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A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7

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ABSTRACT

Alterations in the amino acid sequence of the estrogen receptor (ER) have been shown to have dramatic effects on its function. Recently, mutant ERs have been isolated from both clinical samples and established breast cancer cell lines, primarily through the use of the polymerase chain reaction (PCR). All previously reported mutations have given rise to either alterations or truncations of the ER protein. We determined the structure of a novel 80 kDa ER which is expressed in an estrogen independent subclone of the MCF-7 human breast cancer cell line (MCF-7:2A). This 80 kDa ER was initially detected by Western blot analysis using a variety of ER specific antibodies. PCR mapping and partial PCR mediated subcloning of the ER cDNA were used to demonstrate that this protein was an ER containing an in-frame duplication of exons 6 and 7. This type of duplication has not been previously described for any members of the steroid receptor superfamily. Karyotype analysis coupled with fluorescence *in situ* hybridization (FISH) demonstrated that MCF-7:2A cells contained 4–5 copies of the ER gene in contrast to 2 copies in MCF-7:WS8 cells. The ER gene was localized by FISH analyses in both the MCF-7:WS8 and MCF-7:2A cells on chromosome 6, which is the source of the ER in normal human cells. The relative expression level of 2:1 is consistent with DNA gene dosage analysis. Genomic PCR was then used to demonstrate that the 80 kDa ER mRNA was not derived from the trans-splicing of two ER mRNAs but was the result of a genomic rearrangement in which exons 6 and 7 were duplicated in an in-frame fashion. This variant ER may prove to be useful in elucidating the mechanism of estrogen action in breast cancer cells.

INTRODUCTION

The estrogen receptor (ER) is a ligand activated, transactivating protein that is involved in the growth control of ER positive breast cancer cells and other estrogen target tissues (1). In order to study the mechanisms responsible for the development of estrogen independent growth in the human breast cancer cell line MCF-7, we have derived a subclone (i.e., MCF-7:2A) which grows maximally in the absence of estradiol (2). The long term growth of MCF-7 cells in the absence of estrogens leads to the development of a uniform phenotype characterized by estrogen independent, but antiestrogen sensitive, growth and elevated expresssion of the ER (3,4). The MCF-7:2A clone shares these characteristics in addition to the expression of a unique 80 kDa ER. In this paper we describe the molecular structure and genomic rearrangement responsible for the genesis of this mutant ER.

The cloning of the human ER made possible the analysis of its molecular structure and examination of the expression and function of variant ERs (5–7). The wild type ER is a 66 kDa protein coded by a 6.6 kb mRNA, comprised of five functional domains derived from eight exons. The three domains of the ER which show the greatest degree of sequence homology are the A domain which, in concert with the B domain, contains the transactivation function #1 (AF-1), the C domain which contains two zinc-fingers and is responsible for DNA binding and the E domain which is responsible for ligand binding as well as the ligand inducible transactivation function #2 (AF-2) (8).

A vast literature exists describing mutant ERs with one or more changes (reviewed in ref. 9). Generally, these mutants were derived from either of two sources, mutants found in clinical or laboratory samples that have been initially identified using RT-PCR (10–15) or variant ERs constructed through site directed mutagenesis (6,16–22). Both sources have given insight into the functions of the ER protein.

A number of splicing mutants have been isolated from clinical samples. These mutant mRNAs demonstrated the loss of one or more exons. The activity of some of the proteins derived from these mutant mRNAs were subsequently studied through their expression in heterologous systems. Examples include an exon 3 deletion which abrogates DNA binding and causes complete inactivation of the ER as a sequence specific transcriptional activator. Deletion of exon 5 leads to an ER of ~41 kDa that acts as a constitutive activator of estrogen responsive genes when

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expressed in a yeast system. The exon 7 deletion mutant gives rise to a ~52 kDa ER that behaves as a dominant negative regulator of the wild type ER function in a similar yeast system (23).

In this paper we describe a novel mutant 80 kDa ER that was isolated from a subclone of the widely studied MCF-7 human breast cancer cell line. Our finding is unique in that this mutant protein is expressed at readily measurable levels in the MCF-7:2A cells and has not been seen in any other human breast cancer cell line.

MATERIALS AND METHODS

Tissue culture

MCF-7 cells were obtained from Dean Edwards (at the San Antonio Breast Cancer Group, TX) (originally obtained from the Michigan Cancer Foundation Detroit, MI). T47D (24) and MDA-MB-231(25) cells were obtained from American Type Culture Collection, Rockville, MD. All tissue culture components were obtained from GIBCO Laboratories, Grand Island, NY unless otherwise stated. MCF-7:WS8 and T47D:A18 cells were grown in RPMI 1640 containing 10% heat inactivated fetal bovine serum (Bioproducts for Science Inc., Indianapolis, IN) 6 ng/ml bovine insulin, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (fully estrogenized medium). MCF-7:2A and MDA-MB-231:10A cells were routinely grown in estrogen-free medium which substitutes phenol-red free RPMI and 3× dextran-coated charcoal treated fetal bovine serum. Cells were passed at 1:10–1:20 dilutions once per week using 0.1% trypsin.

Western blotting

Whole cell extracts were prepared by direct lysis of PBS washed cells in sample buffer (10% glycerol, 150 mM Tris–HCl pH 6.8, 0.5 mM EDTA, 0.125% SDS, 1% β-mercaptoethanol and 5 µg/ml bromphenol blue) followed by immersion in a boiling water bath for 5–10 min. Equal amounts of protein were run in a standard Western blot as described previously (2) with the following changes. The secondary antibody used was a horseradish peroxidase (HRP) conjugated goat anti-rat antibody, (HyClone Laboratories, Logan, UT) and visualization was accomplished using the ECL visualization kit (Amersham Corp., Arlington Heights, IL) as per the manufacturers directions. The membrane was then wrapped in plastic film and exposed to Kodak X-Omat film for 15 s and developed.

