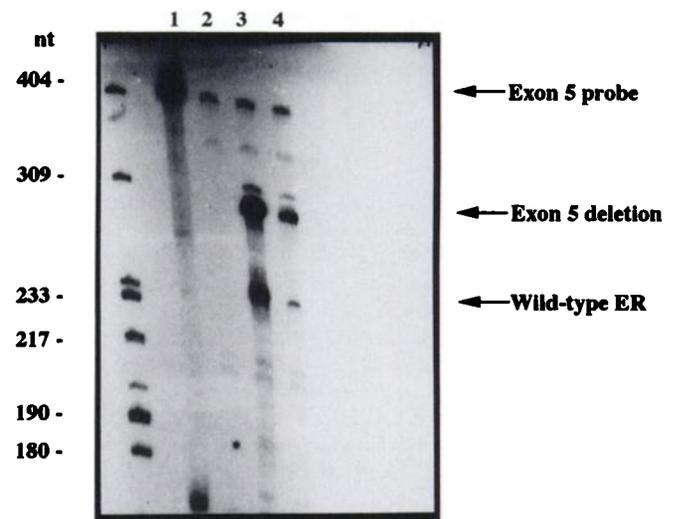


Fig. 3. RNase protection analysis of ER mRNA from MCF-7 and BT-20 cells detecting the exon 5 deletion variant. RNA from these cell lines was hybridized with an ER complementary RNA probe prepared from the variant cDNA with a deletion of exon 5. BT-20 cells (Lane 4) express 5-fold more exon 5 deletion variant (300 nucleotides) than wild-type ER mRNA (221 nt). MCF-7 cells (Lane 3) express 1.4- to 1.5-fold more wild-type than exon 5 deletion variant ER mRNA. pBR322 digested with *Msp*I used as molecular weight marker. Lane 1, probe alone.

cells, the exon 5 deletion variant transcript was expressed at 5-fold higher levels than the wild-type ER mRNA, while MCF-7 cells expressed 1.4- to 1.5-fold more wild-type transcript than the exon 5 deletion variant. Upon visual analysis, it appears that the exon 5 deletion variant and the wild-type ER mRNA are expressed at approximately equivalent levels in the MCF-7 cells. This is misleading, however, since the overall amount of radioactivity (probe) bound to the wild-type protected fragment is approximately 30% less than that bound to the full-length protected fragment of the variant transcript. We have corrected the numbers generated by densitometric analysis to account for the molar differences in size between the 2 protected fragments. MDA-MB-231 cells failed to express any ER transcripts (data not shown).

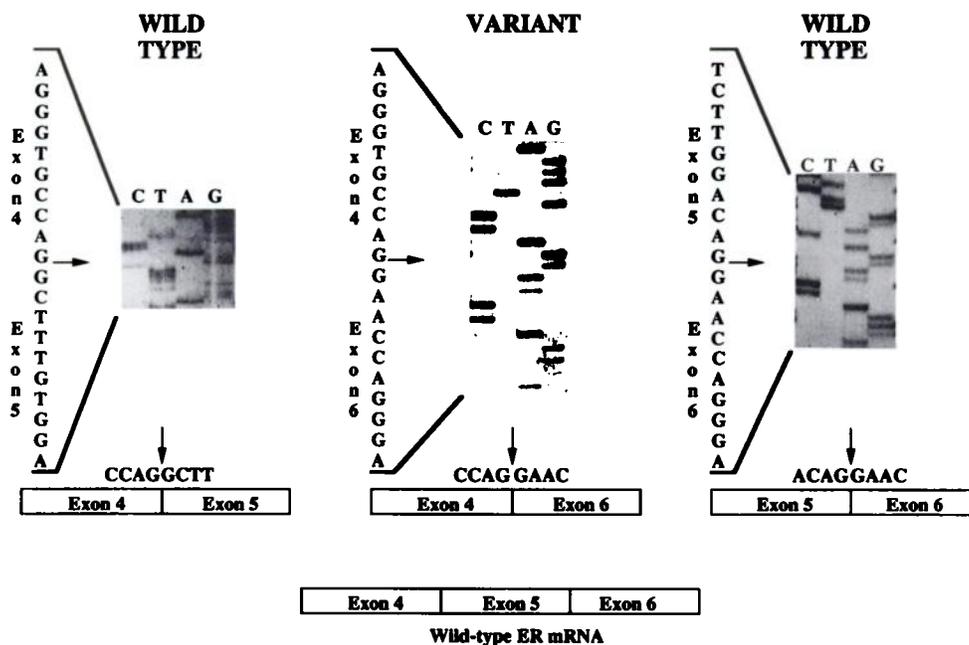


Fig. 2. Sequence analysis of an ER variant lacking exon 5 from BT-20 breast cancer cells. Total cellular RNA from the BT-20 and MCF-7 cell lines was isolated and reverse transcribed, and the ER cDNAs cloned and sequenced as described in "Materials and Methods." Arrows, exon boundaries.

