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BRCA1 Splice Variants Exhibit Overlapping and Distinct Transcriptional Transactivation Activities

Kristen A. McEachern, William B. Archey, Karen Douville, and Bradley A. Arrick*

Departments of Biochemistry and Medicine, Dartmouth Medical School, Hanover, NH 03755

Abstract The global changes in gene expression induced by transient increased expression of full length BRCA1 as well as the spliced variant $BRCA1_S$ were evaluated by cDNA expression array in a human non-tumorigenic mammary epithelial cell line, MCF10A. Over 30 genes were identified that displayed an altered expression pattern in response to the expression of BRCA1 splice variants. The expression of NFκB inducing kinase was markedly down-regulated in $BRCA1_L$ transfected cells. However, a NFκB-responsive promoter construct yielded increased basal activity in $BRCA1_L$ transfected cells, as well as following treatment with tumor necrosis factor-α or lymphotoxin. In addition, nuclear extracts from $BRCA1_L$ transfected cells displayed increased DNA binding to the κB consensus site. The transcriptional activity of a panel of promoter constructs was evaluated following expression of wild type or mutant BRCA1. Full length BRCA1 transactivated the estrogen receptor-α (ERα) and BCL2 promoters as well as AP-1, SRE, and CRE containing promoters. Transactivation activity of the exon 11-deleted $BRCA1_S$ was more limited and usually of lower magnitude. The ability of a pathogenic mutation, 5382insC, to abrogate the transcriptional transactivation by $BRCA1_L$ and $BRCA1_S$ was also investigated. Mutant BRCA1 retained wild type levels of transcriptional activity for the ERα promoter as well as for the NFκB, AP-1, and CRE-responsive promoters but had reduced or no activity with the BCL2 and SRE promoters. These results show that BRCA1 isoforms have both overlapping and distinct transcriptional transactivation activity, and that a mutant form of BRCA1 implicated in carcinogenesis is not devoid of all activity. J. Cell. Biochem. 89: 120–132, 2003.

Key words: BRCA1; breast cancer; gene expression; alternative splicing

Since the cloning of BRCA1 in 1994, the effort to elucidate its function in cells has been intense. Investigators, using several different approaches, have implicated BRCA1 in multiple cellular processes in a wide variety of cell types. More challenging, however, has been identifying those functions that contribute to its role as a tumor suppressor gene. BRCA1-associated cancers arise specifically from the epithelial cells of the breast and ovary following loss of BRCA1. Thus, while BRCA1 functions in important processes in many cell types, the tissue specificity in which its loss becomes carcinogenic must be considered. Many of the published studies of BRCA1 function, however, are based on non-relevant cell types.

Another aspect of BRCA1 function that is typically not considered is that most mammary epithelial cell lines and tissues co-express a shorter isoform, $BRCA1_S$, resulting from an alternative in-frame splicing event [Lu et al., 1996; Thakur et al., 1997; Wilson et al., 1997]. $BRCA1_S$ (also termed BRCA1-Δ11b or BRCA1a) lacks 3,309 nucleotides from exon 11, encoding a protein that shares amino and carboxy termini with full length BRCA1 ($BRCA1_L$) but lacks ~60% of the internal amino acid sequence. Importantly, this alternative splicing of BRCA1 is evolutionarily conserved in murine cells. Interestingly, knockout mice that retain the shorter isoform have a longer survival during embryogenesis than mice in which both isoforms are lost, suggestive of an important role for $BRCA1_S$ in embryogenesis and development [Gowen et al., 1996; Hakem et al., 1996; Xu et al., 1999].

For our study of BRCA1 function, we have focused on its effects on gene expression as a

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*Correspondence to: Bradley Arrick, Department of Medicine, Dartmouth Medical School, Hanover, NH 03755. E-mail: Arrick@dartmouth.edu

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measure of its role as a tumor suppressor in human mammary epithelial cells. The BRCA1 protein has an amino terminal RING domain and a carboxy terminal acidic domain [Miki et al., 1994]. Its capacity for transcriptional transactivation was first demonstrated when the carboxy terminus of BRCA1 was linked to the GAL4 DNA binding domain, yielding a fusion protein active in transcription [Chapman and Verma, 1996; Monteiro et al., 1996]. Subsequent to this finding, BRCA1 was shown to interact with RNA polymerase II holoenzyme [Scully et al., 1997; Schlegel et al., 2000] as well as with components of the histone deacetylase complex [Yarden and Brody, 1999]. BRCA1 has also been linked to chromatin remodeling through an association with the SWI/SNF complex [Bochar et al., 2000]. BRCA1 acts as a co-activator with p53 to enhance p53-dependent transcription and it interacts with and functions with the transcriptional co-activator CBP/p300 [Ouchi et al., 1998; Zhang et al., 1998; Chai et al., 1999; Somasundaram et al., 1999; Pao et al., 2000]. BRCA1 also interacts with ATF1, a member of the CREB/ATF family of transcription factors, and stimulates transcription from cAMP response elements in a human embryonic kidney cell line [Houvras et al., 2000]. BRCA1 represses the transactivation and transforming ability of c-Myc [Wang et al., 1998] and can act as a transcriptional co-repressor through an interaction with ZBRK1 [Zheng et al., 2000]. Comparisons of gene expression profiles within breast tumors has demonstrated that BRCA1-associated cancers display a distinctive pattern of gene expression that differs from that found in BRCA2-associated cancers and sporadic cancers [Berns et al., 2001; Hedenfalk et al., 2001; Van't Veer et al., 2002].

We have previously demonstrated the ability of full length BRCA1, $BRCA1_L$, as well as the splice variant, $BRCA1_S$, to transactivate the p21 promoter [Lu and Arrick, 2000]. In this study, we have evaluated the more extensive effects of $BRCA1_L$ and $BRCA1_S$ isoforms on gene expression in human non-tumorigenic mammary epithelial cells through the use of cDNA array technology. We have identified over 30 genes whose expression is altered in a BRCA1 isoform-specific manner. The expression of one of those genes, NFκB inducing kinase (NIK), was confirmed by Northern analysis to be markedly down-regulated by $BRCA1_L$. While investigating the functional consequences of the loss of NIK expression in MCF10A cells, we uncovered an ability of $BRCA1_L$ to augment basal as well as tumor necrosis factor-α (TNF)- and lymphotoxin (LT)-induced transcription from a NFκB responsive promoter construct. In addition, we have demonstrated that the transcriptional activation by $BRCA1_L$ correlates with an increase in binding of nuclear proteins, including p50, to the κB consensus site. We also studied the effects of BRCA1 wild type and mutant splice variants on the transactivation of activator protein-1 (AP-1)-, serum response element (SRE)-, and the cAMP response element (CRE)-containing reporter plasmids, as well as plasmids driven by the promoters for estrogen receptor-α (ERα) and BCL2, and observed that BRCA1 variants exhibit specific and distinct transcriptional activities.