Fluorescence *in situ* hybridization (FISH) studies

MCF-7:WS8 and MCF-7:2A cells were prepared for FISH by incubating actively growing cultures with 0.1 mg/ml of colcemid for 3 h. After exposure to a hypotonic solution of 0.075 M KCl at 37°C for 20 min, the cells were then fixed in 3:1 methanol-glacial acetic acid three times. Slides were made by dropping the fixed cells onto cold wet slides and air dried. FISH was performed on both interphase and metaphase cells from MCF-7:WS8, MCF-7:2A and normal human peripheral blood, using a digoxingenin-labeled ER DNA probe (Oncor, Gaithersberg, MD). The protocol was based on the manufacturer's recommendations with some modifications. Briefly, the fixed slides were denatured in a solution containing 70% formamide and 2× SSC pH 7.0 for 2 min at 72°C, dehydrated in a pre-chilled ethanol series and allowed to air dry. A hybridization mixture consisting of 65% formamide in 2× SSC, 0.03 mg/ml herring sperm DNA and 10 mg/ml of digoxingenin-labeled ER DNA probe was applied to each slide. The slides were then overlaid with a coverslip and incubated overnight at 37°C. Post-hybridization washes consisted of rinsing the slides for 20 min in 50% formamide in 2× SSC at 43°C, followed by two changes in 2× SSC for 10 min each at 37°C. The hybridized probe was detected with an immunodetection system using a commercial fluorescein-conjugated anti-digoxingenin antibody. Chromosomes and nuclei were counterstained with propidium iodide and the slides were mounted in antifade solution. Hybridization signals were visualized using an MAX-BX40 Olympus fluorescence microscope equipped with a dual color filter. A minimum of 50 metaphases and 100 interphases were examined for each cell line.

PCR analysis of the 80 kDa ER

Total RNA was prepared from MCF-7:WS8 and MCF-7:2A cells grown in estrogen-free medium by lysis in 4 M guanidine isothiocyanate followed by discontinuous cesium chloride gradient centrifugation through a 5.7 M cushion. Total RNA, 5 µg per reaction, was reverse transcribed using MMLV reverse transcriptase primed with oligo $(dT)_{12–18}$ (GIBCO BRL, Gaithersburg, MD). PCR was then performed using ~5% of this reaction and 100 ng of each appropriate primer (Oligos Etc., Wilsonville, OR) in a 50 µl reaction with 2.5 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN). PCR was run for 40 cycles, 1 min at 60°C, 2 min at 72°C and 94°C for 1 min in a DNA Thermal Cycler (Perkin-Elmer-Cetus, Foster City, CA). A portion of this reaction was then run on a 1.4% agarose gel containing 0.5 µg/ml ethidium bromide and photographed. Specific PCR was performed using the same conditions with a downstream primer (D80) that was specific for the junction of the 3′ end of exon 7 and the 5′ end of exon 6.

Partial cDNA cloning of the 80 kDa ER

Following PCR using the primer set U4–D4, the ~800 bp band was gel purified and subcloned into pUC18 using the Sure Clone Ligation kit (Pharmacia Biotech, Piscataway, NJ). Clones containing appropriate inserts were then sequenced using a standard dideoxy chain termination method. The universal and reverse primers were specific for regions flanking the multiple cloning site in pUC18 and were purchased from Pharmacia. The sequencing reaction was performed using Sequenase® T7 DNA Polymerase (version 2.0, United States Biochemical, Cleveland, OH) as per the manufacturers instructions.

Genomic PCR

High molecular DNA was prepared using standard methods (26). Total genomic DNA, 100 ng per reaction, was amplified using the XL PCR kit as per the manufacturer's directions (Perkin-Elmer-Cetus, Foster City, CA). Fifteen cycles were run using 93°C for 1 min followed by 68°C for 12 min. This was followed by 16 additional cycles as described above with a 15 s per cycle extension. A final 20 min extension segment at 72°C was then performed and the samples were run on a 1% TBE agarose gel

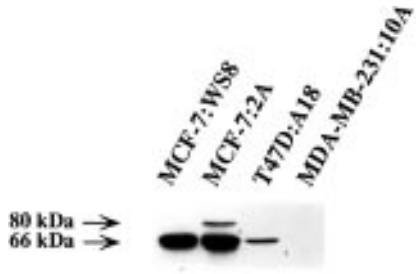

Figure 1. Western blot analysis of human breast cancer cell lines. Whole cell extracts containing equal amounts of total protein were transfered to nitrocellulose and probed with monoclonal antibody, H222. All cells were grown in estrogen free medium for a minimum of 5 days prior to lysis. Shown on the left is the size of the ERs as determined from protein molecular weight markers.

containing ethidium bromide. The gel was illuminated on a Foto/Prep I (Fotodyne Inc, Hartland, WI) and photographed.

RESULTS

Expression of the 80 kDa ER

The expression of the ER can be readily measured through the use of standard Western blotting techniques of unfractionated whole cell extracts from human breast cancer cell lines. As shown in Figure 1, the MCF-7:WS8 and T47D:A18 cell lines express a single ER protein that migrates at 66 kDa in a denaturing SDS polyacrylamide gel. The ER negative cell line MDA-MB-231:10A displays no ER signal, as expected. The MCF-7:2A subclone, however, clearly expresses both the wild type 66 kDa ER as well as a species that migrates at ~80 kDa. This species has been shown to react with three other antibodies to the ER (ref. 2 and data not shown).