Transcriptional Activity of the Exon 5 ER Variant in Yeast. A yeast expression vector system was used to determine the transcriptional regulatory activity of the exon 5 deletion ER variant. As previously shown (6), neither the reporter gene alone (YRPE2) nor wild-type ER (YEPE10) was able to stimulate β -galactosidase activity in the absence of estradiol. However, in the presence of estradiol, ligand-activated wild-type ER showed maximal transcriptional activity (Fig. 4). In contrast, the exon 5 deletion ER variant exhibited 40–45% of fully activated wild-type activity in the absence or presence of estradiol. The relative level of transcription in the yeast cells expressing the exon 5 deletion variant did not vary significantly among the series of six β -galactosidase activity experiments.

Western Blot Analysis of Variant ER Isolated from Cell Lines and Synthesized in Yeast. Sequence analysis of the exon 5 deletion variant ER mRNA suggested that a truncated ER protein, missing most of the hormone-binding domain, would be translated from this transcript. Therefore, immunoprecipitation and Western blot analysis were used to identify variant ER proteins. The H226 ER monoclonal antibody, which recognizes an epitope in the NH₂-terminus of the receptor, was used in these studies. By this approach, 2 immunoreactive proteins, one of approximately M_r 65,000 and the other of approximately M_r 42,000, were identified in the BT-20 cell line (Fig. 5). The smaller M_r 42,000 protein was the principal immunoreactive product in the ER-negative BT-20 cells, while only the M_r 65,000 protein product could be identified in the MCF-7 cell line. The smaller M_r 42,000 protein identified in the BT-20 cells corresponds to the predicted size of a truncated protein containing a stop codon after amino acid 370. The ER protein expressed from our YEPE Δ 5 variant ER cDNA construct in yeast cells was similar in size to the one expressed in the BT-20 cells (Fig. 6).

DISCUSSION

We report here the expression of a variant ER mRNA in the BT-20 human breast cancer cell line previously classified as ER-negative by ligand-binding analysis. We have also previously identified this exon 5 deletion variant ER transcript in the ER-positive MCF-7 cell line. This variant contains a precise deletion of exon 5 and appears to encode a transcriptionally active variant ER protein. Both cell lines, however, coexpressed variant and wild-type ER mRNA, although the levels and the ratio of exon 5 deletion variant to wild-type ER mRNA

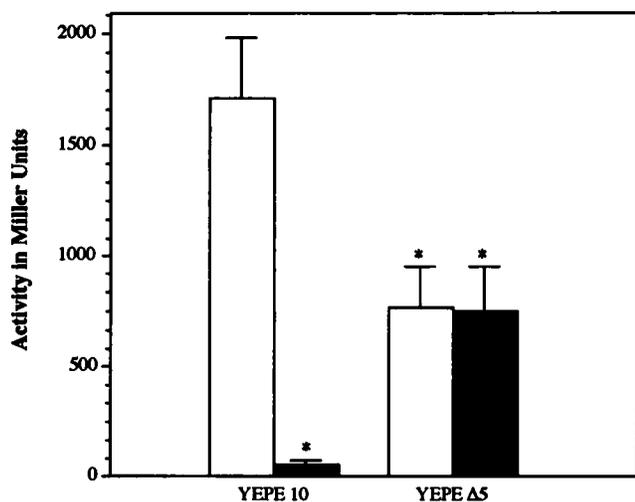


Fig. 4. Yeast cells expressing either wild-type (YEPE 10) or variant (YEPE Δ 5) ER were grown in medium containing 25 μ M CuSO₄ in the presence of 10⁻⁸ M estradiol (□) or absence of estradiol (■). β -Galactosidase activity is expressed in Miller units (33). Values presented are the average of 6 separate experiments. * P < 0.01.

