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BRCA1 is involved in two fundamental cellular processes: DNA repair and transcriptional regulation. BRCA1 C-terminus acts as a transactivation domain and introduction of germ-line mutations, but not benign polymorphisms, abolishes transcriptional activation, suggesting a critical role for this function of BRCA1 in cancer development. Our hypothesis is that BRCA1 transactivation function is regulated by an intramolecular interaction. During the past year we focused on three specific aims: *1) map the interaction sites; 2) define the in vivo dominant negative activity of truncations in BRCA1 that retain the inhibitory domain but disrupt the transactivation domain; 3) To analyze which mutations abolish transcriptional activation.* We performed immunoprecipitations with FLAG-tagged constructs which revealed an in vivo interaction of the C-terminal region with aa 1366-1559, consistent with our hypothesis. We optimized transfection conditions for breast and ovarian cancer cells and are now currently determining if the *trans-inhibition* is differentially regulated in these cells. In addition, we provided validation for our functional assay, a required step before testing the effect of mutations located in the putative interaction region.

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Thomas Lucabeing 6/2/07

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INTRODUCTION

Breast cancer is the most frequent malignancy affecting women in the United States. Hereditary breast and ovarian cancer represent 5-10% of all cases, most of which are attributed to inherited mutations in the tumor suppressor gene *BRCA1*. These mutations are highly penetrant and confer a 56-85% lifetime risk. The human *BRCA1* gene is large and encodes a protein product of 220 kDa, which is likely to be multifunctional. *The exact role of BRCA1 is still unclear but recent evidence points to the involvement of BRCA1 in two fundamental cellular processes: transcriptional regulation and DNA repair.* To date, there is a wealth of evidence implicating BRCA1 in a variety of different DNA repair processes yet none of the evidence indicates a potential biological mechanism by which BRCA1 may act. These functions of BRCA1 may reflect two separate activities or, alternatively, part or all of the functions of BRCA1 in maintaining genome integrity may be an indirect result of BRCA1-mediated regulation of gene expression. Both scenarios are consistent with the current evidence.

We have shown that the BRCA1 C-terminus (aa 1560-1863) has the ability to activate transcription when fused to a heterologous DNA binding domain both in yeast and in mammalian cells [1;2]. Another group has reported a similar finding [3]. Importantly, we have also shown that germ-line mutations found in BRCA1 families abolish this activity while benign polymorphisms maintain wild type activity [1;4]. *Since the mutations found in patients result in loss of transcriptional activation function, this provides a* ***genetic framework*** *to link the transcriptional activation function of BRCA1 to the development of cancer.*

In our proposal we presented preliminary evidence suggesting a critical region for the modulation of BRCA1's transactivation activity. We proposed the existence of an intramolecular interaction domain that masks the activation domain of BRCA1, thus impairing the recruitment of the machinery involved in transcription initiation. Our hypothesis is that BRCA1 function is regulated by an autoinhibitory intramolecular interaction between regions encoded by exon 12 (and possibly part of exon 13) and the C-terminal region comprising the BRCT domains. We suggested that this mechanism might form the basis of the regulation of transcriptional activation by BRCA1. We expect that a region that interacts and masks the activation domain will be identified in detail and will reveal one of the means by which BRCA1 activity is regulated. In particular, it will be important to test whether germ-line mutations in BRCA1 that are suspected of cancer-association will modify this putative intramolecular interaction.

BODY

During the past year we focused mainly on performing experiments proposed in Tasks 2 and 3 described in our proposal and in the work statement. The experiment in Task 1 was only partially completed at the time of the first report:

Task 1. **To map the sites involved in the intramolecular interaction in BRCA1 using the yeast two-hybrid assay (months 1-12).**

- Express the tagged fragments in mammalian cells and check the interaction by immunoprecipitation and western blotting (e.g. IP with FLAG antibody and blot against GST antibody).

Task 2. **To define the in vivo inhibition and the dominant negative activity of truncations of BRCA1 (months 8-24).**

- Optimize transient transfections of breast cancer cell lines and in ovarian cancer cell lines.
- Test if there is a difference in the *trans*-inhibition in breast cancer cell lines versus ovarian cancer cell lines.

Task 3. **To analyze which mutations abolish transcriptional activation (months 12-24).**

- Introduce mutations found in patients with hereditary breast and ovarian cancer, especially those found in the putative interaction domain.
- Test how these mutations affect transcription activation of a reporter gene in yeast cells. If a particular mutation is found to modify BRCA1 activity, develop yeast two-hybrid constructs containing the mutations and test if it modifies the strength of interaction (judged by activation of β-galactosidase production in yeast two-hybrid assays.

KEY RESEARCH ACCOMPLISHMENTS

Progress on goals defined in Task 1.