MATERIALS AND METHODS

Cell Culture and Transfection

The human mammary cell line MCF10A was provided by Dr. Jean Gudas (National Cancer Institute). Cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 2.5 MM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml human recombinant epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, and 8 μg/ml insulin. Cells were incubated at 37°C, 5% CO_2. Cells were routinely plated at a density of 2×10^5 cells in 60 mm dishes the day before transfection. Cells were transfected with BRCA1 expression plasmids that use the cytomegalovirus promoter (pRK7 vector), prepared as previously described [Lu and Arrick, 2000]. Using the FuGENE 6 transfection reagent (Boehringer, Mannheim, Indianapolis, IN) in a ratio of 1.5 μg DNA to 3 μl FuGENE, transfection efficiencies of 60–80% were obtained, as determined by transfection of a GFP reporter plasmid.

cDNA Expression Array

RNA was harvested from cells 20 h after transfection using the CLONTECH Total RNA Labeling Kit. Briefly, cells were lysed with a denaturing solution and total RNA was isolated following two rounds of phenol/chloroform extraction, ethanol precipitation, and treatment with DNase I. RNA was then enriched for poly A^+ RNA using biotinylated oligo(dT) and streptavidin coated magnetic beads. To prepare probes, poly A^+ RNA was eluted from

beads and incubated with the supplied primer mix, α^{32}P-dATP, dNTPs, and MMLV reverse transcriptase. Following purification of labeled probe using column chromatography, the CLONTECH Atlas™ Human Cancer 1.2 Array was hybridized overnight with radiolabeled probes. Following extensive washes, array membranes were exposed to a phosphoimager screen and hybridization intensities were quantified using IPLab Gel software (Signal Analytics, Fairfax, VA). Values for each cDNA position were normalized against controls within each membrane corresponding to the ubiquitin and 40S ribosomal protein S9 genes. For those genes whose expression was induced by BRCA1, the fold inductions were calculated by dividing the normalized value of each gene for each experimental condition (i.e., transfected with $BRCA1_L$ or $BRCA1_S$) over the respective normalized value for the RK7 (empty vector control) transfected cells. Fold repressions were calculated by dividing the RK7 normalized values by the normalized values for $BRCA1_L$ or $BRCA1_S$.

Northern Analysis

Total cellular RNA from cells transfected with BRCA1 expression plasmids was isolated using TRIZOL reagent (GIBCO/BRL, Rockville, MD). For Northern analysis, 20 μg of total RNA was subjected to electrophoresis on a 1% agarose/formaldehyde gel, then electrotransferred and UV crosslinked to nylon membranes (GeneScreen; NEN, Boston, MA). Probes were labeled with α^{32}P-dCTP using a random primed labeling kit (Boehringer, Mannheim, Indianapolis, IN) and purified by Micro Bio-Spin Chromatography Columns (BioRad, Hercules, CA). Prehybridization, hybridization, and washes were performed using the Super Hybridization Buffer System (DNA Technologies, Gaithersburg, MD). Two NIK cDNA probes were generated by restriction digest of a NIK expression construct. Probe 1 was a 380 bp *Xma*I and *Pst*I fragment corresponding to nucleotides 2160-2541 of the NIK coding sequence. To prepare probe 2, a 1,497 bp *EcoR*I and *EcoN*I fragment was isolated and further digested with *Xma*I to generate a 385 bp product corresponding to nucleotides 1332-1718 of the NIK coding sequence.

Transient Transfections and Luciferase Assays

MCF10A cells were transiently co-transfected with luciferase reporter plasmids and BRCA1 expression plasmids. For the experiments with the NFκB-responsive p-55IgκLuc promoter [Fujita et al., 1993], 24 h after transfection, the cells were trypsinized and divided equally into 60 mm dishes for control and treated with either human recombinant TNF (R&D Systems, Minneapolis, MN), or human recombinant lymphotoxin α1/β2 (LTα1/β2) (R&D Systems, Minneapolis, MN). For the experiments with the CRE-responsive promoter (pCRE), cells were treated with 10 μM forskolin 4 h prior to harvest. The pAP-1, pSRE, and pCRE reporter constructs (Stratagene, La Jolla, CA) contain the luciferase gene driven by a TATA box joined to 7 tandem repeats of AP-1 consensus binding site, 5 repeats of the SRE consensus binding sites, or 4 sites of the CRE consensus binding sites, respectively. ER-EHO-210Luc contains ~4 kb fragment of the ER promoter. To construct this reporter plasmid, we obtained and modified the previously published ER-3500-210Luc promoter plasmid from Dr. Ronald Weigel [DeConinck et al., 1995]. Additional upstream genomic sequence was PCR amplified using genomic DNA from MCF-7 cells as template, then subcloned into this construct, resulting in a full length ER promoter luciferase reporter plasmid containing 3,813 bp of sequence upstream of the transcription initiation site, as well as 210 bp of 5′ untranslated region. The BCL2 promoter construct contains 2.5 kb of upstream promoter sequence and the BCL2 5′ untranslated region subcloned into the pGL2-basic vector (Promega, Madison, WI) [Klampfer et al., 1995]. The MMP-13 promoter construct places luciferase under the transcriptional control of 405 bp of the human MMP-13 promoter [Mengshol et al., 2001]. Luciferase lysates were harvested 48 h post-transfection and assayed in triplicate, as described [Tobin et al., 2001].

Western Blot Analysis

MCF10A cells were transfected with BRCA1 expression plasmids and protein lysates were harvested 1 day after transfections. Cell lysates were prepared from trypsinized washed cells by resuspension in cold lysis buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.5% NP40, 9.5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM pefabloc), and stored at −80°C. Thirty micrograms of protein was resolved by SDS–PAGE, transferred to nitrocellulose membranes, then analyzed for BRCA1 expression using the amino terminal BRCA1 MS110 (Ab-1) polyclonal antibody (Oncogene Research Products,

San Diego, CA; OP92-100UG,) at 1μg/ml, as described [Lu and Arrick, 2000].