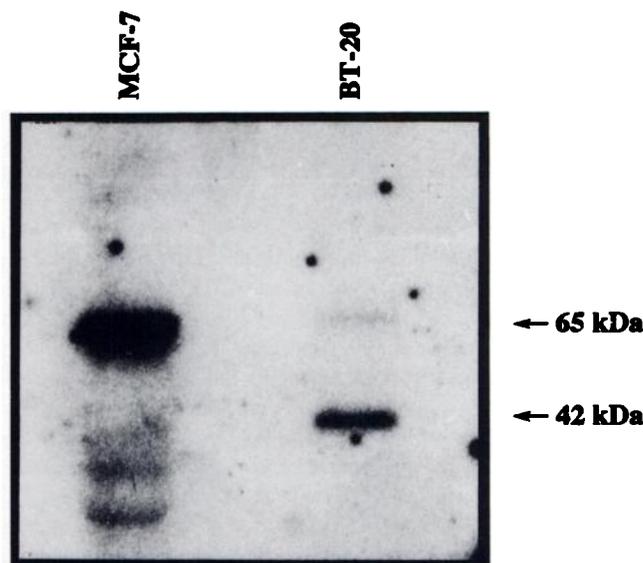


Fig. 5. Western blot analysis of immunoprecipitated ER from MCF-7 and BT-20 cells. Immunoprecipitation of ER with the H226 ER monoclonal antibody followed by separation on 7.5% sodium dodecyl sulfate-polyacrylamide gel and Western blot analysis identifies a truncated ER protein in BT-20 cells which is smaller (M_r 42,000) than the wild-type ER (M_r 65,000) seen in MCF-7 cells. ER protein was obtained from approximately 1.5×10^6 MCF-7 cells and 1.5×10^7 BT-20 cells. Rainbow molecular weight markers (Amersham, Arlington Heights, IL) are not shown.

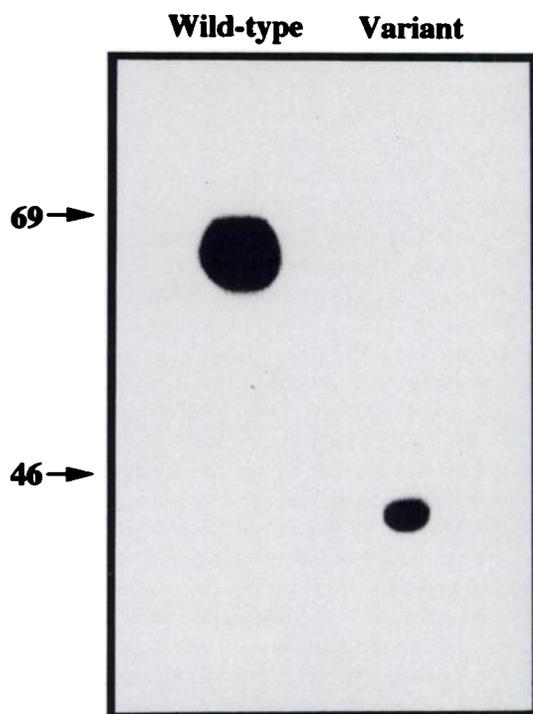


Fig. 6. Western blot analysis of ER recovered from yeast cells which contained plasmids that expressed either the wild-type (YEPE10; Lane 1) or variant (YEPE Δ 5; Lane 2) ER protein. Sizes were determined by Rainbow molecular weight standards.

differed significantly between these 2 cell lines. In the BT-20 cell line, the exon 5 deletion transcript appears to be the predominant message as determined by RNase protection analysis, while the MCF-7 cells express somewhat more wild-type than variant ER. Northern blot analysis of ER transcripts in Fig. 1 did not show the presence of a smaller (variant) transcript in MCF-7 cells or a wild-type transcript in BT-20 cells. This may be due to overloading in the MCF-7 lane and the extremely low expression of wild-type ER in BT-20 cells. The observation of coexpressed ER transcripts, as seen by RNase protec-

tion analysis, is consistent with earlier reports in breast tumor specimens (6), the T47D breast tumor cell line (11), as well as normal uterine tissue (12), suggesting that the ER variant may occur frequently in both normal and cancerous cells.

