

# Novel Consensus DNA-Binding Sequence for BRCA1 Protein Complexes

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Increasing evidence continues to emerge supporting the early hypothesis that BRCA1 might be involved in transcriptional processes. BRCA1 physically associates with more than 15 different proteins involved in transcription and is paradoxically involved in both transcriptional activation and repression. However, the underlying mechanism by which BRCA1 affects the gene expression of various genes remains speculative. In this study, we provide evidence that BRCA1 protein complexes interact with specific DNA sequences. We provide data showing that the upstream stimulatory factor 2 (USF2) physically associates with BRCA1 and is a component of this DNA-binding complex. Interestingly, these DNA-binding complexes are downregulated in breast cancer cell lines containing wild-type BRCA1, providing a critical link between modulations of BRCA1 function in sporadic breast cancers that do not involve germline BRCA1 mutations. The functional specificity of BRCA1 tumor suppression for breast and ovarian tissues is supported by our experiments, which demonstrate that BRCA1 DNA-binding complexes are modulated by serum and estrogen. Finally, functional analysis indicates that missense mutations in BRCA1 that lead to subsequent cancer susceptibility may result in improper gene activation. In summary, these findings establish a role for endogenous BRCA1 protein complexes in transcription via a defined DNA-binding sequence and indicate that one function of BRCA1 is to co-regulate the expression of genes involved in various cellular processes. Published 2003 Wiley-Liss, Inc.†

**Key words:** BRCA1; cyclic amplification and selection of targets (CASTing); USF2

## INTRODUCTION

Individuals with inherited mutations in the breast cancer susceptibility gene, *BRCA1*, have a predisposition for the development of early-onset breast and ovarian cancer. *BRCA1* mutations account for approximately 45% of hereditary breast cancers and 80–90% of combined hereditary ovarian and breast cancers [1]. Compelling data support a multifunctional role for BRCA1 in several different fundamental cellular processes. BRCA1 is proposed to function in the maintenance of genome integrity, DNA repair, and transcriptional regulation. Numerous observations have demonstrated that BRCA1 is a protein component of several different repair complexes. The association of BRCA1 with DNA repair proteins such as Rad51, p53, ATM, BRCA2, and the Rad50-hMre11-p95 complex strongly supports the hypothesis that BRCA1 participates in maintaining genomic integrity [2–5]. In addition, biochemical purification coupled with mass spectrometry analysis leads to the purification of a multi-subunit DNA-repair complex termed the BRCA1-associated genome surveillance

complex. This supercomplex is composed of DNA damage repair proteins, including BRCA1, ATM, MSH2, MSH6, MLH1, and BLM [6]. The association of BRCA1 with these repair proteins also suggests a potential role for BRCA1 in the transcription-coupled repair process. This theory is supported by direct evidence showing that BRCA1-deficient mouse embryonic stem cells are defective in transcription-coupled repair of oxidative DNA damage

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Abbreviations: STAT1, signal transducer and activator of transcription; IFN- $\gamma$ , interferon- $\gamma$ ; IGF-I-R, insulin-like growth factor-I receptor; CASTing, cyclic amplification and selection of targets; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; BRCT, BRCA1 carboxy (C)-terminal; USF, upstream stimulatory factor; DNA-PK, DNA-dependent protein kinase.

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and are hypersensitive to ionizing radiation and hydrogen peroxide [7]. These results strongly implicate BRCA1 in the repair process; however, the precise mechanistic basis by which BRCA1 elicits cellular responses to damage remains unclear.

One possibility is that the BRCA1 protein directly modulates DNA repair; however, BRCA1 could possibly mediate gene expression through spectrum of target genes involved in DNA repair and cellular proliferation. Early observations showed that the BRCA1 protein contains an N-terminus ring finger proposed to be involved in protein-protein or protein-DNA interactions, two nuclear localization signals, and a highly acidic C-terminal with transactivation capabilities [8–11]. Consistent with these data, BRCA1 physically associates with several proteins involved in gene regulation. These proteins include a number of transcription factors (eg, p53, CtIP, Rb, estrogen receptor- $\alpha$ , signal transducer and activator of transcription (STAT1a), MYC, and the RNA polymerase II holoenzyme complex) and chromatin remodeling proteins (e.g., BRG1, RbAP46, RbAP48, HDAC1, HDAC2, p300/CBP, and the SWI/SNF-related complex) [reviewed in 12–15]. Perhaps most important is the involvement of BRCA1 in the activation and/or repression of a variety of genes. BRCA1 transactivates the cell-cycle inhibitor p21<sup>WAF1/CIP1</sup> and the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup>, enhances interferon- $\gamma$  (IFN- $\gamma$ )-stimulated growth arrest via STAT1a, and alternatively represses myc-mediated transcription, insulin-like growth factor-I receptor (IGF-I-R) promoter activity, and estrogen-stimulated estrogen receptor- $\alpha$  activation [16–21]. More recently, the utilization of global gene expression analysis using cDNA arrays has revealed several BRCA1 target genes, such as *GADD45*, *GADD153*, cyclin B1, cyclin D1, *MYC*, *STAT1*, *JAK1*, and *ID4* [13,22–24]. Therefore, it appears that BRCA1 could be directly involved in gene regulation. However, there have been conflicting reports about the ability of BRCA1 to bind specific DNA sequences. One investigative group has presented data showing that BRCA1 interacts with ZBRK1 to bind a specific DNA sequence, whereas another group proposed that BRCA1 binds DNA directly, but without any DNA sequence specificity [25,26]. To determine whether BRCA1 is directly involved in gene regulation through a specific DNA sequence, we used the cyclic amplification and selection of targets (CASTing) method [27,28] with endogenous BRCA1 protein complexes to identify a nonrandom 8-base pair (bp) recognition motif for BRCA1.