Gel Shift Assay

Nuclear extracts were harvested 24 h after transfection, as described [Barchowsky et al., 1996]. Protein concentrations of nuclear extracts were determined by the BCA assay (Pierce, Rockford, IL). The gel shift probe was a double-stranded NFκB consensus oligonucleotides (NFκB: 5′-AGTTGAGGGGACTTTCCCAGG-3′, Promega, Madison, WI). For binding reactions, 3 μg of nuclear extract was incubated with 10×10^5 cpm of 5′-^{32}P-labeled probe and then resolved by electrophoresis in a 10% polyacrylamide gel under non-denaturing conditions. For supershift assays, 2 μg of anti-p50 (Santa Cruz Biotechnology, Santa Cruz, CA, sc114X) or anti-p52 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-298) was added to the binding reactions.

RESULTS

To evaluate the contribution of the alternatively spliced isoforms of BRCA1 to the overall function of BRCA1 with respect to gene expression, we employed cDNA expression array technology to identify genes whose expression was altered in a BRCA1 isoform specific manner in a biologically relevant system. We chose to focus on the effects of transient transfection of $BRCA1_L$ and $BRCA1_S$ expression constructs into non-tumorigenic human mammary epithelial cells. MCF10A cells contain a wild type *p53* gene, express low levels of endogeneous BRCA1 isoforms, and are readily transfected [Lu and Arrick, 2000]. In our hands, transient transfection of a GFP expression plasmid in these cells demonstrated a transfection efficiency of upwards of 80 percent (data not shown). Western analysis with an antibody specific to the amino terminus of BRCA1 confirmed increased expression of both isoforms of BRCA1 in these cells as a function of transient transfection (Fig. 1).

RNA isolated from cells 20 h following transfection with BRCA1 expression constructs was analyzed by cDNA array. Only genes in which consistent results across three independent experiments, with at least a 50% change in the level of expression are reported. Overall, we found that BRCA1 isoforms frequently exhibited distinct, and sometimes opposing effects on gene expression. Of the 1,176 genes on the

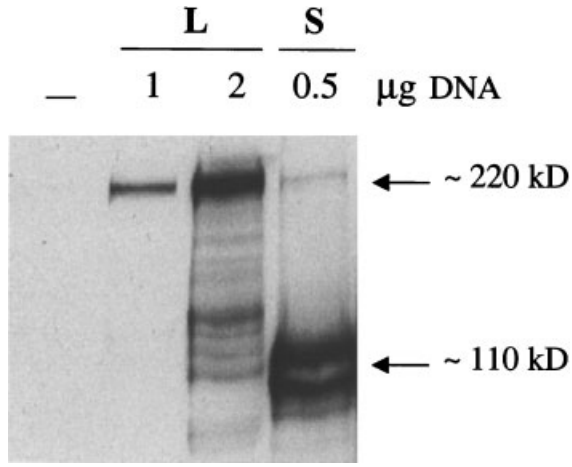

Fig. 1. Western blot analysis of BRCA1 expression in transfected MCF10A cells. MCF10A cells were transfected with 1 or 2 μg of $BRCA1_L$ or 0.5 μg $BRCA1_S$. Thirty micrograms of protein was resolved on an 8% SDS polyacrylamide gel. Blot was probed with the amino-terminal anti-BRCA1 antibody, MS110 (Ab-1) at 1 μg /ml. Signal from endogeneous BRCA1 protein in control-transfected cells was evident in longer exposures.

array, thirty-eight displayed BRCA1-mediated alterations in expression (Table I). One gene was induced following $BRCA1_L$ expression, but not BRCA1 $_S$ expression, while 22 genes were specifically induced following expression of $BRCA1_S$, but not $BRCA1_L$. An additional five genes were induced following expression of either $BRCA1_L$ or $BRCA1_S$. Isoform-specific repression of gene expression was also observed. Specifically, expression of $BRCA1_L$ resulted in the decreased expression of ten genes, nine of which were induced following expression of $BRCA1_S$. The changes in gene expression identified by the cDNA array reflect fairly moderate changes in gene expression levels by transient transfection of BRCA1 constructs. The majority of genes were induced or repressed less than 3-fold. Of note, while $BRCA1_L$ repressed expression of as many genes as it induced, $BRCA1_S$ did not repress the expression of any genes in this array, but rather induced a greater number of genes.

NIK was identified as a gene whose expression was repressed by $BRCA1_L$ and induced by $BRCA1_S$. NIK is structurally related to MAPK3 and has been shown to interact with TRAF2, an adaptor protein that interacts with TNF receptors. In response to TNF, LT, and EGF, NIK is activated and sets off a cascade of phosphorylation events that lead to the activation of NFκB [Malinin et al., 1997; Lin et al., 1998b; Habib et al., 2001; Matsushima et al., 2001; Yin et al.,

TABLE I. Transcriptional Targets of BRCA1 Splice Variants[a]

Induced by $BRCA1_L$	Fold induction[b]	
Guanylate kinase	2.02	
Induced by $BRCA1_S$	**Fold induction**	
Cyclin-dependent kinases regulatory subunit 1	1.61	
Early growth response alpha	1.79	
Cytohesin-1	1.86	
Vimentin	1.87	
Interleukin-13	1.88	
rho HP1	1.90	
Tissue plasminogen activator	1.95	
Cyclin dependent kinases regulatory subunit 2	1.96	
Tenascin-R	2.00	
DR-nm23	2.02	
Replication factor C	2.12	
Extracellular MMP inducer	2.20	
Ninjurin-1	2.25	
lkaros/LyF-1 homolog hlk-1	2.44	
Interferon-regulated resistance GTP-binding protein	2.47	
NFκB DNA binding subunit	2.48	
Cytokeratin 14	2.49	
Inosine-5′-monophosphate dehydrogenase 2	2.55	
Interleukin-6	2.61	
TNF type 1 receptor associated protein	2.68	
Active breakpoint cluster region-related protein	2.98	
Semaphorin E	3.18	
Induced by $BRCA1_L$ and $BRCA1_S$	**L fold induction**	**S fold induction**
Beta catenin	1.26	1.92
Cytokeratin 2E	1.33	2.74
Early growth response protein 1	1.72	1.79
CD59 glycoprotein precursor	1.84	2.31
Interleukin-1	3.50	9.75
Repressed by $BRCA1_L$	**Fold repression[c]**	
Deleted in colorectal cancer, DCC	4.91	
Repressed by $BRCA1_L$ & induced by $BRCA1_S$	**L fold repression**	**S fold induction**
MYT1	1.21	1.58
NFκB inducing kinase, NIK	1.25	1.72
Caspase-9 precursor	1.40	1.72
ABL2	1.44	1.66
Polycystin	1.50	1.51
Thyrotropin-releasing hormone receptor	2.28	2.05
Cyclin I	2.60	1.88
Envoplakin	2.87	2.70
Ribonucleoside-diposphate reductase M1 chain	2.81	1.72

[a]Average fold change from three independent experiments of MCF10A cells transfected with BRCA1 expression plasmids. RNA was harvested 20 h after transfection.
[b]Fold induction was calculated by dividing the normalized value of each gene for each experimental condition (i.e., transfected with $BRCA1_L$ or $BRCA1_S$) by the corresponding normalized value for RK7 (empty vector) transfected cells.
[c]Fold repression was calculated by dividing the RK7 normalized values by the normalized values for $BRCA1_L$ or $BRCA1_S$.