We have performed co-immunoprecipitation experiments in 293T cells. One series of immunoprecipitations was done by overexpressing the following constructs on a pCMV-FLAG (Kodak) vectors described in our 2000 report: BRCA1 aa (1366-1778); (aa 1366-1455); (aa 1366-1559) and (aa 1366-1718). To provide an appropriate control we made additional constructs expressing the N-terminal region of BRCA1 fused to a FLAG tag. These constructs were also transfected into 293T cells and their expression confirmed, although constructs (aa 302-1313) displayed significant lower levels of expression likely due to its size. Cells overexpressing these constructs were lysed and immunoprecipitation performed using α-FLAG (M2 antibody) at 1μg per immunoprecipitation. Western blot was done using α-BRCA1 Ab-1 that recognizes the full length endogenous BRCA1. A second series of immunoprecipitations was done by co-expressing the plasmids described above with a fusion of BRCA1 C-terminal region (aa 1560-1863) to GAL4 DBD. Western blot was done using a monoclonal antibody against the GAL4 DNA binding domain. The FLAG constructs were not able to immunoprecipitate detectable levels of the endogenous full length BRCA1, despite its relative high levels in these cells. We detected a weak interaction between BRCA1 aa 1366-1559 and the GAL4 DBD aa 1560-1863, consistent with our hypothesis. However, we did not detect interaction with aa 1366-1778, which also comprises the region, suggested to participate in the interaction. That may mean that region aa 1366-1559 in the construct aa 1366-1778 is masked. We are also repeating the experiments in cell extracts lysed with milder buffers that do not contain SDS and might interfere with the FLAG immunoprecipitations. We expect to complete and extend these studies in the next few months.

Progress on goals defined in Task 2.

In order to perform the experiments in Task 2 we have gathered a series of cell lines derived from breast and ovarian cancer (see Table 1). In the past year we have tested several transfection

reagents and optimized the transfection conditions. We have tested lipofectin, lipofectamine and lipofectamine 2000 (GIBCO-BRL), Fugene 6 (Roche), and GeneFECTOR and Nova FECTOR (Venn Nova, Inc.) as well as calcium phosphate (Stratagene). Transfection efficiency was evaluated by the percentage of GFP positive cells by transfecting a GFP vector. All cell lines, with the exception of L56Br cells, yielded better transfection efficiency when treated with Fugene 6. The L56Br cells displayed higher transfections efficiency with NovaFECTOR. These reagents were then used for subsequent experiments. We had obtained similar results (better efficiency with Fugene 6) in other cell lines as well, as described in our past report. Furthermore, we have confirmed the expression of the constructs containing the activation domain (GAL4 DBD:BRCA1 aa 1396-1863) and the "*trans*-inhibitory" construct (FLAG-tagged BRCA1 aa 1366-1718) in these cell lines. We are currently starting the experiments to directly test the *trans*-inhibition in breast and ovarian cells.

Table 1. Human cell lines used in this proposal.

CELL LINES	BRCA1 STATUS	DESCRIPTION
HCC1937	Hemizygous for a BRCA1 5382insC mutation.	ATCC CRL-2336. The only commercially available human cell deficient for BRCA1[5]. Derived from breast ductal carcinoma.
L56Br-C1	Hemizygous for a BRCA1 1806C>T, ter 553, mutation.	A recently described BRCA1 deficient cell line. Derived from breast cancer. Myb amplification. [6].
HeLa	wild type	ATCC CCL-2. Derived from carcinoma of the cervix.
SKOV-3	wild type	Ovarian adenocarcinoma [7]. Overexpresses the p110α catalytic subunit of phosphoinositol 3-kinase and HER-2/neu. Lacks p53. Gift from Jeff Boyd (Memorial Sloan Kettering).
CAOV-2	wild type	Established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary. Estrogen and Androgen receptor positive. Gift from Jeff Boyd.
MCF-7	wild type (1 allele)	ATCC HTB-22. Derived from Estrogen-receptor positive breast adenocarcinoma.
NIH-OVCAR-3	wild type	Overexpresses the p110α catalytic subunit of phosphoinositol 3-kinase. Derived from ovarian carcinoma. Gift from Jeff Boyd.
293T	wild type	Chosen for its high efficiency of transfection and ability to achieve high levels of expression.

Progress on goals defined in Task 3.

The lack of knowledge concerning the precise biochemical function of BRCA1 is a major hurdle in developing a functional test to provide reliable presymptomatic assessment of risk for breast and ovarian cancer. The available data derived from linkage analysis indicate that all mutations that cause premature termination (even relatively subtle mutations such as the deletion of 11 amino acids from the C-terminus) will confer high risk. However, a considerable number of mutations result in amino acid substitutions that, in the absence of extensive population-based studies or a functional

assay, do not allow assessment of risk. *BRCA1* missense mutations detected in healthy women may be polymorphisms with no functional significance or cancer predisposing mutations. Unless the particular mutations have been found in the general population (polymorphisms) or in afflicted individuals (predisposing mutations) there is no predictive value in the diagnostics.