Cytogenetics studies

In order to investigate the chromosomal location of the ER genes, karyotype analysis was performed on both the MCF-7:2A and MCF-7:WS8 cells. Giemsa banding studies confirmed that the MCF-7:WS8 and MCF-7:2A cells are both very evolved hypotetraploid lines with model chromosomes numbers of 68 and 69, respectively. While 2/3 of these chromosomes are marker chromosomes, ~70% of these markers are found in both cell lines, strongly indicating that both cell lines are derived from a common parent line (data not shown). The human ER has been previously mapped to the long arm of chromosome 6 at band 6q25.1 (27; Fig. 2A). (The accepted designation for the estrogen receptor gene in cytogenetic studies is ESR. However, in this section we will continue to use the abbreviation ER in order to maintain consistency with the rest of the paper.) Karyotpe analysis determined that each cell line expressed four copies of chromosome 6. Of these four copies, one is cytogenetically normal and the other three have dramatic alterations. These data verify the common lineage of the MCF-7:WS8 and MCF-7:2A cells.

FISH was then performed in order to determine the location and copy number of ER genes in these cell lines. One copy of the ER gene was found on each chromosome 6 in normal peripheral blood cells (data not shown). The MCF-7:WS8 cell line also contained two copies of the ER gene which were located on the normal chromosome 6 and on the derivative chromosome 6; der(6)^{1} (Fig. 2). The chromosome der(6)^{1} has an interstitial deletion from q21 to q24, resulting in a shorter long arm, but still retaining 6q25, where the ER gene was found. No ER signal has been found on either der(6)^{2} or der(6)^{3} in the MCF-7:WS8 line.

In the MCF-7:2A cell line, 4–5 copies of the ER gene were found in the majority of cells (Fig. 2B). One copy of the ER was found on the normal chromosome 6 and one copy on the derivative chromosome der(6)^{6}. There are 2–3 copies of the ER gene in the middle of the long arm of the large derivative chromosome der(6)^{5}. The breakpoint in the long arm of this chromosome is located at the band 6q25 with unidentified material translocating onto it. No ER signal was found in the der(6)^{4} chromosome.

The finding of a 2:1 ratio of ER gene copy number in the MCF-7:2A as compared with the MCF-7:WS8 cells was confirmed by genomic dot blot analysis using a human ER cDNA probe. Additionally, the gross structure of the ER genes in the MCF-7:2A cells was not visibly altered, as assessed by Southern blots utilizing more than 15 different restriction enzymes (data not shown).

Table 1. Primers used in PCR mapping of the ER

Primer[a]	Position[b]	Sequence[c]
U1	351–370	GCC ACG GAC CAT GAC CAT GA
D1	726–707	CTG CAG GAA AGG CGA CAG CT
U2	565–584	AAC GCG CAG GTC TAC GGT CA
D2	1047–1028	AAT GGT GCA CTG GTT GGT GG
U3	831–850	ACG CCA GGG TGG CAG AGA AA
D3	1317–1298	CAA GGC ACT GAC CAT CTG GT
U4	1211–1230	GAG ACA TGA GAG CTG CCA AC
D4	1756–1737	GGG TGC TGG ACA GAA ATG TG
U5	1684–1703	GAA GAG GAG TTT GTG TGC CT
D5	2167–2148	TGT GGG AGC CAG GGA GCT CT
D80[d]	1910–1891	TAC ACA TTT TCC CTG GTT CCT CA
U7[e]	1748–1768	CCA GCA CCC TGA AGT CTC TGG
D6[e]	1705–1685 (2022–2002)[f]	GAG GCA CAC AAA CTC CTC TCC

[a]Primer names refer to which region of the ER they were used to amplify, U and D indicate upstream and downstream primers, respectively.

[b]Numerical base positions refer to the GenBank sequence for the wild type 66 kDa human ER, unless otherwise noted. The nucleotide corresponding to the 5′ end of the primer is listed first.

[c]All sequences are listed 5′→3′.

[d]This specific primer binds only to the 80 kDa ER cDNA and the position designations refer only to this sequence.

[e]These primers were used for the genomic PCR only.

[f]This refers to the binding site in the duplicated exon 6.

Estrogen receptor cDNA analysis

Northern analysis of the MCF-7:2A cells was unable to resolve a larger mRNA which could code for the 80 kDa ER. An RT-PCR based strategy was subsequently utilized to analyze the structure of the 80 kDa ER mRNA. Using primer sets that encompass the entire coding sequence of the human ER (Fig. 3A and Table 1) PCR was performed on oligo(dT) primed MCF-7:WS8 and MCF-7:2A cDNAs (Fig. 3B). Primer set #1 typically gave poor results due to the very high (>65%) G + C content in this region of the ER cDNA. Subsequent studies using a modified PCR protocol (28) demonstrated no differences in the sizes of the PCR product in this region of the ER cDNA from MCF-7:WS8 or