2001]. Northern analysis of RNA from MCF10A cells transiently transfected with BRCA1 expression plasmids confirmed that increased expression of full length BRCA1 results in the down-regulation of NIK mRNA (Fig. 2). The exon 11-deleted isoform of BRCA1, $BRCA1_S$, however, did not alter NIK mRNA levels. We also tested the ability of a pathogenic mutant form of BRCA1, 5382insC that results in a prematurely truncated protein lacking 34 amino acids from the carboxy terminus, to down-regulate NIK expression. We observed that mutant $BRCA1_L$ was also active in the regulation of NIK expression, suggesting that this activity by $BRCA1_L$ is not essential to its role as a tumor suppressor.

We next investigated the functional consequences of BRCA1-mediated down regulation of NIK in MCF10A cells by using a NFκB-responsive luciferase promoter construct. This promoter construct places the luciferase gene under the control of three tandem repeats of the

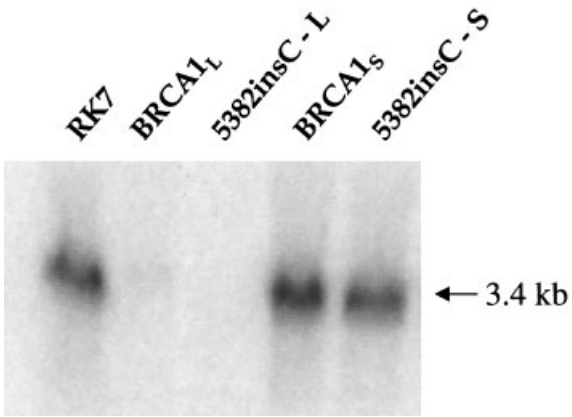

Fig. 2. $BRCA1_L$ expression results in the down-regulation of NFκB inducing kinase (NIK) message. MCF10A cells were transfected with 7.5 μg empty vector (RK7), **lane 1**; expression plasmids for $BRCA1_L$ or $BRCA1_S$, **lanes 2 and 4** respectively; or mutant BRCA1 expression plasmids (5382insC-L or 5382insC-S), **lanes 3 and 5**. RNA was harvested 24 h later and 20 μg was subjected to electrophoresis on a 1% agarose/formaldehyde gel. RNA blots were probed with a α^{32}P-labeled NIK cDNA probe corresponding to nucleotides 1332-1718 of the NIK coding sequence. Similar results were obtained when a probe corresponding to nucleotides 2160-2541 of NIK coding sequence was used. Ethidium bromide staining determined determine equal loading of lanes (not shown).

consensus NFκB recognition site immediately upstream of the minimal interferon-β promoter [Fujita et al., 1993]. It was expected that the $BRCA1_L$-mediated down regulation of NIK would result in a decrease in NFκB activation following the appropriate stimulus. Surprisingly, $BRCA1_L$ expression resulted in a 3–4-fold increase in luciferase activity, and this activation was further increased to 8-fold following TNF stimulation ($P < 0.001$) (Fig. 3A). In contrast, expression of $BRCA1_S$ did not significantly increase basal or TNF-stimulated levels of NFκB activity over that of control. Mutant BRCA1 isoforms were also able to augment NFκB activity to the same degree as wild type, suggesting that this activity as well is not essential to BRCA1 tumor suppressor function.

Recent reports have questioned the role of NIK in TNF-induced NFκB activation. Specifically, it has been reported that TNF-α-induced NFκB activation is not abrogated in NIK deficient mice suggesting that NIK is not required for TNF signaling [Yin et al., 2001]. Rather, it was shown that NFκB induction resulting from stimuli that engage the LT-β receptor occurred through a pathway involving NIK [Matsushima et al., 2001]. Therefore, we next investigated the effect of $BRCA1_L$-mediated down regulation of NIK on LT-induced NFκB activation. As shown in Fig. 3B, $BRCA1_L$ increased basal levels as well as LTα1/β2-induced NFκB activity ($P < 0.006$). However, the overall relative effect of LTα1/β2 treatment on NFκB activity was not significantly different in $BRCA1_L$ transfected cells versus control cells. These results, therefore, suggest that $BRCA1_L$ does not alter LTα1/β2-induced NFκB activation.

To determine if the increased transcriptional activity of NFκB correlated with an increase in binding of nuclear proteins to the NFκB recognition sequence, an electrophoretic mobility shift assay was performed with a double stranded DNA oligonucleotide probe containing the NFκB consensus binding site. Expression of $BRCA1_L$ did in fact result in a modest increase in binding of nuclear proteins to the NFκB binding sequence (Fig. 4). Treatment of cells with LTα1/β2 for 45 min prior to isolation of nuclear proteins resulted in an overall increase in binding for both RK7 and $BRCA1_L$ transfected cells. However, there was no significant difference in basal levels or LTα1/β2-stimulated binding of nuclear proteins between $BRCA1_L$ transfected cells and RK7 transfected cells. Supershift analysis with antibodies to NFκB subunits confirmed the identification of one protein in the binding complex as p50, the product of NFκB1 p105 processing.