Our first step was to use site-directed and random mutagenesis (error prone PCR and transposon mediated) to generate mutations in the BRCA1 C-terminal region that disrupt transcription activation. Our goal was to define critical residues for BRCA1 function and to derive general rules to predict the impact of a particular mutation. An article describing our main findings was published in *Cancer Research*[2]. Secondly, to evaluate the usefulness of the proposed assay we needed to provide validation with population-derived data. This was obtained in a study involving families from Lund, Sweden. This study, which is the first validation of a functional assay for *BRCA1* was recently published in *Human Molecular Genetics* [8]. We believe that the publication of these two studies lays a solid basis for the use of our transcriptional assay. The experiments described above were a prerequisite to provide validation for our studies using mutations that might disrupt the putative interaction domain.

Our next step was to analyze the region in exons 12 and 13 and identify variants recorded in the Breast Cancer Information Core (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). The missense mutations found are depicted in Table 2. We then analyzed each mutation for its predicted ability to form coiled-coil structure as had been determined in the region aa 1366-1455 of the wildtype BRCA1 (as described in the Original proposal). This analysis was done using the algorithm developed by Peter Kim and colleagues (Multicoil scoring form; http://nightingale.lcs.mit.edu/cgi-bin/multicoil). The results are listed in table 2. We identified variant H1402Y as reducing the probability of forming the coiled coil structure and variant L1407P a completely disrupting the structure. These variants were chosen for further studies. As a control we selected variant H1421Y that did not affect coiled core structure because it is localized outside the small region directly involved in forming the coiled coil structure. We have designed PCR primers to introduce these mutations and are now in the process of obtaining the constructs.

Table 2. Missense variants in exon 12 and 13 of human BRCA1.

Exon	Mutation	Dog[a]	Mouse[b]	*Rat*[c]	Nucleotide[d]	Base change	Comments and probable secondary structure elements[e]
12	C1372Y	Y	Y	Y	4234	G to A	Probably a benign polymorphism.
12	V1378I	L	L	L	4251	G to A	Probably a benign polymorphism.
13	H1402Y	D	Y	D	4323	C to T	Reduces the probability to form coiled coil structure.
13	I1405V	I	I	I	4332	A to G	Conserved stretch.
13	L1407P	L	L	L	4339	T to C	Conserved stretch. Disrupts coiled coil formation.
13	H1421Y	H	R	H	4380	C to T	
13	R1443G	L	P	P	4427	T to C	Residue is not conserved.

[a] Amino acids correspond to predicted translation from canine *Brca1* cDNA (GenBank accession # U50709); [b] murine *Brca1* cDNA (GenBank accession # U68174); [c] rat *Brca1* cDNA (GenBank accession # AF036760); [d] Nucleotide numbering corresponds to human *BRCA1* cDNA (GenBank accession #U14680); [e] According to prediction by the Multicoil scoring form (*Protein Science* 1997; 6:1179-1189 - http://nightingale.lcs.mit.edu/cgi-bin/multicoil).

ADDITIONAL ACHIEVEMENTS

During our analysis of BRCA1 mutations we came across a naturally occurring *BRCA1* allele identified in a family from Lund that displayed unusual behavior in the transcriptional activation assay. The clinical data suggests that the variant (Arg to Trp substitution) is likely to have a deleterious effect *in vivo* and predispose carriers to cancer [8]. Disease association is further emphasized by the presence of the variant in a large pedigree with several women diagnosed with ovarian cancer, through three generations and across four degrees of relatedness (Åke Borg, personal communication). Nonetheless, our initial yeast-based tests of this variant showed that it retained wild type activity, an apparent divergence between clinical data and the transcription activation assay. To further investigate this mutant we assessed transcription activity of the GAL4 DBD fusion to BRCA1 (aa 1560-1863) in human 293T cells. Considering the conservation of basal transcription machinery in yeast and human cells, we hypothesized that the discrepancy was due to differences in the temperature in which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. To test this idea directly, we cultured yeast cells at 30°C and 37°C and quantified their transcription activity using an integrated reporter gene (6 *lexA* binding sites; LEU2). Activation of this reporter gene allows growth in medium lacking leucine. Cells carrying the wild-type BRCA1 construct were able to grow in selective medium at both temperatures. Conversely, cells carrying the two disease associated mutants did not show any detectable growth at either temperature. Interestingly, cells carrying this variant were able to grow at levels comparable to the wild type at 30°C but growth was dramatically impaired at 37°C, indicating a marked reduction in transcriptional activity. Similar to the experiment in yeast cells, the transcriptional activity of the variant was restored when human cells were cultured at 30°C. *Taken together, this data indicated that this mutant acted as a temperature-sensitive allele of BRCA1.* Further characterization of this temperature-sensitive mutant is important because it constitutes an invaluable tool to probe for the function of a particular protein as exemplified in other areas such as study of p53 [9;10] or tyrosine kinases [11].