## MATERIALS AND METHODS

### Cell Lines and Whole Cell Extracts

In this study, 184 human mammary epithelial cells (normal primary breast epithelial cells) were provided by Dr. Martha Stampfer's laboratory

(Lawrence Berkeley Laboratory, University of California, Berkeley, CA) and cultured as previously described [29]. HBL100 (Simian virus-40 [SV-40] transformed immortalized), B5 (chemically immortalized breast epithelial cells) and breast cancer cell lines SKBR3, T47D, ZR751, MDAMB231, MDAMB436, MDAMB453, MDAMB175, BT459, BT474 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured at 37°C with 5% CO<sub>2</sub> and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.2 mM serine, 0.1 mM aspartate, and 1.0 mM pyruvate and 50 U/mL of penicillin/streptomycin. In addition, cells were routinely tested and found to be negative for mycoplasma contamination. Cells were grown to 80% confluence and were re-fed with fresh medium 24 h before harvesting for cell extracts. Whole cell extracts were prepared as described previously [30]. For serum induction experiments, cells were starved in 0.5% serum for 72 h and restimulated with 10% serum for 24 h before harvest. Estrogen depletion experiments were performed as described previously [31].

### Cyclic Amplification and Selection of Targets (CASTing)

The CASTing method was performed as described [32]. The target CAST oligonucleotide contained a 30-bp random core flanked by EcoRI/HindIII restriction sites and  $\lambda$ gt10 polymerase chain reaction (PCR) primer binding sites. These random oligonucleotides (5  $\mu$ g) were converted to double-stranded products by extension of the 3' primer with Taq DNA polymerase through one PCR cycle (94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 72°C for 7 min). The DNA was ethanol precipitated and resuspended in 30  $\mu$ L of 1 $\times$  Shift buffer (200 mM HEPES, pH 7.6; 50 mM EDTA; 200 mM dithiothreitol, 400 mM KCl, 200 mM MgCl<sub>2</sub>, 0.003 mg/mL bovine serum albumin). The first round of CASTing was initiated with 100  $\mu$ g BRCA1 immunoprecipitated complexes from HBL100 whole cell extracts (rounds 2–6 contained 50  $\mu$ g) in 200  $\mu$ L total volume of 1 $\times$  Shift buffer. These random DNA oligos were mixed with BRCA1 immunoprecipitated protein complexes to isolate specific BRCA1-bound sequences. BRCA1 complexes were isolated by immunoprecipitation of whole cell extracts with BRCA1 monoclonal antibody Ab-3 (Oncogene Research, Boston, MA). Briefly, protein lysate was pre-cleared with 50  $\mu$ L of Protein G agarose slurry (Boehringer-Mannheim, Indianapolis, IN) for 1 h. Then, 6  $\mu$ g of BRCA1 antibody was added to the supernatant and incubated overnight at 4°C; 30  $\mu$ L of Protein G slurry was added back to the antibody/protein mix and incubated for 45 min. The Protein G beads were then washed four times with 1 $\times$  Shift buffer and incubated in a 27- $\mu$ L binding reaction with the double-stranded random oligonucleotide for 1 h at room temperature. The Protein G beads

were washed four times in 1× Shift buffer and resuspended in 100 µL PCR mix. The mix was heated at 95°C for 5 min to release DNA from the beads, and Taq polymerase was added followed by 30 PCR cycles (94°C for 1 min, 58°C for 1 min, 72°C for 1 min). This amplified DNA was analyzed by agarose gel electrophoresis to avoid overamplification and was used in subsequent CASTing rounds. After six rounds of selection, retained DNA sequences were amplified with λgt10 primers, digested with EcoRI/HindIII, and cloned into pBluescript (Stratagene, La Jolla, CA). Individual clones were isolated, sequenced, and analyzed for enriched sequences.

#### Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) experiments were performed by incubating 3 µg of whole cell extract with <sup>32</sup>P-labeled double-stranded oligonucleotide as described previously [30]. The DNA-protein binding reaction was incubated at room temperature for 20 min and resolved on a 4% polyacrylamide gel that was run at 320 V for 1 h, 20 min. Supershift assays were performed by preincubating whole cell extract with the indicated antibody before the addition of labeled oligonucleotide. BRCA1 Ab1 (aa 1–303), Ab3 (1843–1863), and BR64 (aa 80–100) [33] may be purchased from Oncogene Research and Chemicon International (Temecula, CA), respectively; 115 (aa 673–1365) [34] and AP16 (1313–1863) [35] were as published.

#### Immunoprecipitations and Western Blotting

Whole cell extracts were used for immunoprecipitation experiments. Extracts (100 µg) were pre-cleared with Protein G-Sepharose (Amersham Pharmacia, Piscataway, NJ) for 1 h while rotating at 4 degrees to reduce nonspecific binding. Samples were collected by centrifugation, and supernatants were transferred to a new tube containing the appropriate immunoprecipitating antibody and incubated overnight at 4 degrees with rotation. Agarose beads were added to the protein/antibody sample and incubated for 45 min. The beads were collected by centrifugation, washed 4× with 1× Shift buffer (200 mM HEPES, pH 7.6, 50 mM EDTA, 200 mM DTT, 400 mM KCl, 200 mM MgCl<sub>2</sub>, (0.003 mg/mL BSA)), and resuspended in 50 µL of 2× sodium dodecyl sulfate Sample Buffer. Samples were boiled and loaded onto a 6% polyacrylamide gel, and were run at 120 V for 2 h. Proteins were transferred onto 0.45 µm nitrocellulose at 100 V for 1 h and then blocked overnight in 10% milk/Tris-buffered saline containing Tween 20. Western blotting was performed using BRCA1 AB-1 (Oncogene) at a dilution of 1:3,000. Proteins were visualized using the enhanced chemiluminescence system (Amersham Pharmacia).