Given the substantial evidence in the literature of a role for BRCA1 in transcription, as well as our own findings identifying BRCA1-mediated changes in gene expression, we next wanted to investigate whether other promoter elements, in addition to the NFκB recognition sequence, also mediate BRCA1 effects on gene expression. Specifically, we tested the ability of BRCA1 isoforms to transactivate luciferase reporter constructs driven by promoters containing the sites AP-1, SRE, and CRE. $BRCA1_L$ and $BRCA1_S$ were both able to transactivate a canonical AP-1 reporter construct 4–5-fold ($P < 0.001$) (Fig. 5A). Mutant BRCA1 isoforms exhibited similar activities. Conversely, only full length BRCA1 was able to transactivate a SRE containing promoter construct ($P < 0.01$) and, importantly, introducing a mutation into $BRCA1_L$ largely abrogated this activity (Fig. 5B). Both wildtype and mutant $BRCA1_L$ transactivated the CRE-responsive promoter 4–5-fold ($P < 0.01$), while $BRCA1_S$ had a more moderate effect (Fig. 5C). Following forskolin

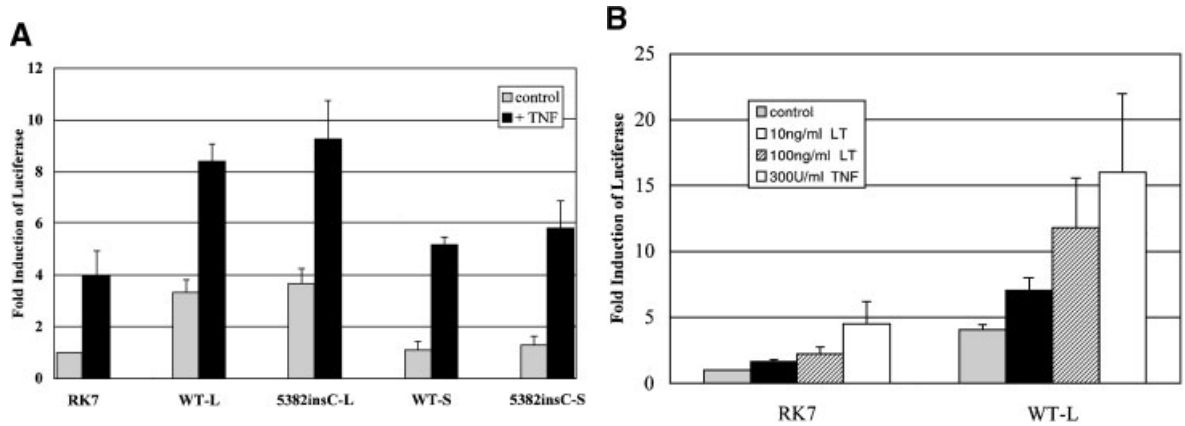

Fig. 3. BRCA1$_L$ transactivates basal levels, and TNF and LTα1/β2 induced levels of a NFκB responsive promoter. **Panel a**. MCF10A cells were co-transfected with the NFκB-responsive luciferase reporter plasmid, p55IgκLuc, and either empty vector control (RK7), BRCA1 expression plasmids (BRCA1$_L$ or BRCA1$_S$), or mutant BRCA1 expression plasmids (5382insC-L or 5382insC-S). The next day, cells were treated with 30 U/ml human recombinant TNF for 18–20 h and assayed for luciferase activity. Values were normalized against RK7 value for calculation of fold induction of luciferase activity. Values shown are the means + SEM of eight independent experiments for RK7, WT-L, and 5382insC-L; 4 for WT-S and 5382insC-S. **Panel b**. MCF10A were co-transfected with p55IgκLuc, and either empty vector control (RK7) or BRCA1 $_L$. The next day, cells were treated with human recombinant LTα1/β2 at either 10 or 100 ng/ml or 300 U/ml recombinant human TNF for 18–20 h and assayed for luciferase activity. Values were normalized against RK7 value for calculation of fold induction of luciferase activity. Values shown are the means + SEM of three independent experiments.

treatment to further induce the cAMP-dependent protein kinase A pathway, required for CRE activity, promoter activity was increased by ~30–60%.

BRCA1's transcriptional activity was also studied on two biologically relevant promoters. One of the most notable phenotypic features of breast cancers that arise in BRCA1 mutation carriers is the higher percentage of ERα-negative tumors than that seen in non-BRCA1-associated tumors [Jóhannsson et al., 1997; Karp et al., 1997; Loman et al., 1998]. Furthermore, there is also a higher proportion of ERα-negative tumors among sporadic cancers that have decreased expression of BRCA1 [Catteau et al., 1999; Lee et al., 1999]. More recently, BRCA1-associated tumors and sporadic breast cancers with diminished expression of BRCA1 have been found to have decreased expression of the anti-apoptotic gene BCL2 when compared to control tumors [Lee et al., 1999; Freneaux et al., 2000]. Constructs with the luciferase coding sequence downstream of the promoters for ERα and BCL2 were co-transfected with BRCA1 isoforms into MCF10A cells. BRCA1$_L$ was able to transactivate the ERα promoter (Fig. 6A, $P < 0.006$), as was mutant BRCA1$_L$ (not shown). Activity of BRCA1$_S$ with the ERα promoter was, by comparison, minimal. BRCA1$_L$ was able to transactivate the BCL2 promoter ($P < 0.005$) while BRCA1$_S$ was inactive (Fig. 6B). Importantly, a mutant BRCA1$_L$, 5382insC-L, exhibited significantly reduced activity in the transactivation of the BCL2 promoter, suggesting that this activity may be relevant to BRCA1's role as a tumor suppressor in mammary epithelial cells. A summary of the transcriptional transactivation activities of BRCA1 splice variants on a number of gene promoter as well as on several promoter elements is provided in Table II.

DISCUSSION

Our lab has previously presented evidence detailing the BRCA1 splice variant-specific transactivation of the p21 promoter [Lu and Arrick, 2000]. In that work, we showed that the exon 11-deleted isoform of BRCA1, in addition to the full length protein, displayed a transcriptional transactivation activity in that it functions in the transactivation of the p21 promoter in a p53-dependent manner. In this current study, we have expanded our investigation into BRCA1 isoform-specific transcriptional regulation to include additional biologically relevant promoters, as well as a more global analysis of gene expression through the use of cDNA expression arrays. The use of cDNA expression arrays has proven to be an invaluable approach to the study of the function of many gene products with respect to transcription. Several