CONCLUSIONS

In the second year of our project we were again able to achieve most of the goals defined in our proposal. We have made significant progress towards testing our hypothesis, in particular generating important reagents and optimizing conditions ideal to perform the experiments. This will be crucial to test the activity of the constructs in cells derived from breast and ovarian cancer and determine whether there is differential regulation of BRCA1 in these cell types. Unfortunately, the immunoprecipitation experiments seem to suggest that other additional experiments will be needed to carefully define the interaction region. In addition to the progress made in direct connection to the proposal, we have provided validation for our functional assay for BRCA1 and uncovered a conditional mutant that may be very useful to dissect the function of BRCA1.

REPORTABLE OUTCOMES

- Additional FLAG-tagged plasmids for mammalian expression

pCMV-FLAG (aa 1-302) pCMV-FLAG (aa 302-1313)

- Article on the mechanism of transcription activation by BRCA1 in *EMBO reports* [12](DoD support is acknowledged; copy attached).

- Article on a structure function analysis of BRCA1 in *Cancer Research* [2](copy attached).

- Review article on transcription activation of BRCA1 in *Trends in Biochemical Sciences* [13](DoD support is acknowledged; copy attached).

- Article on the development of a functional assay for BRCA1 in *Human Molecular Genetics*[8](DoD support is acknowledged; copy attached).

- Funding from DoD (Concept Award # BC996964) was obtained during the past year.

- Funding for a postdoctoral fellowship (#BC000959) in the laboratory was obtained during the past year.

REFERENCES

1. **Monteiro,A.N.**, August,A., and Hanafusa,H. (1996) Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc Natl Acad Sci U S A,* 93,13595-13599.

2. Hayes,F., Cayanan,C., Barilla,D., and **Monteiro,A.N.** (2000) Functional assay for BRCA1: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation. *Cancer Res*, 60,2411-2418.

3. Chapman,M.S., Verma,I.M. (1996) Transcriptional activation by BRCA1. *Nature*, 382,678-679.

4. **Monteiro,A.N.**, August,A., and Hanafusa,H. (1997) Common BRCA1 variants and transcriptional activation. *Am J Hum Genet*, 61,761-762.

5. Tomlinson,G.E., Chen,T.T., Stastny,V.A., Virmani,A.K., Spillman,M.A., Tonk,V., Blum,J.L., Schneider,N.R., Wistuba,I.I., Shay,J.W., Minna,J.D., and Gazdar,A.F. (1998) Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. *Cancer Res*, 58,3237-3242.

6. Johannsson, O., Gudjonsson, T., Kytola, S., Hedenfalk, I. A., Persson, K., Duggan, D. J., Adeyinka, A, Kjellén, E., Wennerberg, J., Baldetorp, B., Pedersen, O. W., Trent, J. M., Borg, A., Isola, J., and Olsson, H. Establishment and characterization of normal and breast carcinoma cell lines derived from BRCA1 and BRCA2 germ-line mutation carriers. Cold Spring Harbor Meeting on Cancer Genetics and Tumor Suppressor Genes, Abstract#100, 2000.

7. Buick,R.N., Pullano,R., and Trent,J.M. (1985) Comparative properties of five human ovarian adenocarcinoma cell lines. *Cancer Res*, 45,3668-3676.

8. Vallon-Christersson,J., Cayanan,C., Haraldsson,K., Loman,N., Bergthorsson,J.T., Brondum-Nielsen,K., Gerdes,A.M., Moller,P., Kristoffersson,U., Olsson,H., Borg,A., and Monteiro,A.N. (2001) Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. *Hum Mol.Genet*, 10,353-360.

9. Martinez,J., Georgoff,I., Martinez,J., and Levine,A.J. (1991) Cellular localization and cell cycle regulation by a temperature- sensitive p53 protein. *Genes Dev.*, 5,151-159.

10. Michalovitz,D., Halevy,O., and Oren,M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, 62,671-680.

11. Mayer,B.J., Jove,R., Krane,J.F., Poirier,F., Calothy,G., and Hanafusa,H. (1986) Genetic lesions involved in temperature sensitivity of the src gene products of four Rous sarcoma virus mutants. *J Virol.*, 60,858-867.

12. Nadeau,G., Boufaied,N., Moisan,A., Lemieux,K.M., Cayanan,C., **Monteiro,A.N.**, and Gaudreau,L. (2000) BRCA1 can stimulate gene transcription by a unique mechanism. *EMBO Reports*, 1:260-265.

13. **Monteiro,A.N.** (2000) BRCA1: exploring the links to transcription. *Trends Biochem.Sci,* 25,469-474.