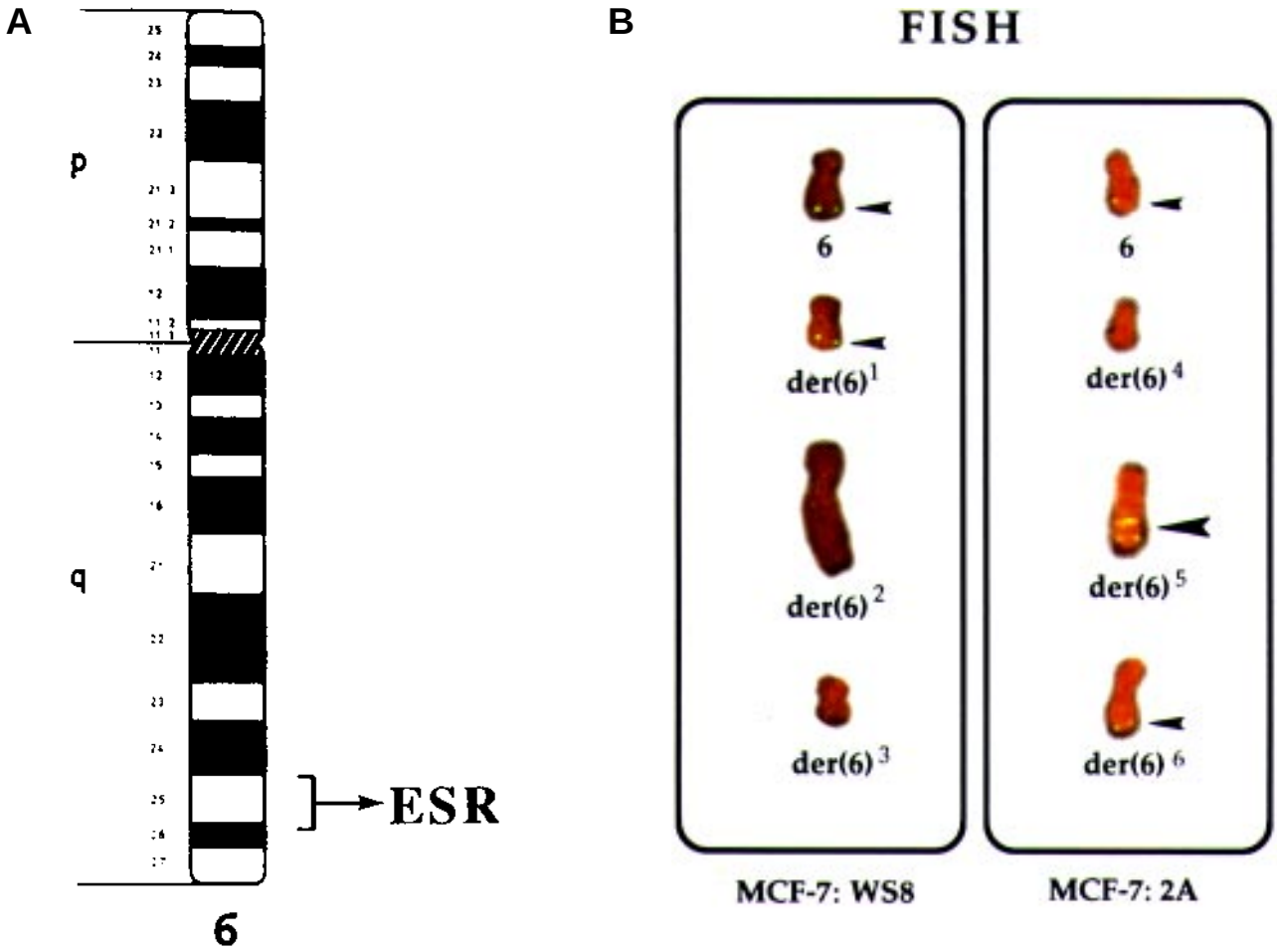

Figure 2. FISH analysis of the MCF-7:WS8 and MCF-7:2A cells. (**A**) Schematic diagram of Giemsa (G) banded chromosome 6, indicating the region at which the estrogen receptor (ESR) is mapped. (**B**) Partial karyotype obtained from FISH using an ER DNA probe shows the location and approximate copy number of the ER in MCF-7:WS8 and MCF-7:2A cell lines. Arrowheads indicate the location of the ER gene on normal chromosome 6s and the derivative chromosome 6s (small arrow heads: one copy; large arrow head: 2–3 copies).

MCF-7:2A cell lines (see Discussion). The PCR products using primer sets #2 and #3 were indistinguishable in MCF-7:WS8 and MCF-7:2A groups. The products from primer sets #4 and #5, however, show numerous extra bands in the MCF-7:2A lanes that are not present in the MCF-7:WS8 lanes. Together these two primer sets amplify the entire ligand binding domain of the ER. The smaller of the additional bands from primer sets #4 and #5 in the MCF-7:2A groups are ~300–400 bp larger than the wild type product. This is consistent with an insertion necessary for a 14 kDa increase in the size of the ER protein. Primer sets #4 and #5 amplify products that overlap by ~70 bases in the wild type 66 kDa ER. The fact that both sets gave rise to larger products in addition to the expected products suggested that the insertion was in the region of their overlap. These data demonstrated the existence of a unique mRNA in the MCF-7:2A cells which contains an insertion in the ligand binding domain.

Analysis of the insertion sequence in the 80 kDa ER

Further examination of the structure of the mutant ER mRNA was undertaken by sequencing the ~800 bp band which was produced using primer set 4 in the MCF-7:2A cDNA. This ~800 bp band was gel purified and subcloned into pUC18. This clone was then sequenced using standard dideoxynucleotide methodology. Analysis of the sequence of this fragment demonstrated that it contained an in-frame duplication of exons 6 and 7 (Fig. 4). This duplication is unique in that it does not cause a disruption in the reading frame of the protein and leads to the addition of 318 bases which code for 12 kDa of additional protein sequence.

To confirm the presence of a mutant mRNA with a duplication of exons 6 and 7 in the MCF-7:2A cells we used PCR amplification of specific alleles (PASA) (29). A unique primer (D80) was designed that could only bind to a cDNA that contained a duplication of exons 6 and 7. As seen in Figure 5 the MCF-7:2A cells contain RNA with this unique sequence and give rise to the amplified product of the expected size, 690 bp. Neither wild type MCF-7:WS8 nor the ER positive cell line T47D:A18 (30) contain an mRNA with this unique sequence. The ER negative cell line MDA-MB-231 (25) was included as a negative control. This conclusively verifies that the larger product observed in the original PCR is a result of amplification of a unique mRNA containing a duplication of exons 6 and 7, which is expressed exclusively in the MCF-7:2A cells.