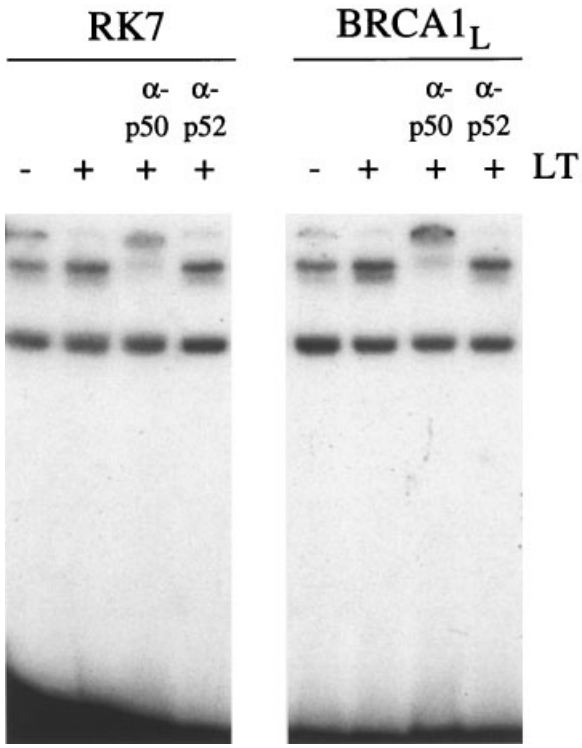

Fig. 4. $BRCA1_L$ transactivation of a NFκB-responsive promoter correlates with increased binding of nuclear proteins. MCF10A cells were transfected with empty vector control (RK7) or $BRCA1_L$ expression plasmid. Cells were either left untreated or were treated with 10 ng/ml LTα1/β2 45 min prior to harvest of nuclear extracts. Three micrograms of nuclear protein were incubated for 20 min with a radiolabeled double stranded DNA oligonucleotide containing the consensus κB binding site. Supershift binding reactions had also 1 μl of 2 mg/ml antibody. Binding reactions were then subjected to electrophoresis on a non-denaturing 10% polyacrylamide gel.

groups have used expression array technology in the study of BRCA1 function and the identification of its downstream targets in cell systems [Harkin et al., 1999; MacLachlan et al., 2000; Aprelikova et al., 2001; Mullan et al., 2001; Welcsh et al., 2002]. However, these studies are often performed in less biologically relevant cell systems (i.e., non-mammary cells) and/or ignore the contribution of the functionally important exon 11-deleted splice form, $BRCA1_S$. In addition to applying array analysis to determine alterations in gene expression in BRCA1 transfected cells, arrays have also been used to determine the gene expression profiles in BRCA1-associated versus non-BRCA1-associated tumors [Berns et al., 2001; Hedenfalk et al., 2001; Jazaeri et al., 2002; Van't Veer et al., 2002]. Remarkably, the gene expression profiles from BRCA1-associated tumors are distinct from that of non-BRCA1-associated tumors. Moreover, sporadic tumors that have decreased expression of BRCA1 have a gene expression profile that is similar to that of BRCA1-associated tumors.

Our analysis of gene expression by cDNA expression array following the increased expression of BRCA1 splice variants in non-tumorigenic human mammary epithelial cells has identified many genes whose expression is altered in a BRCA1 isoform-specific manner. We have identified genes whose expression is induced by either $BRCA1_L$ or $BRCA1_S$, or both, as well as genes whose expression is repressed following $BRCA1_L$ expression. Perhaps most interesting were genes whose expression is both induced by $BRCA1_S$ and repressed by $BRCA1_L$, suggesting that for some effects these two major splice variants counteract. We did not perform co-transfections augmenting expression of both isoforms to determine which isoform's effect would dominate. In addition, given that relatively low levels of BRCA1 are endogeneously expressed in these cells, we have not explored changes in gene expression the arise in the complete absence of BRCA1 in MCF10A cells.

Several of the genes we identified have previously been shown to be regulated by BRCA1, however, our study uncovers the isoform specificity of this regulation. For example, early growth response protein 1 (EGR1) was reported to be induced by full length BRCA1 [Harkin et al., 1999]. In our study, EGR1 was induced following expression of both BRCA1 isoforms. Vimentin was identified as a gene down-regulated in BRCA1 null mouse embryonic stem (ES) cells, and therefore concluded to be a BRCA1-responsive gene [Aprelikova et al., 2001]. We observed that induction of vimentin expression was specific to $BRCA1_S$. Additional genes identified in our study as well as in others include cytokeratin 2E and CD59. The expression levels of rhoGDI 1 and ROCK, previously identified as BRCA1 targets [MacLachlan et al., 2000; Aprelikova et al., 2001], were also altered by BRCA1 in our system, but did not meet our designated cutoff of a 50% or greater change.

In our cDNA array experiments, increased expression of $BRCA1_S$ correlated with augmented expression of approximately three dozen genes, but no examples of significant gene repression. In contrast, expression of $BRCA1_L$ was associated with the reduced expression of a number of genes, as well as the increased