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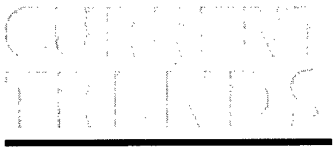

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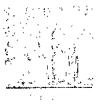

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BRCA1: exploring the links to transcription

Alvaro N.A. Monteiro

Progress on determining the function of the breast and ovarian cancer susceptibility gene *BRCA1* suggests it might be involved in two fundamental cellular processes: DNA repair and transcriptional regulation. Recent developments indicate that BRCA1 is a multifunctional protein, and disruption of its transcriptional activity could be crucial for tumor development.

MUTATIONS IN *BRCA1* account for approximately 45% of families with high incidence of breast cancer and for the majority of families with high incidence of both breast and ovarian cancer[1]. Several lines of evidence indicate that BRCA1 is a tumor suppressor, but a role as a negative regulator of cell proliferation is yet to be unambiguously demonstrated. *BRCA1*-linked tumors arising in carriers of germ-line mutations display loss of heterozygosity in the *BRCA1* locus with retention of the mutant allele[2]. BRCA1 induces the expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/CiP1}$, causing cell-cycle arrest[3]. Conversely, inhibition of *BRCA1* expression with antisense oligonucleotides results in accelerated proliferation in a mammary epithelial cell line[4]. It is still not clear whether these effects of BRCA1 on cell proliferation correspond to a physiological function or represent a response to abnormal levels of the protein induced by experimental conditions.

Human *BRCA1* codes for an 1863-amino-acid nuclear protein (Fig. 1a) with no detectable similarity to known proteins, with the exception of a RING-finger domain located in the N terminus and two BRCT (BRCA1 C-terminal) domains in tandem (aa 1653–1736 and aa 1760–1855)[5,6]. The BRCT is a globular domain found in proteins involved in repair and cell-cycle control[6]. Most of the documented cancer-associated mutations (Breast Cancer Information Core; http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/) cause truncations of the C-terminal region, a highly evolutionarily conserved region of the protein comprising the BRCT domains, underscoring the importance of this region for function.

Brca1 disruption in mouse leads to early embryonic death[7,8], and cells isolated from $Brca1^{-/-}$ embryos were shown to accumulate chromosomal abnormalities[9]. Cell-biological and genetic experiments have implicated BRCA1 in the maintenance of genome stability and DNA repair. BRCA1 has been found in large complexes that contain proteins involved in DNA repair[10–12], and human cells lacking BRCA1 display high sensitivity to γ-irradiation[13]. In addition, BRCA1 seems to be required for efficient homologous recombination[14]. To date, the evidence implicating BRCA1 in a variety of DNA-repair processes are based largely on genetic experiments and do not reveal by which mechanism BRCA1 acts. Although many scenarios remain possible at this stage, it is plausible that these effects are indirectly mediated through transcription activation.

Presence of a transcriptional activation domain in BRCA1

An early hint that BRCA1 might be involved in transcriptional activation came from the observation that the C-terminal region has a high content of negatively charged residues[5]. Highly acidic regions usually correlate with the transactivation domain in several eukaryotic transcription factors[15]. The hypothesis that BRCA1 could act as a transcription factor was tested by making fusions to a heterologous DNA-binding domain (DBD) and measuring the activation of a