Sequence analysis of the BT-20 ER transcript with the exon 5 deletion revealed a frame-shift alteration resulting in a stop codon at the 5' end of exon 6, corresponding to amino acid 370 in the wild-type ER. Even though we and others have identified variant ER mRNAs in primary tumors, variant receptor proteins have not previously been identified in breast tumor samples or breast cancer cell lines. The inability to identify variant receptor proteins thus far from tumor samples may be due to sample limitations and cross-reactivity of commonly used monoclonal antibodies with IgG rather than the actual failure of these cells to express the variant protein product. By performing Western blot analysis with immunoprecipitated ER from large quantities of cellular material, we were able to identify in BT-20 cells a major variant ER protein of approximately M_r 42,000 as well as minimal amounts of a larger M_r 65,000 protein product. This variant is the expected translated size of the cloned exon 5 deletion variant. Parallel studies using the H222 ER monoclonal antibody for immunoprecipitation yielded only minimal amounts of the apparently wild-type M_r 65,000 protein in the BT-20 cells (data not shown). This antibody recognizes an epitope at the carboxy-terminal portion of the hormone-binding domain of the ER, and this portion of the receptor would be expected to be lost in the truncated M_r 42,000 ER. Based on the apparent loss of approximately M_r 23,000 in this variant receptor as well as our sequencing data indicating a stop codon in exon 6, it appears that translation of this variant ER transcript prematurely terminates leading to the expression of a truncated form of the receptor lacking the majority of the hormone-binding domain as we originally predicted (6). This deleted region contains various subdomains necessary for proper dimerization, binding of HSP 90, and even transcriptional inactivation (26). Our ability to detect this variant ER protein reflects the stability of this translation product and points to its possible physiological significance.

It is possible and even likely that the BT-20 cell line, like many other breast tumor cell lines, displays some heterogeneity. Clearly there are different degrees of ER expression in these cells, with some cells failing to express ER altogether. Immunocytochemical analysis of BT-20 cells using a panel of anti-ER monoclonal antibodies has revealed that this is indeed the case (data not shown).

In order to examine the transcriptional regulatory activity of this BT-20 ER deletion variant, we used an estrogen-responsive transcriptional activation system in *Saccharomyces cerevisiae* which we have previously used to study this and other ER variants (6, 7). Our results are consistent with those reported for the exon 5 deletion variant identified in primary breast tumors (6), insofar as our variant shows constitutive transcriptional activation. However, the level of β -galactosidase activity induced by our variant was significantly higher (40–45% of wild type) than the activity we previously reported for the exon 5 deletion variant (10–15%) (6). Several possibilities may account for this discordance, including the incorporation of a greater number of copies of the yeast plasmid construct into the BJ3505 yeast cells during the transformation process or possible clonal selection of yeast cells leading to an increase in transcriptional/translational efficiency. Cotransformation with a constitutively active reporter plasmid to control for transformation efficiency, however, was not possible, as these yeast cells are unable to accommodate a third plasmid. It is possible that the exon 5 deletion variant retains full transcriptional regulatory activity, as White *et al.* (27) showed with a truncated ER lacking the entire hormone-binding domain. In addition to these possibilities, our assay conditions may not be fully optimized to allow maximal activity of this ER variant construct. So while we have

confirmed the previous observation that this ER variant was able to constitutively activate gene transcription in a yeast expression system, we may have not yet accurately determined the absolute level of transcriptional activity of this variant in comparison to the wild-type ER. Ultimately, this may only be resolved by further analysis of ER protein in breast tumors.

In the studies presented here, we were unable to detect the expression of any variant ER protein in MCF-7 cells. It is possible that the wild-type ER transcript is preferentially translated by MCF-7 cells, with the variant ER transcripts playing a minimal role (if any) in activating gene transcription. This may help explain the continued estrogen-responsive nature of our MCF-7 cells even though significant levels of the variant ER transcript are expressed. However, MCF-7 cells stably transfected with the exon 5 deletion ER construct do express this variant ER protein at levels equivalent to wild-type ER (28). Analysis of these cells showed the development of tamoxifen resistance with retention of estrogen responsiveness, suggesting that the expression or possibly overexpression of this variant ER may be related to the development of a tamoxifen-resistant phenotype. Studies are currently under way to determine whether there are any factors which modulate the expression ratio of variant and wild-type ER in breast cancer cell lines.