#### Reporter Assays

Transient transfections were performed with Lipofectamine Reagent (Gibco; Frederick, MD) according

to the manufacturer's instructions. Cells were plated at  $3.4 \times 10^5$  cells in 60-mm dishes and transfected the following day. Each transfection included 2–10-µg control vector or cytomegalovirus-driven wild-type or mutant BRCA1 expression plasmids [34] 1–5 µg of chloramphenicol acetyltransferase (CAT) reporter vector (Promega, Madison, WI) and 0.5 µg of the pGL3-control plasmid (Promega). CAT reporter constructs contained the consensus BRCA1 binding site in the presence of minimal SV-40 promoter/enhancer sequences (Promega-pCAT<sup>®</sup>3-Promoter Vectors). Co-transfections with an empty pcDNA3 vector and the pGL3 control constructs were used as normalization controls. Equal amounts of protein lysates were measured for CAT activity 48 h post-transfection according to the manufacturer's instruction (Promega).

## RESULTS

### Identification of Specific DNA-Binding Sequence for BRCA1 Protein Complexes

To identify potential specific sequences that could be bound by BRCA1, we used the CASTing method, using endogenous BRCA1 complexes as bait to determine DNA sequence binding specificity. Immunoprecipitated BRCA1 protein complexes were incubated in a binding reaction with a mixture of target double-stranded oligonucleotides containing a 30-bp random core flanked by restriction sites and PCR primer binding sites (Figure 1A). Specifically retained DNA was denatured, PCR amplified, and mixed with fresh immunoprecipitated BRCA1 protein complexes to initiate additional rounds of CASTing. After six rounds of enrichment, the BRCA1 complex bound sequences were isolated, cloned, and sequenced. Figure 1B lists the specific DNA sequences of 43 clones that were obtained using this approach. These sequences were aligned and the frequency at each position yielded a nonrandom 8-bp TTC(G/T)GTTG sequence (Figure 1C).

The physical interaction of BRCA1 complexes with this consensus sequence was examined using EMSA and supershift assays. The sequences of the DNA probes used to demonstrate binding specificity by BRCA1 protein complexes are shown in Figure 1D. EMSA experiments with labeled probes containing the BRCA1 binding site (Figure 1D, Consensus probe or clone 25) produced four distinct DNA-protein complexes (Figure 2). Two of these complexes (BRCA1 complexes I and II) were ablated by competition with excess unlabeled consensus oligonucleotide (Figure 2, lane 2), but not with unrelated oligonucleotides (Figure 2, lane 3), indicating that these two DNA-binding complexes were sequence specific. The remaining DNA/protein complexes appear to be nonspecific because the addition of consensus DNA oligonucleotides failed to compete for binding specificity, and BRCA1 antibodies had no



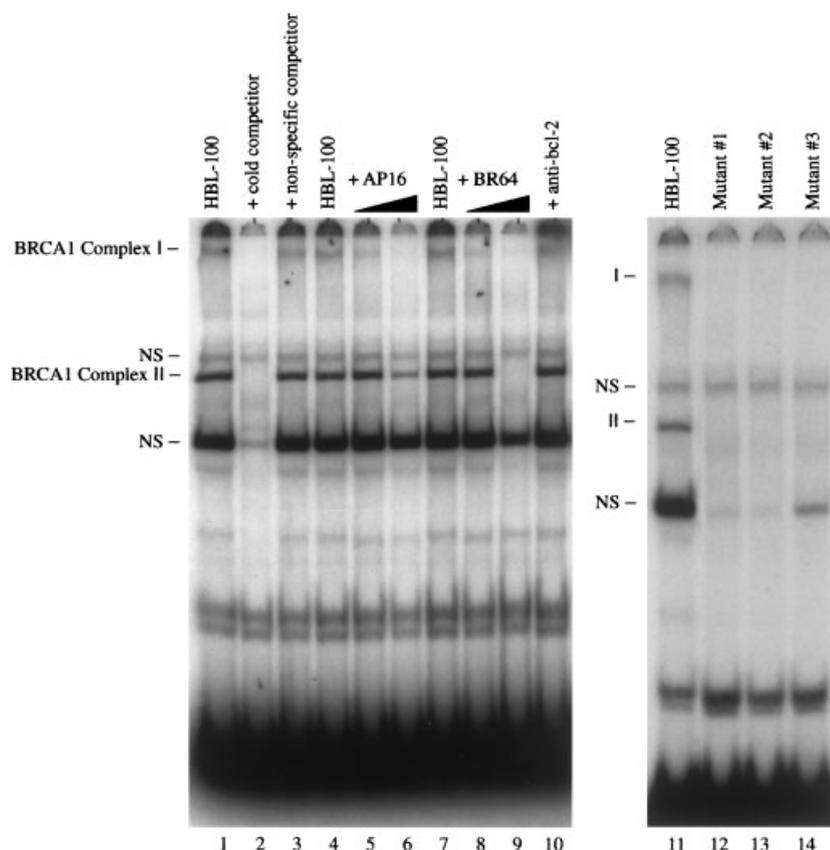


Figure 2. Sequence-specific DNA binding by BRCA1 protein complexes. EMSA experiments were performed using 3  $\mu$ g of HBL100 whole cell extract incubated with  $^{32}$ P labeled oligonucleotide containing the BRCA1 consensus sequence. Lane 1, binding of protein complexes in the absence of competitor sequences. Bands I and II were competed with 100-fold excess unlabeled oligonucleotide (lane 2), but not with an unrelated sequence competitor (lane 3). Antibody supershifts assays were performed using anti-BRCA1 monoclonal antibodies. The addition of increasing amounts (1–2  $\mu$ l) of BRCA1