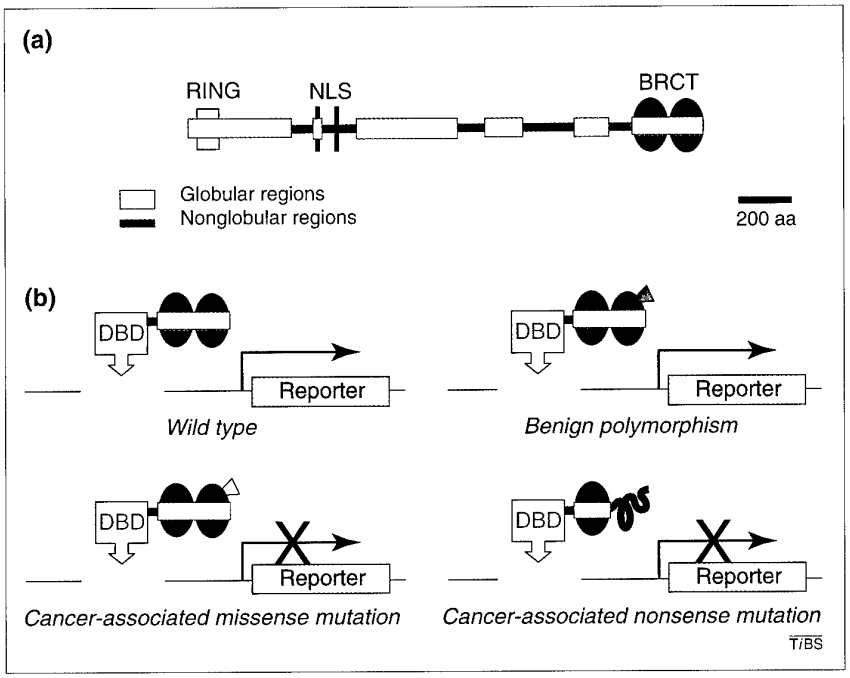

Figure 1

Transcriptional activation by BRCA1. **(a)** Domain structure of human BRCA1. Light-blue boxes and dark-blue lines correspond to predicted globular and nonglobular regions, respectively. The gray box represents the zinc-binding RING domain, and red circles represent the BRCA C-terminal (BRCT) domains. NLS, nuclear localization signal. Bar represents 200 amino acids. **(b)** Transcriptional activity by wild-type and mutant BRCA1 C-terminal region fused to a heterologous DNA-binding domain (DBD) (yellow boxes). Introduction of cancer-associated mutations (but not benign polymorphisms) abolishes reporter activation. Fusion to GAL4 DBD, a well-characterized heterologous DBD, enables the fusion protein to recognize specific sequences in the promoter of a reporter gene.

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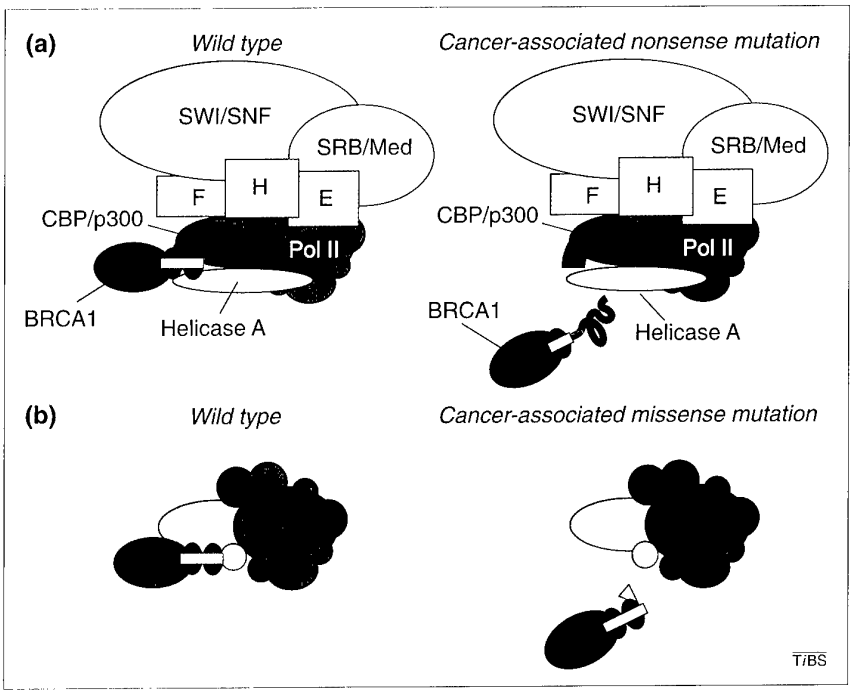

Figure 2

Interaction of BRCA1 with RNA polymerase II holoenzyme and with the core polymerase. **(a)** Physical interaction of BRCA1 with the RNA polymerase II holoenzyme via RNA helicase A and CBP/p300. The holoenzyme also contains the core polymerase subunits (dark-green circles), general transcription factors (blue squares), the SRB–Med complex and the chromatin remodeling SWI/SNF complex. Introduction of cancer-associated mutations abolishes interaction with RNA helicase A. **(b)** Physical interaction of BRCA1 with components of the core RNA polymerase II subunits, hRPB2 and hRPB10α (light-green circles). Introduction of cancer-associated mutations (open triangle) abolishes interaction with the core RNA polymerase II.

reporter gene by the fusion protein[16,17] (Fig. 1b). This kind of analysis is possible due to the modular nature of transcription factors in which DBDs and transactivation domains (domains that recruit the general transcription factors and RNA polymerase II) can function independently[15]. Results showing that the BRCA1 C terminus (aa 1560–1863) has the ability to activate transcription when fused to a GAL4 DBD (Refs 16,17) provided the initial experimental evidence that BRCA1 could be involved in transcription and pointed to a role for the BRCT domains. Fusion to GAL4 DBD, a well-characterized heterologous DBD, enables the fusion protein to recognize specific sequences in the promoter of a reporter gene and is used when a DNA-binding sequence for a particular protein is unknown (Fig. 1b).

Assays involving overexpression of fusions of heterologous DBD with truncated proteins can generate artifactual results. In addition, transcription activation could be a physiological function of BRCA1 but still be unrelated to its role in tumor suppression. Introduction of cancer-associated mutations abolished transactivation[16,17], whereas benign polymorphisms did not[18] (Fig. 1b), suggesting that transactivation function is important to prevent tumor development. Extensive mutagenesis analysis of the C-terminal region confirmed that the BRCT domains correspond to the core transactivation domain of BRCA1 (Ref. 19). The fact that cancer-associated mutations result in loss of transcriptional activation function, in particular those that are not predicted to disrupt the BRCT fold, provided a genetic framework to link the transcriptional activation function of BRCA1 to the development of cancer. However, although BRCA1 can act as a transcriptional regulator, it is unclear which precise biochemical function is performed by BRCA1.