Due to the precise nature of these deletions across known exon/intron boundaries, it seems probable that this variant ER mRNA transcript, as well as others containing complete deletions of whole exons, are the result of alternative splicing of the ER mRNA. There is strong evidence in other members of the steroid hormone superfamily that alternative splicing results in the expression of variant transcripts. For example, the thyroid hormone receptor α -subunit transcript in the rat is translated into a protein which fails to bind hormone, although it is able to bind to the thyroid-response element. This thyroid hormone receptor variant is, however, unable to activate gene transcription (29–31). Whether this alternative splicing pattern, which apparently produces these variant ER transcripts, is the result of genomic alterations remains to be answered. However, based on several studies including that of hypoxanthine phosphoribosyl transferase mRNA by Andersson *et al.* (32), it appears that genomic mutations may play a central role in these splicing errors. There are a number of mutations in the hypoxanthine phosphoribosyl transferase gene which can lead to alternative splicing, including a G:C to T:A transversion in the last base of intron 5 which causes exon 6 to be entirely omitted, as well as a deletion in the acceptor site of intron 7 which leads to the deletion of exon 8. Based on these data, genomic mutations may be one of the mechanisms by which ER mRNA variants with complete deletions of exons are generated. However, it also appears possible, based on observation of this exon 5 deletion ER variant in uterine tissue as well as breast tumors and breast tumor cell lines, that this variant ER mRNA may be the result of alternative splicing and a normal component of routine cellular function.

In breast cancer, the expression or possible overexpression of exon 5 deletion ER variants, which possess a dominant-positive function leading to constitutive transcriptional activation, may reflect the hormone-independent, antiestrogen-resistant status of the tumor. The relative overexpression of this exon 5 deletion variant as compared to wild-type ER in the BT-20 cell line, may serve to allow low to moderate transcriptional activation of normally estrogen-regulated growth factor genes (*e.g.*, transforming growth factor- α or insulin-like growth factor-I) even in the absence of ligand. We are currently undertaking studies to determine whether this variant ER is expressed in other cell lines and to determine its effects on cell growth and function, especially with respect to the activation of estrogen-responsive genes and the development of hormone independence and antiestrogen resistance in breast tumor cells.

ACKNOWLEDGMENTS

The authors thank Dr. Geoffrey Greene and Abbott Laboratories for kindly providing the H226 and H222 ER monoclonal antibodies. We also thank Yolanda Jupiter, Shelly Krieg, and Chye-Ning Weng for their excellent technical assistance.