antibodies AP16 (lanes 5,6) and BR64 (lanes 8,9) resulted in the disruption of bands I and II. Nonspecific antibody  $\alpha$ -bcl2 (lane 10) had no effect on these protein complexes. The synthetic consensus oligonucleotide displayed the same binding pattern as the retained CASTing clone 25 (lane 11). Site-directed mutagenesis of consensus sequence resulted in loss of binding (mutant probe sequences; Fig. 1D). Mutant probes 1, 2, and 3 lost their ability to bind BRCA1 DNA-specific protein complexes (lanes 12–14).

the antibody recognizes a region of the protein involved in DNA-complex interaction, it might disrupt or block protein-DNA interactions, resulting in a reduction of DNA-binding protein complex formation [36]. Antibody supershifts with the BRCA1 antibodies AP16 (Figure 2, lanes 5 and 6) and BR64 [33] (Figure 2, lanes 8 and 9), as well as Ab-1 (Oncogene Research) and 115 [37] (data not shown), specifically diminished complexes I and II, thus supporting the presence of BRCA1 in these complexes, whereas the negative control, bcl-2 antibody, failed to affect the formation of these complexes (Figure 2, lane 10). Cumulatively, these results strongly indicated that DNA-binding complexes I and II were indeed sequence-specific complexes that contained the BRCA1 protein.

#### Specific Binding of BRCA1 Protein Complexes to the Newly Defined BRCA1 Binding Sequence

Recent data suggest that the BRCT region of BRCA1 binds the termini of DNA fragments in a nonspecific

fashion [38]. To address the issue of sequence specificity, we performed site-directed mutagenesis on the defined consensus sequence (Figure 1D, mutants 1–3) and repeated EMSA experiments to determine the critical DNA bases required for the DNA binding. Mutants 1, 2, and 3 completely abolished BRCA1 protein complexes I and II (Figure 2, lanes 12–14), indicating that certain bases within the consensus binding site were essential for DNA binding. In fact, a minimal 2-bp substitution (see mutant 3, Figure 1D) was sufficient to disrupt completely the binding of these complexes. These observations demonstrate that the ability of these BRCA1 protein complexes to bind DNA is not random and is dependent on specific DNA sequences.

#### BRCA1 DNA-Binding Protein Complex Contains Upstream Stimulatory Factor

We wanted to identify additional components of the BRCA1 DNA-binding protein complexes. In an attempt to identify potential cofactors, we used

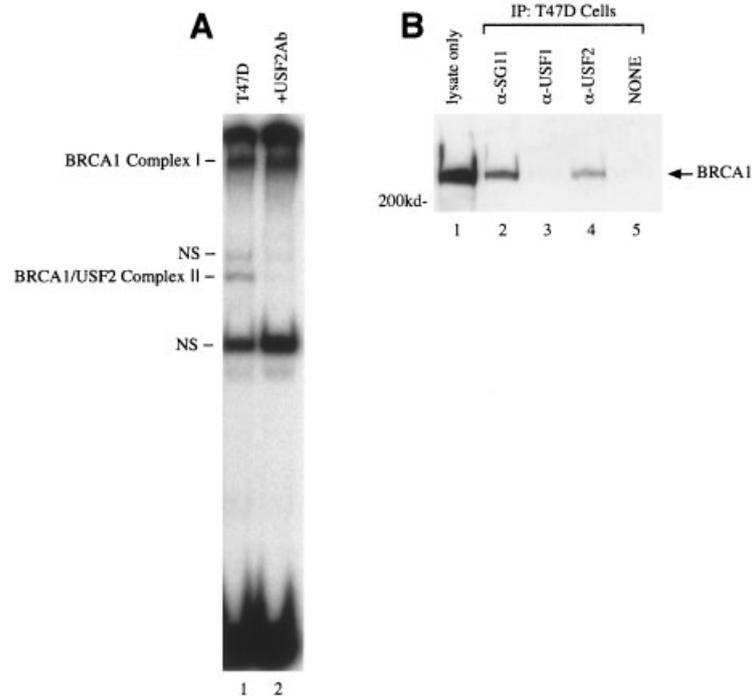
antibody supershift analysis to test for the presence of more than 20 individual proteins known to be associated with BRCA1. We were unable to identify any of these proteins as BRCA1 DNA-binding partners in complexes I or II (data not shown). In addition, we tested several candidate DNA-binding factors of interest as potential BRCA1 DNA-binding co-partners. We examined the upstream stimulatory factor (USF) family of general transcription factors as potential interacting cofactors because of their antiproliferative properties and role in breast carcinogenesis [39]. Interestingly, previous work demonstrates that there is a loss of USF transcriptional activity in breast cancer cell lines (including MCF7, HBL100, and T47D cells); however, there are no significant variations in USF1 or USF2 protein levels in these cells compared with normal breast epithelial cells [39].