BRCA1 interacts with RNA polymerase II

Mammalian RNA polymerase II (pol II) has been shown to exist in two distinct multiprotein complexes: the 'core', composed of 12 RNA pol II subunits (~500 kDa), and the 'holoenzyme' (>1 MDa), containing the core plus the SRB–Med complex [this complex was isolated both genetically, as suppressors of RNA pol II deletions in yeast (SRB), and biochemically, as proteins required for activated transcription in a partially purified system *in vitro*], general transcription factors and factors involved in chromatin remodeling (Fig. 2). Support for a physiological role of BRCA1 in transcription came from results showing that BRCA1 co-purified with pol II holoenzyme under conditions in which other transcription factors such as YY1, TFII-I, p65 Rel and RBPJ-κ did not[20] (Fig. 2a). BRCA1 interacts directly with RNA helicase A, a component of the holoenzyme that also interacts with the coactivator CBP/p300 (Ref. 21). Importantly, an ectopically expressed epitope-tagged BRCA1 carrying a cancer-associated mutation failed to interact with the holoenzyme[20]. Interestingly, GAL4 DBD–BRCA1 C terminus fusions can activate transcription *in vitro* in the presence of core pol II (Ref. 22; Fig. 2b). This *in vitro* system was later used, in transcription reconstitution experiments, to define hRPB10α (a homolog of yeast ABC10 α shown to be essential in yeast) and hRPB2 (homologous to the prokaryotic pol II β subunit) as the interaction partners in the core polymerase[23]. Recently, it was shown that the coactivator CBP/p300 interacts with and acts as a coactivator for BRCA1 (Ref. 24) (see Table 1 for BRCA1-interacting proteins involved in transcription). The interaction does not seem to be mediated by RNA helicase A, because a GST-tagged CBP fragment (aa 451–721) lacking a RNA-helicase-A-binding site (aa 1805–1890) can co-precipitate BRCA1 (Ref. 24). Although the results discussed so far revealed a function for BRCA1 in transcription, and the *in vitro* experiments suggest potential mechanisms through which BRCA1 activates transcription, they provide no clear idea of the physiological outcome of BRCA1-mediated transcription.

Transcriptional activation by BRCA1

In 1997, experiments showing that ectopically expressed wild-type BRCA1 caused cell-cycle arrest via transactivation of the cell-cycle inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner suggested a basis for BRCA1 tumor suppressor action and provided the first hint of putative downstream effectors of BRCA1 (Ref. 3; Fig. 3a). Importantly, cancer-predisposing, transactivation-deficient mutants failed to cause either cell-cycle arrest or $p21^{WAF1/CIP1}$ induction. However, the experiments did not discriminate between a direct or indirect effect of BRCA1 on transactivation of $p21^{WAF1/CIP1}$.

It was also found that BRCA1 physically interacts with p53 and enhances p53-mediated activation of p53-responsive genes including $p21^{WAF1/CiP1}$, suggesting a more direct effect of BRCA1 on transcription[25,26] (Fig. 3a). These results are important, because they link the p53 pathway and BRCA1 function and raise intriguing questions. Does binding of BRCA1 to p53 induce changes in p53 DNA-binding affinity for different p53-responsive elements, therefore conceivably shifting p53 response from a set of target genes to another? To demonstrate a direct involvement of BRCA1 in the activation, it would also be important to test if antibodies against BRCA1 can induce supershift of p53-containing complexes in an electromobility shift assay (EMSA). In EMSA, nuclear extracts are incubated with a radiolabeled DNA probe containing the sequence-specific binding site. Proteins that recognize and bind the probe appear as a labeled band on a gel. The identity of the protein is determined by adding a specific antibody that, upon binding to the protein–DNA complex, will retard its migration (mobility shift) on a gel.

In a different system, BRCA1 was implicated in enhancing IFN-γ-stimulated growth arrest, most likely by binding to activated signal transducer and activator of transcription 1 (STAT1)[27] (Fig. 3b). STAT1 is phosphorylated by the Janus kinase (JAK) and mediates the biological effects of IFN-γ. In cells stimulated with IFN-γ, growth arrest is mediated by induction of $p21^{WAF1/CiP1}$ via an IFN-γ-responsive element. Experiments in HCC1937, a cell line carrying only one copy of a truncated version of BRCA1, indicate that BRCA1 is required for IFN-γ-mediated $p21^{WAF1/CiP1}$ induction[27]. BRCA1 differentially regulates some IFN-γ target genes by enhancing its growth-arrest response, but not other IFN-γ target genes such as IRF-1, SMAD7 and IP10, suggesting that BRCA1 displays promoter selectivity (Fig. 3b).