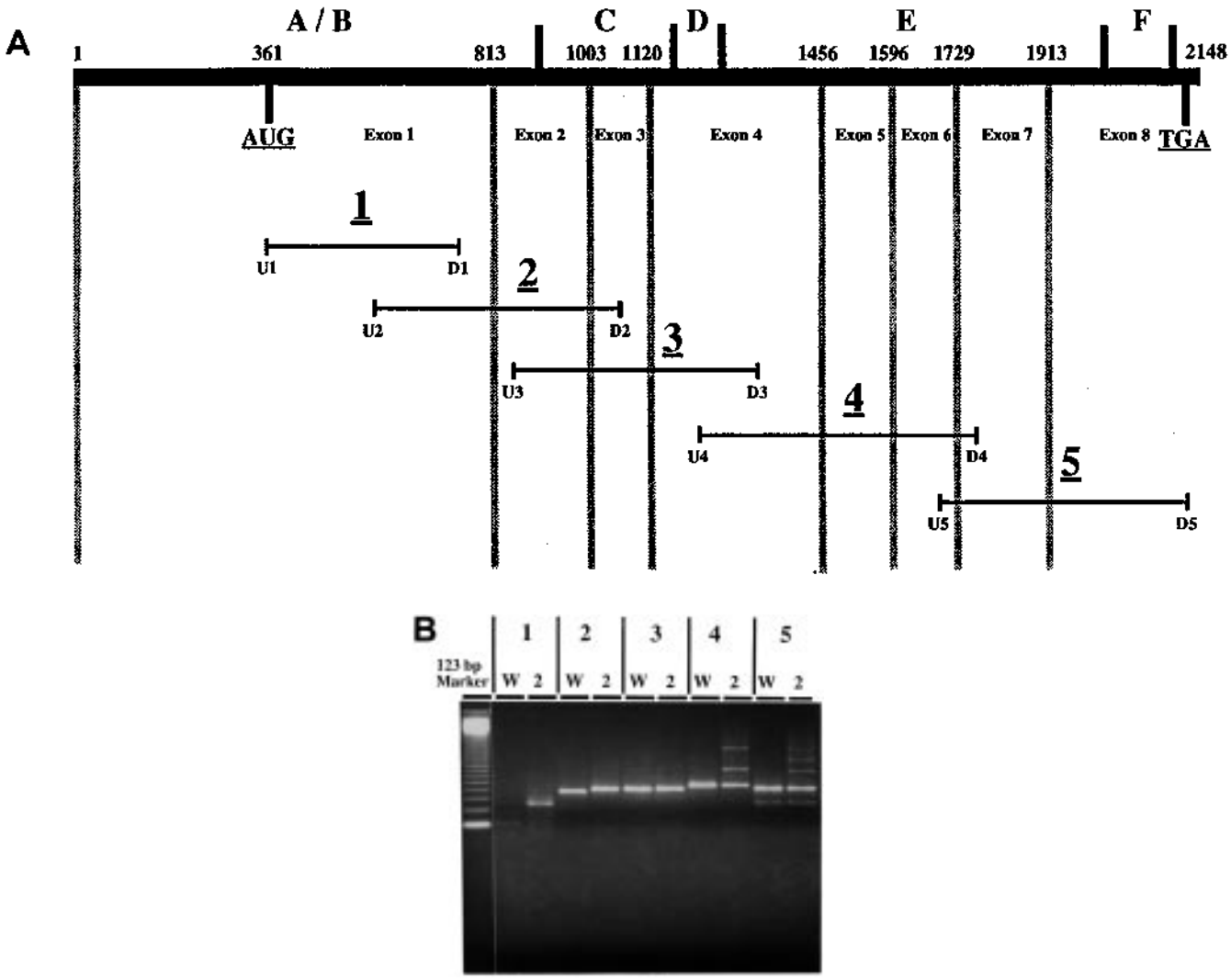

Figure 3. PCR mapping of the ER cDNA in MCF-7:WS8 and MCF-7:2A cells. (**A**) Representation of the ER cDNA and the regions amplified with the primers described in Table 1. The letters denote the domains of the ER as described in (6). The numerical designations refer to the location of the exon borders as found in the GenBank sequence, start of translation is at bp #361 and termination of translation is at bp #2148. The primer sets used to amplify the entire coding region of the ER cDNA are shown as the regions 1–5. (**B**) PCR mapping of the cDNAs from MCF-7:WS8 (W) and MCF-7:2A (2) cells. 25% of each PCR was loaded into each lane.