REFERENCES

- DeSombre, E. R., Greene, G., and Jensen, E. V. Estrogen receptors and the hormone dependence of breast cancer. *In: M. J. Brennan, C. M. McGrath, and M. A. Rich (eds.), Breast Cancer: New Concepts in Etiology and Control*, pp. 69–87. New York: Academic Press, 1980.
- Edwards, D. P., Chamness, G. C., and McGuire, W. L. Estrogen and progesterone receptors in breast cancer. *Biochim. Biophys. Acta*, **560**: 457–486, 1979.
- Leake, R. E., Laing, L., Calman, K. C., MacBeth, F. R., Crawford, D., and Smith, D. C. Oestrogen-receptor status and endocrine therapy of breast cancer: response rates and status stability. *Br. J. Cancer*, **43**: 59–66, 1981.
- MacFarlane, J. K., Fleiszer, D., and Fazekas, A. G. Studies on ERs and regression in human breast cancer. *Cancer (Phila.)*, **45**: 2998–3003, 1980.
- Raam, S., Robert, N., Pappas, C. A., and Tamura, H. Defective estrogen receptors in human mammary cancers: their significance in defining hormone dependence. *J. Natl. Cancer Inst.*, **80**: 756–761, 1988.
- Fuqua, S. A. W., Fitzgerald, S. D., Chamness, G. C., Tandon, A. K., McDonnell, D. P., Nawaz, Z., O'Malley, B. W., and McGuire, W. L. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.*, **51**: 105–109, 1991.
- Fuqua, S. A. W., Fitzgerald, S. D., Allred, C. D., Elledge, R. E., Nawaz, Z., McDonnell, D. P., O'Malley, B. W., and McGuire, W. L. Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. *Cancer Res.*, **52**: 483–486, 1992.
- McGuire, W. L., Chamness, G. C., and Fuqua, S. A. W. Abnormal estrogen receptor in clinical breast cancer. *J. Steroid Biochem. Mol. Biol.*, **43**: 243–247, 1992.
- Dotzlaw, H., Alkhalaf, M., and Murphy, L. C. Characterization of estrogen receptor variant mRNAs for human breast cancers. *Mol. Endocrinol.*, **6**: 773–785, 1992.
- Graham, M. L., Krett, N. L., Miller, L. A., Leslie, K. K., Gordon, D. F., Wood, W. M., Wei, L. L., and Horwitz, K. B. T47D_{co} cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res.*, **50**: 6208–6217, 1990.
- Wang, Y., and Miksicek, R. J. Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.*, **5**: 1707–1715, 1991.
- Fuqua, S. A. W., Allred, D. C., Elledge, R. M., Greene, G. L., Nawaz, Z., and McGuire, W. L. Estrogen receptor mRNA variants in breast cancer (Abstract). *In: Program of the 73rd Annual Meeting of The Endocrine Society*, p. 428. Washington DC: The Endocrine Society, 1991.
- Hall, R. E., Lee, C. S. L., Alexander, I. E., Shine, J., Clark, C. L., and Sutherland, R. L. Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels. *Int. J. Cancer*, **46**: 1081–1087, 1990.
- Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.*, **162**: 156–159, 1987.
- Merkel, D. E., Fuqua, S. A. W., Tandon, A. K., Hill, S. M., Buzdar, A. U., and McGuire, W. L. Electrophoretic analysis of 248 clinical breast cancer specimens for P-glycoprotein overexpression or gene amplification. *J. Clin. Oncol.*, **7**: 1129–1136, 1989.
- Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. Sequence and expression of human estrogen receptor complementary DNA. *Science (Washington, DC)*, **231**: 1150–1154, 1986.
- Greene, G. L., and Press, M. F. Structure and dynamics of the estrogen receptor. *J. Steroid Biochem.*, **24**: 1–7, 1986.
- Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)*, **227**: 680–681, 1967.
- Fuqua, S. A. W., Falette, N. F., and McGuire, W. L. Sensitive detection of estrogen receptor RNA by polymerase chain reaction assay. *J. Natl. Cancer Inst.*, **82**: 858–861, 1990.
- Chen, E. Y., and Seeburg, P. H. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA*, **4**: 165–170, 1985.
- Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**: 5463–5467, 1977.
- McDonnell, D. P., Nawaz, Z., Densmore, C., Weigel, N. L., Pham, T., Clark, J. H., and O'Malley, B. High level expression of biologically active estrogen receptor in *Saccharomyces cerevisiae*. *J. Steroid Biochem. Mol. Biol.*, **39**: 291–297, 1991.
- Mak, P., McDonnell, D. P., Weigel, N. L., Schrader, W. T., and O'Malley, B. W. Expression of functional chicken oviduct progesterone receptors in yeast (*Saccharomyces cerevisiae*). *J. Biol. Chem.*, **264**: 21613–21618, 1989.
- McDonnell, D. P., Pike, J. W., Drutz, D. J., Butt, T. R., and O'Malley, B. W. Reconstitution of the vitamin D-responsive osteocalcin transcription unit in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **9**: 3517–3523, 1989.
- Ponglikitmongkol, M., Green, S., and Chambon, P. Genomic organization of the human estrogen receptor. *EMBO J.*, **7**: 3385–3388, 1988.
- Green, S., and Chambon, P. The estrogen receptor: from perception to mechanism. *In: M. G. Parker (ed.), Nuclear Hormone Receptors*, pp. 15–38. San Diego: Academic Press, 1991.
- White, J. H., Metzger, D., and Chambon, P. Expression and function of the human estrogen receptor in yeast. *Cold Spring Harbor Symp. Quant. Biol.*, **53**: 819–828, 1988.
- Fuqua, S. A. W., Allred, D. C., Nawaz, Z., Greene, G. L., and McGuire, W. L. A dominant-positive variant estrogen receptor confers resistance to endocrine therapy in human breast cancer cells (Abstract). *In: Program of the 74th Annual Meeting of The Endocrine Society*, p. 323. San Antonio, TX: The Endocrine Society, 1992.
- Koenig, R. J., Lazar, M. A., Hodin, R. A., Brent, G. A., Larsen, P. R., Chin, W. W., and Moore, D. Inhibition of thyroid hormone action by a non-hormone binding *c-erbA* protein generated by alternative mRNA splicing. *Nature (Lond.)*, **337**: 659–661, 1989.
- Mitsuhashi, T., Tennyson, G. E., and Nikodem, V. M. Alternative splicing generates messages encoding rat *c-erbA* proteins that do not bind thyroid hormone. *Proc. Natl. Acad. Sci. USA*, **85**: 5804–5808, 1988.
- Izumo, S., and Mahdavi, V. Thyroid hormone receptor *alpha* isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature (Lond.)*, **334**: 539–542, 1988.
- Andersson, B., Hou, S., and Lambert, B. Mutations causing splicing in the human hprt gene. *Environ. Mol. Mutagen.*, **20**: 89–95, 1992.
- Miller, J. H. (ed.). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1972.