We performed gel-shift assays to investigate a potential interaction between the USF family members and BRCA1. Antibody supershifts using a USF2 antibody (Figure 3A, lane 2) specifically diminished a nonspecific complex and BRCA1 complex II, indicating that USF2 is at least one of the components of the BRCA1 DNA-binding protein complex II. The specific physical interaction between USF2 and BRCA1 was confirmed by immunoprecipitating endogenous USF2 protein complexes in breast

cancer T47D whole cell extracts with an anti-USF2 antibody (Santa Cruz, Santa Cruz, CA) followed by immunoblotting with BRCA1 Ab1 (MS110) antibody (Oncogene). BRCA1 complex association was detected when USF2 antibody was used for the immunoprecipitation (Fig. 3B, lane 4), but not with USF1 or negative control antibodies (Fig. 3B, lanes 3 and 5). Whole cell lysates and BRCA1 Ab3 (SG11) immunoprecipitated complexes served as positive controls (Figure 3B, lanes 1 and 2).

#### BRCA1 Co-Regulation Via the Defined BRCA1 DNA-Binding Sequence

Because BRCA1 functions as a transcriptional activator when fused to the GAL4 DNA-binding domain [8,10], we examined whether BRCA1 complexes could activate transcription via the defined consensus binding site using reporter assays. The BRCA1 consensus-binding site (Figure 1D) was cloned into pCAT reporter vectors in the presence of minimal promoter or enhancer sequences. The resulting plasmids were co-transfected with cytomegalovirus-driven wild-type BRCA1 or mutant BRCA1 expression plasmids into MCF7 cells (contain wild-type BRCA1) and assayed for CAT activity 48 h posttransfection (Figure 4). The ability of BRCA1 to transactivate the pGL3 Promoter vector (TATA box present) constructs containing the BRCA1 DNA-



**Figure 3.** BRCA1 and USF2 physical association. (A) Gel-shift experiments were performed with 3  $\mu$ g of T47D whole cell extract incubated with clone 25 consensus probe. Lane 1, levels of BRCA1 DNA-binding protein complexes in T47D cells. Antibody supershift experiments using USF2 antibody (Santa Cruz) resulted in the ablation of complex II, indicating that USF2 is one component of the BRCA1 DNA-binding protein complex (lane 2). (B) T47D whole cell

lysates were subject to immunoprecipitations followed by Western blots probed with BRCA1 Ab-1 (MS110). USF2 successfully immunoprecipitated BRCA1 protein, demonstrating a specific physical interaction; however, BRCA1 was not detected with USF1 or negative control antibodies (lane 3,5). Whole cell lysate only and BRCA1 Ab3 (SG11) immunoprecipitated complexes were used as BRCA1-positive controls (lanes 1,2).