Genomic PCR

To address the possibility that the mRNA coding for the 80 kDa ER is the product of a trans-splicing event (31,32), XL PCR (28) was performed on genomic DNA from the MCF-7:WS8 and MCF-7:2A cells. Primers were designed for this study that would give rise to products containing a single intron separating two neighboring exons. An upstream primer (U7) that bound in exon 7 (see Table 1 and Fig. 6B) was paired with a downstream primer that bound in exon 6 (D6). This set would amplify sequences separating exon 7 and exon 6′ in the 80 kDa ER gene and should not amplify any sequences in the wild type ER gene. The primer U5 was paired with D4 to amplify the sequences separating exons 6 and 7 (or 6′ and 7′). In the wild type ER gene this intron has been shown to be >24 kb (7). Finally, PCR using the primers U7 and D5 would amplify the intron separating exons 7 and 8 in the MCF-7:WS8 ER gene and exons 7′ and 8 in the MCF-7:2A ER gene. This set of primers should give rise to identical products in both the MCF-7:WS8 and MCF-7:2A groups. As shown in Figure 6, primers U7 and D5 give rise to identical ~4.2 kb products in both the MCF-7:WS8 and MCF-7:2A groups. The PCR utilizing primers U5 and D4 did not give rise to any visible product. This is presumably due to poor amplification of sequences of this length (>24 kb). PCR using primers U7 and D6 gives rise to a specific band in the MCF-7:2A group that is ~6 kb. This fragment contains 274 bp of exon sequence, therefore the intron separating exons 7 and 6′ can be estimated to be ~5.7 kb. This is clearly longer than the wild type intron downstream of exon 7 (3.5 kb) and also much shorter than the 17 kb wild type intron upstream of exon 6. Consequently, the sequence separating exon 7 from exon 6′ appears to be the site of the gene rearrangement responsible for the genesis of the 80 kDa ER. This also confirms that the 80 kDa ER mRNA is not the result of a trans-splicing event.

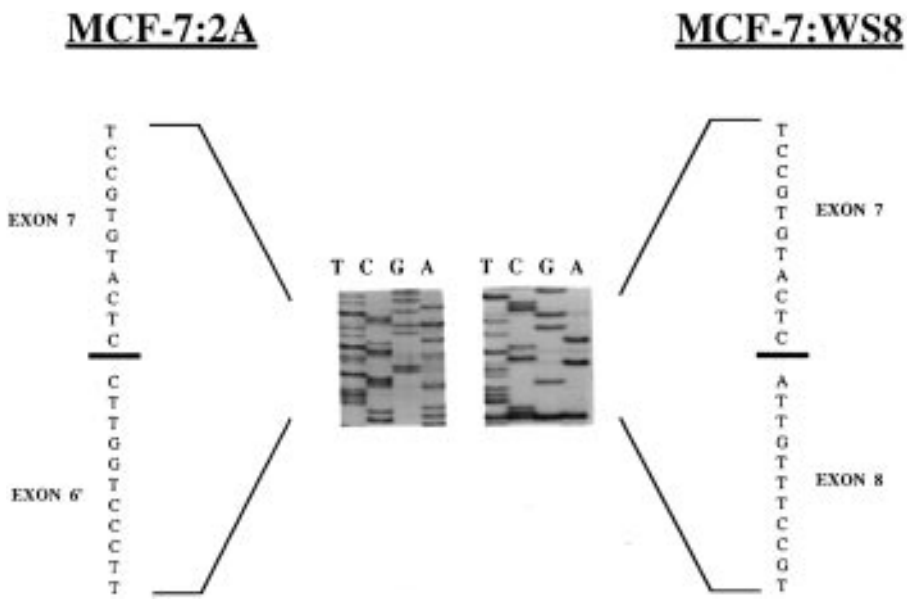

Figure 4. Sequence analysis of the exon 7–6′ junction in the MCF-7:2A ER cDNA. Following the cloning of the ~800 bp band from primer set #4 in the MCF-7:2A cDNA dideoxynucleotide sequencing was performed. Shown here is the unique region in the MCF-7:2A cDNA where exon 7 is spliced to a second copy of exon 6 (exon 6′). The correct splicing of exon 7 to exon 8 is shown from the MCF-7:WS8 cDNA for comparison.

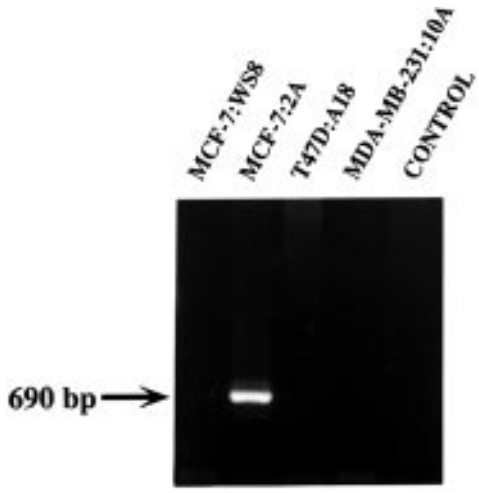

Figure 5. Specific PCR analysis of the 80 kDa ER cDNA. PCR was performed on cDNAs from the denoted cell lines using the primers U4 and D80. The primer D80 was designed to specifically bind only the 80 kDa ER cDNA. The resulting product is 690 bp. As seen here only the MCF-7:2A cDNA served as a template for this amplification.

DISCUSSION

We identified a novel 80 kDa ER that is expressed in the MCF-7:2A human breast cancer cell line in addition to the wild type 66 kDa ER. This 80 kDa protein is the result of translation of an ER mRNA that contains a duplication of exons 6 and 7. While many mutant ER mRNAs have been discovered using the techniques of RT-PCR and RNAse protection, to our knowledge no mutant ER protein has been detected in the cells from which the mutant mRNA was originally isolated. The 80 kDa ER is easily detected using Western blot analysis of unfractionated whole cell extracts. Uniquely, it was Western blot analysis which led to the initial discovery of the 80 kDa ER. While the 80 kDa ER is a small proportion of the total ER complement in the MCF-7:2A cells it may account for enough transcriptional activity to regulate proliferation in the absence of their primary mitogen (estradiol). We have previously described the growth characteristics of these cells as well as their ability to induce the transcription of a luciferase reporter gene and both these activities, while considerably greater than that of the wild type MCF-7:WS8 cell in the absence of estrogen, were significantly less than the maximal activity seen in MCF-7:WS8 cells in the presence of estrogen (2). We surmise that the selection process has allowed the outgrowth of a clone which can use this marginal activity in order to allow sustained growth in estrogen free media.