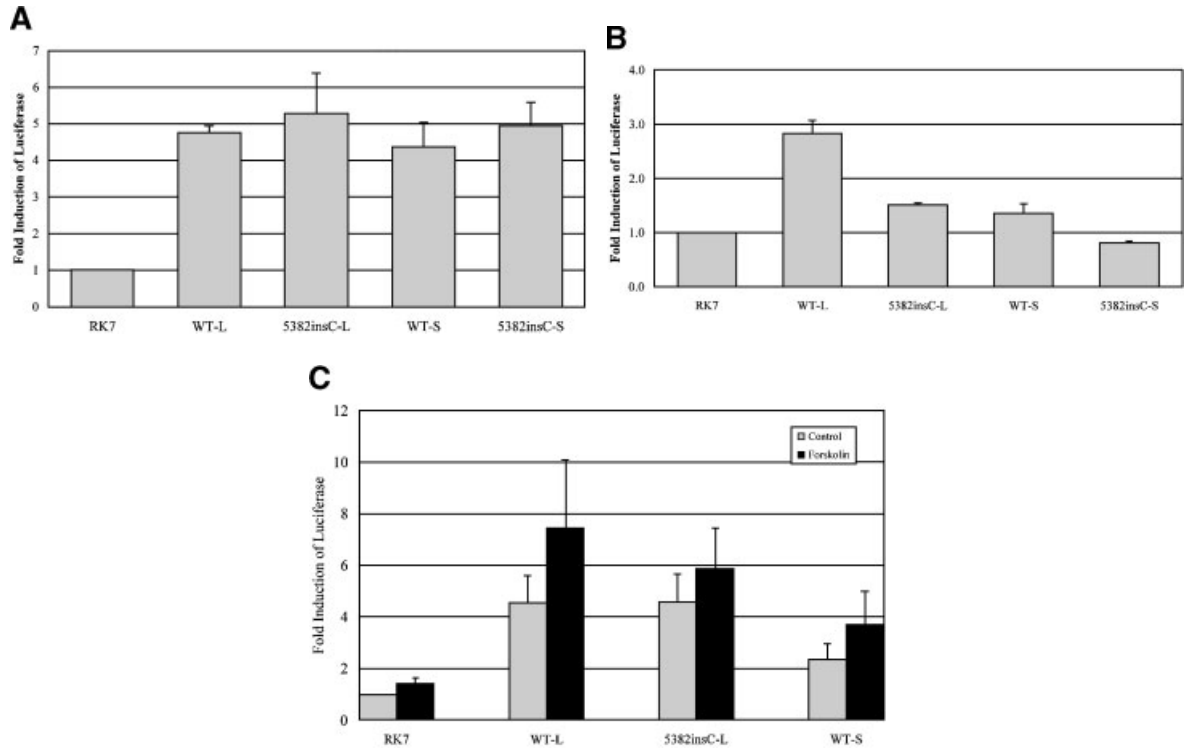

Fig. 5. BRCA1 isoforms transactivate promoters containing AP-1, SRE, and CRE binding sites. MCF10A cells were co-transfected with a luciferase reporter plasmid and either empty vector control (RK7), BRCA1 expression plasmids ($BRCA1_L$ or $BRCA1_S$), or mutant BRCA1 expression plasmids (5382insC-L or 5382insC-S). Lysates were assayed for luciferase activity 48 h post-transfection. Values were normalized against RK7 value for calculation of fold induction of luciferase activity. **Panel a**. AP-1-responsive reporter plasmid. Values shown are the means + SEM of eight independent experiments for RK7 and WT-L; seven for 5382insC-L; and three for WT-S and 5382insC-S. **Panel b**. SRE-responsive reporter plasmid. Values shown are the means + SEM of five independent experiments for RK7, WT-L, and WT-S; two for 5382insC-L and 5382insC-S. **Panel c**. CRE-responsive reporter plasmid. Cells were treated with 10 μM forskolin 45 min prior to harvest. Values shown are the means + SEM of five independent experiments.

expression of other genes. This observation of multiple genes down-regulated by BRCA1 contrasts with published data from BRCA1 null ES cells [Aprelikova et al., 2001], but is analogous to gene expression analyses in other cell systems, although the specific lists of genes whose expression was repressed by BRCA1 exhibited minimal overlap [MacLachlan et al., 2000; Welcsh et al., 2002].

Among the genes whose expression was repressed in MCF10A cells by $BRCA1_L$ is NIK. From experiments utilizing fibroblasts from NIK deficient embryos, or 293 cells transfected with NIK mutant constructs, a central role for NIK as a mediator of NFκB activation following stimulation by TNF, LT, and EGF has been reported [Malinin et al., 1997; Lin et al., 1998b; Habib et al., 2001; Matsushima et al., 2001; Yin et al., 2001]. Therefore, we hypothesized that a loss of NIK expression following expression of $BRCA1_L$ might result in a significant impairment in NFκB activation in MCF10A cells. Our results show, however, that both BRCA1 isoforms increased basal levels of NFκB activity, as well as TNF- and LT-stimulated NFκB activity. NFκB activation by EGF was not increased following expression of BRCA1 isoforms (data not shown). Gel shift analysis correlated the increase in transcriptional activity of NFκB with an increase in nuclear protein binding to the NFκB recognition sequence. Moreover, we show by supershift analysis that a member of the binding complex is the NFκB1 subunit, p50. In addition to activation of NFκB, NIK has also been implicated in the processing of the NFκB2 p52 precursor, p100 [Xiao et al., 2001]. While the processing of the precursor p105 to the NFκB1 subunit p50 is constitutive [Lin et al., 1998a], the processing of p100 to p52 seems to be a regulated process. Western analysis of p100 and p52 levels in MCF10A cells expressing $BRCA1_L$, however, did not show any change in

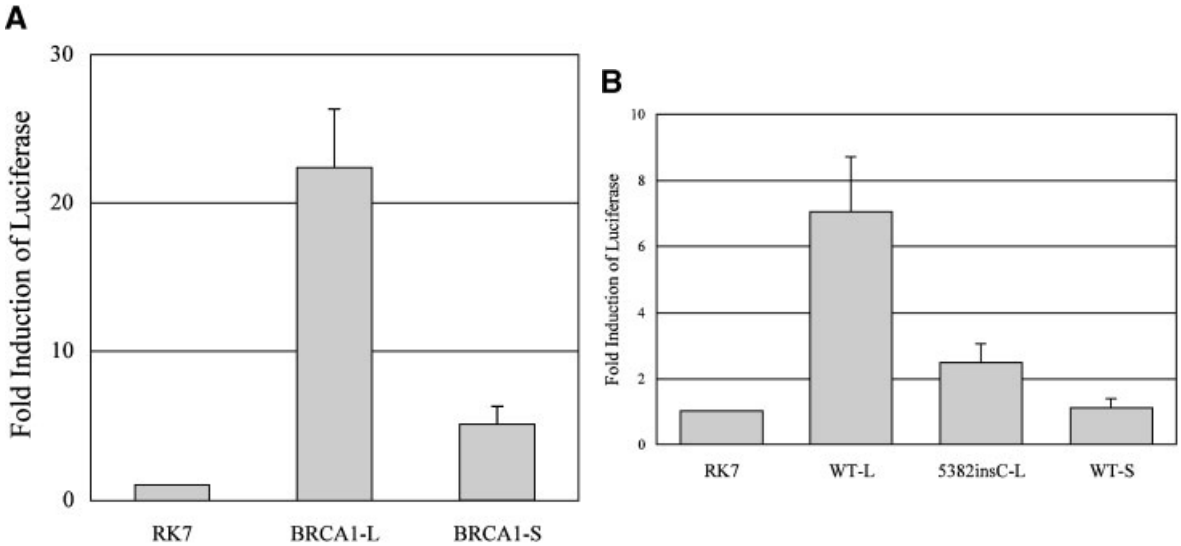

Fig. 6. $BRCA1_L$-mediated regulation of ERα and BCL2 promoters. MCF10A cells were co-transfected with a luciferase reporter plasmid and either empty vector control (RK7), BRCA1 expression plasmids ($BRCA1_L$ or $BRCA1_S$), or mutant BRCA1 expression plasmids (5382insC-L). Luciferase lysates were harvested 48 h post-transfection and assayed for luciferase activity. Values were normalized against RK7 value for calculation of fold induction of luciferase activity. **Panel a**. ERα promoter luciferase reporter plasmid. Values shown are the means + SEM of three independent experiments. **Panel b**. BCL2 promoter luciferase reporter plasmid. Values shown are the means + SEM of seven independent experiments for RK7 and $BRCA1_L$ (WT-L); 4 for mutant $BRCA1_L$ (5382insC-L) and $BRCA1_S$ (WT-S).

the levels of the p52 protein (data not shown). In summary, based on these results, it would appear that although $BRCA1_L$ expression in MCF10A cells resulted in the down-regulation of NIK, there was no attenuation in the transcriptional activity of NFκB (indeed, the opposite) and no discernable effect on the processing of the NFκB2 precursor p100.