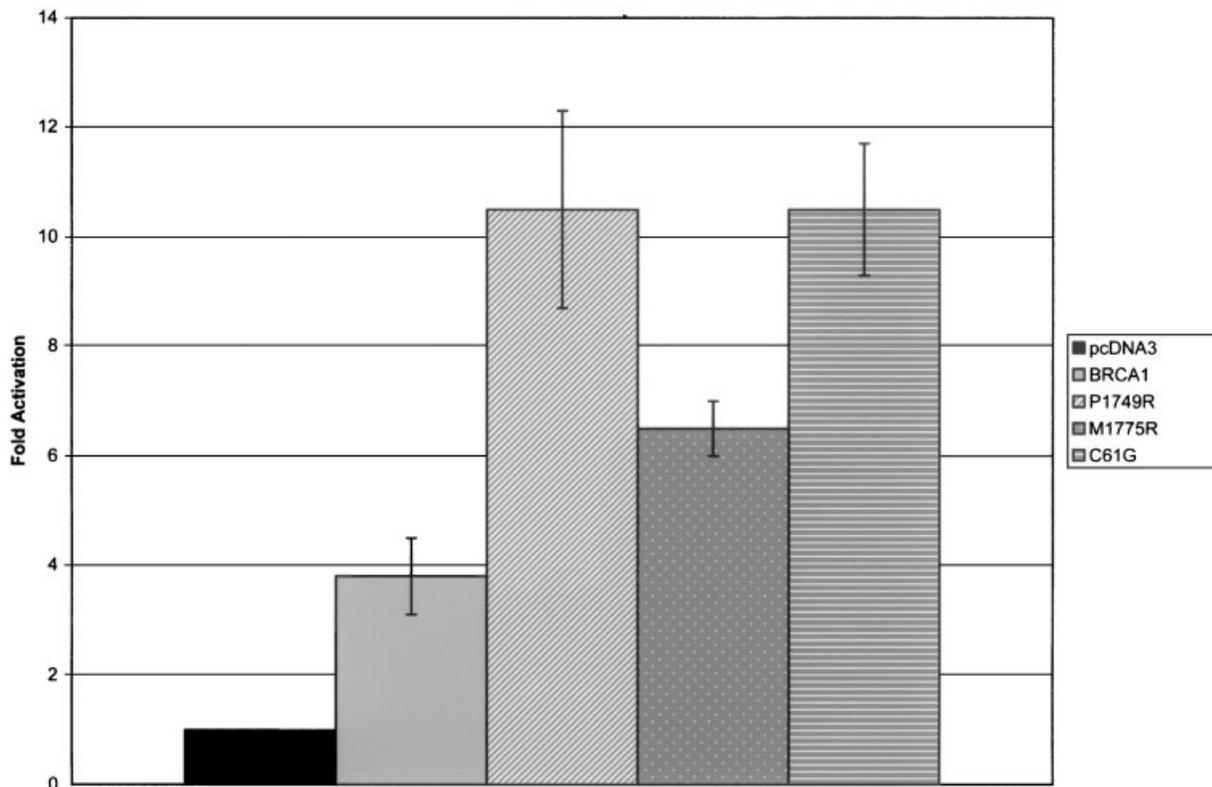


Figure 4. BRCA1 sequence-specific transcription. Transient transfection assays in MCF7 cells demonstrate transactivation by BRCA1 via the BRCA1 consensus site. The consensus sequence was cloned into a CAT reporter vector in the presence of minimal promoter or enhancer sequences. This CAT reporter vector containing the consensus sequence were co-transfected with CMV-driven wild-

type BRCA1 construct, mutant P1749R, mutant M1775R, or mutant C61G, or an empty pcDNA3 control vector. Cells were harvested and assayed 48 h posttransfection for CAT activity. Four samples were used to calculate the standard deviation for each co-transfectant. Data shown are an average of at least two independent experiments. Bars, standard deviation.

binding sequence was compared with the amount of transactivation induced by a control expression plasmid (pcDNA3). Wild-type full-length BRCA1 induced the CAT activity approximately 3.0–4.5-fold when the consensus-binding site was placed upstream of a minimal promoter sequence, clearly indicating that BRCA1 may function in a sequence-specific fashion by modulating transcription via the BRCA1 consensus site. The level of BRCA1-transactivation of the pCAT promoter vector in the absence of the BRCA1 consensus binding sequences was approximately twofold (data not shown). Interestingly, co-transfection of mutant BRCA1 constructs (P1749R, M1775R, and C61G) resulted in a dramatic 6–12-fold transcriptional activation. These data suggest that mutant BRCA1 may lead to inappropriately high levels of some transcripts that may be linked to the susceptibility associated with these mutations.

To explore the biological significance of the BRCA1 binding site, we examined a panel of normal breast epithelial and breast tumor cell lines for their ability to form BRCA1 DNA-binding complexes. We found that normal breast epithelial 184 HMEC (Figure 5A, lane 2) and chemically immortalized B5 breast

epithelial cells (Figure 5A, lane 3) have abundant levels of BRCA1 DNA-binding complexes I and II compared with the breast cancer cell lines examined (Figure 5A, lanes 4–13; 5C, lane 4). This is particularly interesting, considering that all the cell lines examined here express comparable levels of wild-type BRCA1 protein as evidenced by Western blotting (data not shown). HCC1937, a cell line that has a truncation of the BRCA1 protein [40] retains DNA-binding capabilities, indicating that the extreme C-terminal domain of the protein is not necessary for binding (Figure 5C, lane 4). Therefore, decreased levels of BRCA1 DNA-binding protein complexes may ultimately reflect a role for downregulation of BRCA1 directed transcription in hereditary, as well as sporadic (nonfamilial) cancer development perhaps due to limiting amounts of other complex components.

Previous studies have shown that BRCA1 mRNA and protein levels are regulated with the cell cycle in response to serum and estrogen [41–43]. Therefore, we examined the effects of serum and estrogen on modulation of the formation of the BRCA1 DNA-binding complexes. EMSA analysis was performed on protein extracts from quiescent (Figure 5B, lane 1) and log-phase cells (Figure 5B, lane 2). Complex I was