The Western blot presented in Figure 1 demonstrates the presence of the mutant ER in the MCF-7:2A cells and not the quantitative expression of the wild type and mutant ERs in these cell lines. We have performed detailed studies on the steady state expression of the ER mRNA and protein in these two cells lines as well as the T47D:A18 cell line (33). We have shown that changes in the levels of the ER are quite complex, particularly in the MCF-7:2A cell line in which the two forms of the ER appear to be differentially regulated. For the study presented in Figure 1 we used whole cell extracts from cells which had been grown in estrogen-free media for 5 days. This treatment leads to an increase in the steady state ER levels in the MCF-7:WS8 cells, however this is the standard culture media for the MCF-7:2A cells and the ER is already maximal. The ratio of the 80 kDa ER to the 66 kDa ER does not reflect the apparent gene copy number of the mutant versus wild type ER. We have observed that this ratio can fluctuate between nearly equal expression (2) to the ~ 1/10 ratio seen here. It is unclear as to what is the primary determinant of these alterations in expression, however the expression of the ER has been shown to be regulated by sequences in the coding sequence of the ER (34) and the mutation which has caused the appearance of the 80 kDa ER may have given rise to aberrant regulation of this protein which causes its lower steady state

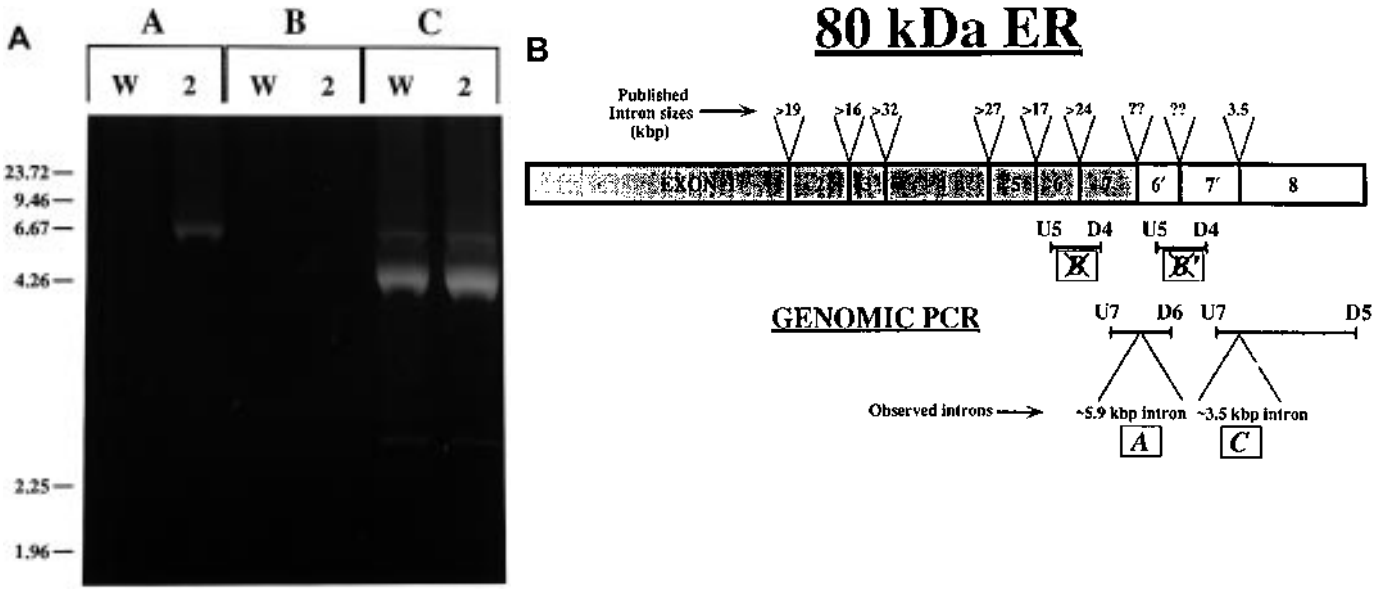

Figure 6. Genomic PCR of the ER in MCF-7:WS8 and MCF-7:2A cells. (**A**) 100 ng of total genomic MCF-7:WS8 (W) and MCF-7:2A (2) DNA was subjected to PCR using the XL PCR kit (Perkin-Elmer-Cetus, Foster City, CA). Each primer set is designed to span at least one intron in the human ER gene.The primer sequences and binding sites are listed in Table 1, see (B) for a schematic representation of the genomic PCR products. Primers U7 and D6 are used in A, this set is designed to amplify intron sequences separating exons 7 and 6. Primers U5 and D4 are used in B and are expected to amplify the intron separating exons 6 and 7 and also exons 6′ and 7′ in the 80 kDa ER. Primers U7 and D5 are used in C and amplify the intron between exons 7 and 8 in the wild type ER and exons 7′ and 8 in the 80 kDa ER. Shown on the left are the positions, in kb, of the molecular weight marker λ-*Hin*dIII. (**B**) The structure of the 80 kDa ER as determined from the partial PCR mapping and sequencing data. The calculated molecular weight of this protein is 77 kDa. Also shown are the fragments produced in the genomic PCR.

expression level. Further studies will be necessary to determine the mechanism of regulation of the two ERs in the MCF-7:2A cells.