In a series of co-transfection experiments, we have demonstrated that BRCA1 can transactivate AP-1, SRE, and CRE containing promoters in an isoform specific manner. Both isoforms of BRCA1 were able to transactivate a canonical AP-1 responsive promoter construct and mutants of the BRCA1 isoforms were also active. While BRCA1 expression increased

TABLE II. Transcriptional Transactivation Activities of Wild-Type and Mutant BRCA1 Splice Variants

	Fold induction[a]				
	RK7	WT-L	5382insC-L	WT-S	5382insC-S
Gene promoter					
p21[b]	1	2.5–10	2	3.5	1.2
ERα[c]	1	22	n.d.	5.1	n.d.
BCL2	1	7.1	2.5	1.1	n.d.
MMP-13	1	3.2	3.9	1.5	1.7
Promoter element					
NFκB	1	3.3	3.6	1.1	1.3
+TNF-α	4	8.4	9.3	5.2	5.8
+LTα1/β2	1.7	7.1	n.d.	n.d.	n.d.
AP-1	1	4.8	5.3	4.4	4.9
SRE	1	2.8	1.5	1.3	0.81
CRE	1	4.5	4.6	2.4	n.d.
+Forskolin	1.4	7.4	5.9	3.7	n.d.

[a]Average fold inductions from three to eight experiments; n.d., not determined.
[b]As reported in [Lu and Arrick, 2000]. Luciferase activities for WT-L correspond to a range of microgram amounts of BRCA1 expression plasmid DNA transfected (0.6–1.2 μg). Luciferase induction of 5382insC-L was comparable to that seen for WT-L for all experiments. Value provided for 5382insC-L is for 0.6 μg of DNA transfected.
[c]In one experiment, transactivation activity of mutant full length BRCA1 (5382insC-L) was equivalent to that observed with wildtype $BRCA1_L$.

transcription from an AP-1 containing promoter, DNA binding of nuclear proteins to a double-stranded oligonucleotide containing the AP-1 consensus site did not increase following BRCA1 expression (data not shown). The AP-1 family of transcription factors are ubiquitously expressed transcription factors that regulate the expression of many genes involved in cell proliferation, survival, and death and, therefore, certainly play an important role in carcinogenesis. Like NFκB, AP-1 proteins are comprised of various homodimer and heterodimer complexes that exhibit specific DNA binding affinities and transcriptional activities for a large number of gene targets [Shaulian and Karin, 2001]. Likewise, BRCA1-mediated effects on AP-1 responsive promoters may be specific to certain AP-1 dimers as well as to certain AP-1 containing promoters. Indeed, while $BRCA1_S$ does transactivate a canonical AP-1 responsive promoter, it is unable to transactivate the AP-1 containing promoter of MMP-13 (Table II). Thus, BRCA1's role in AP-1 transactivation may be specific to certain AP-1 dimers and the DNA sequences they recognize.

The transactivation of a CRE reporter plasmid by $BRCA1_L$ was previously studied in a kidney cell line, 293 cells, with the observation that full length BRCA1 ($BRCA1_S$ was not included) activated this promoter 3-fold over control following forskolin treatment [Houvras et al., 2000]. In our study, we demonstrated a 7-fold induction of a CRE-responsive promoter by $BRCA1_L$ and a 3.7-fold induction by $BRCA1_S$. Promoters containing CRE are regulated by members of the CREB/ATF family of transcription factors as well as by CBP/p300 [Chrivia et al., 1993; Kwok et al., 1994]. BRCA1 interacts with CBP/p300 via the CREB binding domain on CBP [Pao et al., 2000] and with ATF1 via its ring finger domain [Houvras et al., 2000], so it seems logical to deduce that these interactions may mediate the transcriptional activation of CRE by $BRCA1_L$.

We also show that $BRCA1_L$ specifically transactivates a reporter construct driven by a SRE containing promoter, and that the pathogenic mutation 5382insC abrogates this activity. SRE is recognized by a dimer of the serum response factor, which, once bound, facilitates assembly of a transcription activation complex. Important partners in SRE-mediated transcription include Elk-1 and CBP/p300 [Ramirez et al., 1997; Nissen et al., 2001]. As noted above, BRCA1 interacts with CBP, suggesting that the transcriptional transactivation activity of BRCA1 on SRE-containing promoters may in part be mediated by this interaction. Of note, however, is that the exon 11-deleted isoform of BRCA1 has also been shown to interact with CBP [Cui et al., 1998], and yet $BRCA1_S$ does not transactivate the SRE containing promoter. Recently, Chai et al. determined that $BRCA1_S$ antisense expression resulted in an increase of SRE promoter activity. Furthermore, they showed that $BRCA1_S$ interacts with Elk-1 in vivo and inhibits ERK-1 mediated induction of the c-Fos promoter via SRE [Chai et al., 2001]. It is possible, then, that this same mechanism prevents $BRCA1_S$ induction of a SRE promoter construct in MCF10A cells.

Increased expression of $BRCA1_L$ significantly augmented transcription from both the ERα and BCL2 promoters, whereas expression of $BRCA1_S$ had a notably weaker effect. Indeed, BRCA1-mediated transcriptional activation of these promoters may, in part, provide a mechanism by which loss of BRCA1 in breast cancer cells results in the decrease in expression of these genes. Our lab has also demonstrated an increase in methylation at critical CpGs within the ERα promoter in ERα-negative BRCA1-associated cancers as compared to ERα-negative non-BRCA1-associated cancers [Archey et al., 2002]. Current investigation into the regulation of both BCL2 and ERα expression by $BRCA1_L$ is focusing on the specific promoter elements within those promoters that mediate the transcriptional transactivation activity of $BRCA1_L$. The notion that regulation of gene expression by BRCA1 underlies its role as a tumor suppressor gene in female breast cancer suggests that a transcriptional assay based on a BRCA1-responsive reporter construct in mammary epithelial cells can serve as an accurate functional assay, capable of distinguishing benign polymorphisms in BRCA1 from truly pathogenic mutations. Such an assay would greatly enhance counseling of families undergoing genetic testing, and facilitate research into the structure and function of BRCA1.

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