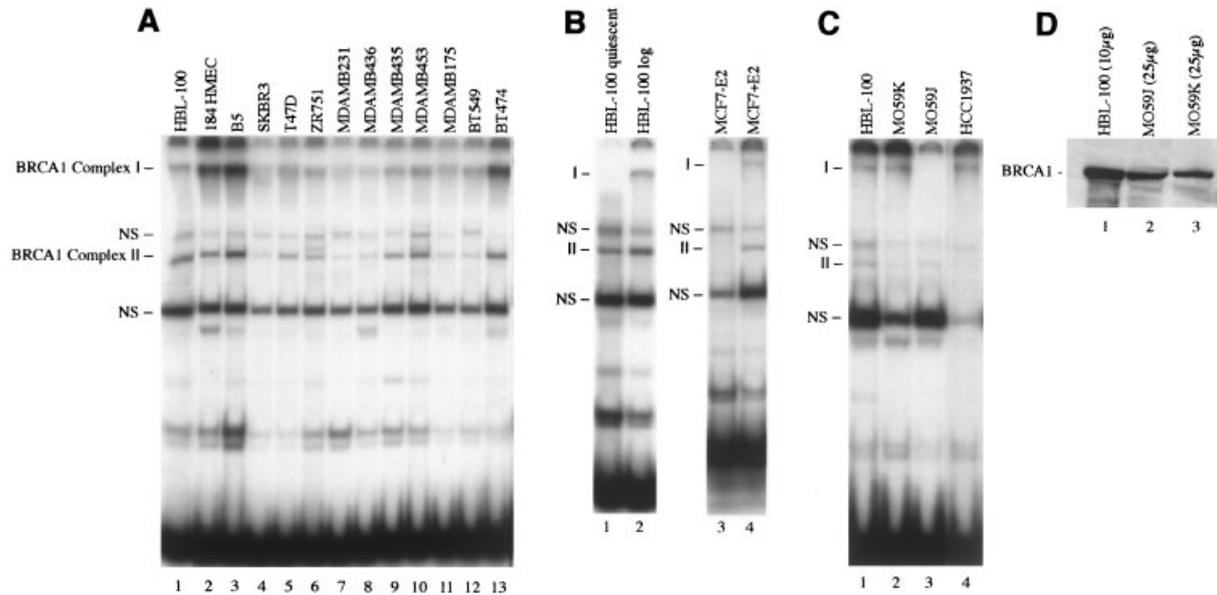


Figure 5. Varying levels of BRCA1 DNA-binding protein complexes. (A) BRCA1 DNA-binding complexes vary between normal and tumor cell lines. Serum and estrogen modulate BRCA1 DNA-binding complexes. Gel-shift experiments were performed with 3 µg of whole cell extract incubated with radiolabeled clone 25. Lane 1, levels of DNA-binding complexes in SV-40-immortalized HBL100 breast epithelial cells. Normal 184 HMEC and chemically immortalized B5 breast epithelial cells (lanes 2,3) express abundant levels of BRCA1 DNA-binding protein complexes. Most breast cancer cell lines (lanes 4–13,17) showed decreased binding of these complexes. (B) Whole

cell extracts from quiescent HBL100 cells (lane 1) were compared with 24-h serum-stimulated cells (lane 2). BRCA1 DNA-binding complexes were compared in estrogen-deprived (lane 3) and stimulated (lane 4) estrogen receptor-positive MCF7 cells. (C) DNA-PK is required for BRCA1 sequence-specific DNA-binding activity. DNA-PK-deficient MO59J cells (lane 3) demonstrated a loss of DNA-binding activity in comparison with DNA-PK-proficient control MO59K cells (lane 2). (D) MO59J and MO59K cells express comparable BRCA1 protein levels.

completely abolished with the removal of serum, and restored upon addition. Furthermore, comparison of estrogen-deprived and stimulated MCF7 cells showed that complexes I and II were able to bind the DNA in the presence of estrogen (Figure 5B, lane 4), but not in its absence (Figure 5B, lane 3). However, while the binding complex is completely decreased, some BRCA1 protein is still expressed in low serum and low estrogen conditions. Therefore, other BRCA1 complex components, such as USF2, or modifying enzymes may be the limiting factors for DNA-binding activity in growth-arrested states.

Finally, because BRCA1 is implicated in double-strand break repair [2,44] we speculated that the BRCA1 DNA-binding complex might be a target for the DNA-dependent protein kinase (DNA-PK). DNA-PK is a nuclear serine/threonine protein kinase involved in mammalian DNA double-strand break repair and V(D)J recombination [45]. EMSA experiments using extracts from DNA-PK-deficient MO59J glioblastoma cells and DNA-PK wild-type control MO59K cells [46] suggest that DNA-PK is required for BRCA1 sequence-specific DNA-binding. MO59J cells (Figure 5C, lane 3) were unable to form BRCA1 DNA-binding complex I, in contrast to MO59K cells (Figure 5C, lane 2), which are DNA-PK proficient. Interestingly, both MO59J and MO59K express comparable levels of BRCA1 protein (Figure 5D); therefore, DNA-PK may be at least partially respon-

sible for the phosphorylation of BRCA1 or other proteins in the complex making the DNA interaction possible. In contrast to what was observed in the DNA-PK-deficient cells, we observed normal DNA-binding complexes in ataxia-telangiectasia mutant (ATM) cell lines. These data indicate that although ATM interacts with BRCA1 in some protein complexes [47], ATM is most likely not involved in the BRCA1 DNA-binding complex formation (data not shown).

## DISCUSSION

This report describes the discovery that BRCA1 protein complexes are capable of controlling gene expression by interacting with a specific DNA sequence TTC(G/T)GTTG. This BRCA1 DNA-binding site differs from any previously reported transcription factor binding site [48], indicative of a novel DNA-binding protein function for the BRCA1 protein complex. In this study, we have shown that BRCA1 physically associates with USF2 to form DNA-binding protein complexes. USF2 belongs to the USF family (USF1 and USF2) of transcription factors that are characterized by a highly conserved basic-helix-loop-helix-leucine zipper (bHLH-zip) DNA-binding domain. USF family members are ubiquitously expressed proteins but appear to play a specific role in breast carcinogenesis. The loss of USF transcrip-