In studies described here we have used the technique of PCR mapping to establish the gross structure of the 80 kDa ER cDNA. The principal change in the structure of the 80 kDa ER cDNA was observed using primers that amplified the steroid binding domain of the cDNA. In primer set 4 (U4–D4) (see Fig. 3) we observed a number of additional bands in the MCF-7:2A group. We believe that only one of these additional bands is responsible for the 80 kDa ER. Subsequent studies demonstrated that the ~800 bp band contains an ER cDNA with a duplication of exons 6 and 7. This observation can explain the presence of the additional larger bands in primer sets 4 and 5. Templates that contain duplicated sequences can give rise to additional spurious products due to the phenomenon of long range jumping in PCR, whereby incompletely extended products anneal to the duplicated sequences that lie outside the original area of interest. This hybridization leads to undesired amplification and the appearance of these larger bands (35).

Functional studies on an artificially created 80 kDa ER was not possible by simply inserting the duplicated exon sequences into the wild type ER expression vector due to the lack of suitable cloning sites in the regions flanking exons 6 and 7 in the wild type ER. Additionally, this duplication could not be definitively shown to be the only change in the coding sequence of the 80 kDa ER until the entire cDNA had been sequenced. Subsequent to the studies presented here we have succeeded in cloning the entire coding sequence of this mutant ER, as well as a wild type ER from the MCF-7:2A cells through the use of XL-PCR. The functional properties of this mutant ER are described in a submitted report (36) in which we show that the 80 kDa ER does not appear to function as a stimulatory transcription factor but does appear to inhibit the activity of the wild type ER in transient transfection studies.

The alteration in conditions used with this procedure also allowed us to confirm that the size and DNA sequence of the fragments in primer sets 1 and 2 were the same in the wild type and 80 kDa ER. More importantly, the entire coding sequence of both ERs have been sequenced and have been found to be wild type other than the exact duplication of exons 6 and 7 in the 80 kDa ER (36).

The presence of the smaller (~300 bp) band in primer set 5 (U5–D5) can be attributed to the deletion of exon 7 from the ER mRNA. This amplified product has been consistently observed in the PCR of MCF-7 cDNAs by ourselves and others as well as in clinical samples including breast cancers and meningiomas (37,38). Fuqua *et al.* have investigated the function of this mutant and have shown that it acts as a dominant negative repressor of normal ER function in yeast (11). While we can demonstrate the presence of this cDNA in both the MCF-7:WS8 and MCF-7:2A cells by PCR we do not observe any measurable amounts of this protein in whole cell extracts by Western blot analysis using the monoclonal antibody H226, which recognizes an epitope in the A/B domain of the ER (6). We believe that this type of splicing error occurs rarely and therefore the amount of protein which is present is below the level of detection of the Western blot, however, the exquisite sensitivity of PCR allows the detection of these rare transcripts. The biologic activity of mutant ERs expressed at these low levels is unclear.

Karyotype and FISH analysis confirm that the MCF-7:2A and MCF-7:WS8 cells share a common lineage. These data, along with genomic PCR data, suggest that the MCF-7:2A cells have undergone a chromosomal rearrangement which has caused a duplication of the entire ER gene as well as a duplication of the portion of the gene which contains exons 6 and 7. The most likely scenerio would be a recombination event between ER genes in which intron 5 exchanged with intron 7 causing a duplication of

exons 6 and 7 and the exchange of exon 8. This would also suggest that at the time of the recombination a gene missing exons 6 and 7 arose. However, this deletion has not been observed in any RT-PCR that we have performed with the MCF-7:2A RNA. It is possible that the gene missing exons 6 and 7 was lost during the growth and selection of the MCF-7:2A cells. It also appears from our data that the 80 kDa ER is coded by only one of the four copies of the ER gene present in the MCF-7:2A cells. The remaining three copies do not appear to be significantly different from the wild type 66 kDa ER. This is confirmed by Southern blot data which suggests that the wild type gene is in such excess that the altered fragments cannot be resolved using this technique.

We also performed genomic PCR on the MCF-7:WS8 and MCF-7:2A cells and found that the segment of the wild type ER gene from exon 7 to exon 8 could be amplified. This segment has been shown to contain the smallest (~3.5 kb) ER intron (7). The majority of introns in the ER gene are >10 kb and are not readily amplified using XL PCR. Genomic PCR of the MCF-7:2A ER gene, however, did demonstrate that the segment of the 80 kDa ER gene from exon 7 to exon 8′ does include an intron of ~5.9 kb. This suggests that this mutant gene includes either part of the wild type intron which separates exons 5 and 6 (~17 kb in the wild type ER) or else an addition to the intron which separates exons 7′ and 8 (~3.5 kb in the wild type ER). This confirms that the 80 kDa ER protein is not the result of a trans-splicing event, but is a consequence of the genetic rearrangement of an ER gene, most likely located on the derivative chromosome der(6)[5].

The fact that the 80 kDa ER protein is present in a cell line that has developed estrogen independent growth suggests that it may be involved with the evolution of this phenotype. The finding that the 80 kDa ER is expressed in all 10 subclones of the MCF-7:2A cell line thus studied and has been maintained in the MCF-7:2A cells for over 100 passages further supports the importance of this protein (2). However, the presence of an excess of 66 kDa ER in this cell line makes direct elucidation of the activity of the 80 kDa ER quite difficult. While expression of mutant ERs in heterologous systems has proven useful in investigating the properties of these proteins it does not directly address the function of these proteins in the cells from which they originate. Further analysis of the MCF-7:2A cell line should provide greater understanding of the properties of this protein in its 'natural' environment.

The appearance of a mutant ER containing a duplication of two exons is an extremely unusual event that has never been observed in any members of the steroid hormone receptor superfamily. Study of the changes associated with this extraordinary genetic rearrangement should provide unique insights into the phenomon of ER regulated growth and the biochemical properties of the ER.

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