Table 1. Potential BRCA1 Target Genes

Gene name	BRCA1 DNA target	Position <sup>a</sup>	Accession no.	Orientation <sup>b</sup>
Fibroblast growth factor 9 (FGF9)	CT <b>TTGGGTTGGG</b>	-1250	D14838	←
Transforming growth factor-β-stimulated (TSC22)	CA <b>TTGTGTTGGG</b>	-965	U35048	←
Epithelial protein lost in neoplasm β (EPLIN)	CT <b>TTGGGTTGTG</b>	-166	AA487557	←
FOXJ2 forkhead factor (FHX)	GG <b>TTGGGTTGTG</b>	799	AA621179	←
BCL2-like 2 (BCL2L2)	CC <b>TTGGGTTGGG</b>	-931	D87461	←
H2B histone family, member C (H2BFC)	GA <b>TTGAGTTGAG</b>	-1185	Z83740	→
SWI/SNF related, subfamily C (SMARCC1)	TT <b>TCGTGTTGAG</b>	-855	U66615	←
RING1 and YY1 binding protein (RYBP)	TT <b>TTGGGTTGAG</b>	267	W67456	←
ras homologue gene family, member G (ARHG)	GT <b>TTTGTGTTG</b>	823	X61587	→
Estrogen-related receptor-γ (ESRRG)	CT <b>TTCTGTTGAG</b>	350	AA018040	→
Flap structure-specific endonuclease 1 (FEN1)	CT <b>TTCTGTTGAG</b>	1081	G14611	→
Signal transducer and activator of transcription (STAT5A)	TG <b>TTCTGTTGGG</b>	-212	L41142	→
Growth arrest and DNA damage-inducible (GADD45G)	GC <b>TTTGTGTTGGG</b>	-195	BC000465	←
Cyclin B1 (CCNB1)	AT <b>TTTGTGTTGGC</b>	-1979	BC006510	←
APEX nuclease (APEX)	GC <b>TTTCTGTTGGG</b>	-4524	D13370	?
ERCC2	CA <b>TTATGTTGTC</b>	-1084	X52221	→
ERCC5	AT <b>CTTGTGTTG</b>	-765	G62084	?
Origin recognition complex, subunit 4 (ORC4L)	CT <b>TTGTTGTTGTA</b>	637	NM_002552	?
Somatostatin (SST)	TG <b>CCCTGTTGCT</b>	-2450	J00306	?
Estrogen receptor 2, ER β (ESR2)	CT <b>GCCTGTTGCT</b>	-3731	AF051427	?
Sterol regulatory element binding factor 2 (SREBF2)	GT <b>TTGGGTTGCG</b>	-30	G22594	←
2', 5'-oligoadenylate synthetase 1 (OAS1)	AG <b>TTCTGTTGCC</b>	-2117	G60392	→
GCLJ5 (GCN5L2)	CT <b>TTTTGTTGAG</b>	-514	G25692	→

<sup>a</sup>Position is relative to start codon.<sup>b</sup>Orientation is in accordance with the direction of transcription.

tional activity in breast cancer cell lines is a common event, even though there is no significant variation in endogenous protein levels [39]. These proteins generally recognize E-box elements containing a central CACGTG motif; however, USF proteins have the ability to mediate transcriptional repression via noncanonical E-box sequences [49]. It is certainly possible that some BRCA1/USF2 protein complexes bind to E-box sequences, suggesting a role for BRCA1 in E-box-mediated transcription. Recent studies have shown that BRCA1 plays a role in the regulation of the human telomerase reverse transcriptase gene (*hTERT*) by interacting with the E-box binding protein c-Myc [50,51]. BRCA1 physically associates with c-Myc at the E-box site within the *hTERT* promoter and participates in the downregulation of *hTERT* expression by inhibiting the binding activity of c-Myc. Whether BRCA1 participates similarly with USF2 to modulate E-box mediated gene expression remains to be determined.

Interestingly, wild-type BRCA1 led to a minimal transactivation via the consensus sequence, using reporter assays, while BRCA1 constructs containing missense mutations led to a dramatic activation. Germline mutations have been reported throughout the entire BRCA1 gene and include all classes of mutations: frameshift, nonsense, missense, deletions, and insertions. Most of these mutations result in the truncation of the 1,863-amino acid protein product and a loss of function, possibly because of the deleted BRCT motifs required for transactivation. Therefore, the repressive mechanisms of BRCA1 could become defective during the cancer progression process as a result of mutation. This loss of function could possibly lead to the constitutive activation of genes responsible for triggering the cancer cascade, as evidenced by our data suggesting that such mutations could lead to the activation of genes that are normally suppressed by BRCA1. Another possibility is a gain-of-function effect for BRCA1 mutants. The substitution of one amino acid with another is sufficient to trigger the stimulation of genes inappropriately rather than a loss of activity that is often seen with deleterious mutations. This gain-of-function effect was observed more than 10 years ago for the mutant p53 tumor suppressor gene [52]. Although p53 harbors a wide mutation spectrum, 75% of the mutations are missense mutations, allowing for an accumulation of the mutant form of the p53 protein. These gain-of-function mutants often exert their oncogenic effects by improperly stimulating a variety of genes involved in cellular proliferation and form stable complexes with other members of the TP53 family, impeding their normal tumor suppressor functions [53]. A similar gain of function could also be possible for BRCA1 and would provide an explanation for the overt amplification observed using reporter assays in conjunction with BRCA1 missense mutants.

Ninety percent of hereditary BRCA1 mutations in affected families result in an alteration or deletion of the BRCT motifs that facilitates interactions with members of the histone deacetylase complex (HDAC1, HDAC2, RbAp46, and RbAp48) [54–57]. We propose that BRCA1 cancer-predisposing mutations disrupt BRCA1-USF2 and/or BRCA1/HDAC protein interactions, resulting in deregulation of targeted promoters containing the BRCA1 consensus DNA-binding sequence. We are currently identifying target genes that contain a BRCA1 binding site within their regulatory regions and examining the effects of BRCA1 on their expression (Table 1). Genes previously identified as BRCA1-regulated target genes that contain potential BRCA1 binding sites include cyclin B1, GADD45, and p21<sup>WAF1/Cip1</sup> [13,16]. Other genes of immediate interest are those involved in DNA repair processes and cell proliferation. We propose that BRCA1 could be responsible for regulating the expression of a repertoire of growth-regulatory and DNA damage-responsive genes. Imprecise regulation of these genes by either deletion or mutation of BRCA1 or downregulation of USF2 could lead to increased genetic instability and/or inappropriate proliferation. Further studies to characterize additional components of the BRCA1 DNA-binding complexes and confirmation of potential target genes should provide insight into the precise function of BRCA1 via the consensus sequence and its role in genomic surveillance and tumor suppression.

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