Lately, investigators have undertaken a global approach to define BRCA1 target genes using cDNA arrays. Inducible expression of BRCA1 in cultured cells led to programmed cell death and revealed that a major BRCA1 target gene is the DNA-damage-responsive gene *GADD45* (Ref. 28). Induction of *GADD45* has been shown to trigger JNK/SAPK-dependent apoptosis[29]. JNK/SAPK (Jun N-terminal kinase/stress-activated protein kinase) is a member of the mitogen-activated protein kinase (MAPK) family used by cells to relay signals triggered by growth factors and extracellular stimuli. Concomitant inhibition of JNK/SAPK by ectopic expression of a dominant-negative mutant of its upstream regulator SEK1 prevented BRCA1-induced apoptosis but not *GADD45* induction, indicating that BRCA1 induces apoptosis via JNK/SAPK. A similar approach using adenovirus-mediated BRCA1 infection also showed induction of cell-cycle-controlling genes (e.g. $p21^{WAF1/CiP1}$) and DNA-damage-response genes (e.g. *GADD45*) but failed to demonstrate induction of apoptosis, suggesting that this effect could be cell-type specific[30]. One caveat of these experiments is the fact that most of them, but not all, rely on overexpression of the protein in cells that express endogenous BRCA1. Establishment of HCC1937 cells carrying a stably transfected inducible BRCA1 will provide a powerful tool for these analyses. Also, as in other experiments involving *in vivo* expression of BRCA1, we cannot distinguish whether the effect on transcription is direct or indirect.

Table 1. BRCA1-interacting proteins involved in transcription

Interacting protein	BRCA1-binding site	Function of interacting protein	Refs
BARD1	RING finger[a]	Unknown; repair (?), mRNA processing (?)	37,44
CBP/p300	1–303 and 1314–1863[b]	Transcriptional coactivator	24
CtiP	1651–1863[a,c]	Binds to CtBP transcription repressor	33,34
HDAC1 and HDAC2	1536–1863[d]	Histone deacetylases involved in chromatin remodeling	32
c-Myc	433–511[c]	Helix-loop-helix transcription factor	31
p53	224–500[e]	Transcription activation	25,26
Rb	1536–1863[f]	Retinoblastoma tumor suppressor gene. Interacts with E2F and represses E2F-mediated transcription	32
RbAp46 and RbAp48	1536–1863[g]	Rb-interacting protein. Component of histone deacetylase complexes involved in chromatin remodeling	32
RNA helicase A	1650–1800[h]	Component of the RNA polymerase II holoenzyme. Also interacts with CBP/p300	21
RPB2	1560–1863[i]	Component of the core RNA polymerase II. Homolog of bacterial RNA polymerase β subunit	23
RPB10α	1560–1863[i]	Component of the core RNA polymerase II. Homolog of yeast ABC10α	23
STAT1α	502–802[j]	Transcription activation induced by interferon	27

Binding site was identified by: [a]Mammalian two-hybrid assay; [b]Binding of glutathione-*S*-transferase (GST)–CBP to Myc-tagged BRCA1 fragments in pull-down assays; [c]Yeast two-hybrid assays; [d]Binding to GST–BRCT in pull-down assays; [e]Interaction of *in vitro* translated BRCA1 fragments with GST–p53 and GST–BRCA1 fragments in pull-down assays; [f]Interaction of *in vitro* translated BRCA1 fragments with GST–RB; [g]Interaction of *in vitro* translated BRCA1 fragments with GST–RbAp; [h]Interaction of *in vitro* translated RHA with GST–BRCA1 fragments; [i]Interaction of biotin-binding PinPoint domain fusion of BRCA1 to core polymerase; [j]*In vitro* binding assay using GST–BRCA1 fragments.
Abbreviations: BARD1, BRCA1-associated RING-domain protein 1; BRCT, BRCA1 C-terminal domain; CBP, CREB-binding protein; CtiP, CtBP-interacting protein; HDAC, histone deacetylase; Rb, retinoblastoma; RbAp, retinoblastoma-associated protein; STAT, signal transducer and activator of transcription.

Transcriptional repression by BRCA1

The data discussed above show that BRCA1 can activate transcription, but it might also be able to repress transcription. In a yeast two-hybrid approach, BRCA1 was shown to interact with the helix-loop-helix transcription factor c-Myc (Ref. 31) (Fig. 3c). In addition, co-transfection studies demonstrated that BRCA1 repressed Myc-mediated transcription and suppressed the number of transformed foci in Ras- or c-Myc-transformed embryo fibroblasts. These results suggest that BRCA1 can negatively regulate transcription by c-Myc. Because overexpressing a particular protein might repress the activity of a transcription factor through physiologically irrelevant protein–protein interactions, it is important to establish whether BRCA1 deficiency in cells facilitate c-Myc-mediated transformation.

The fact that BRCA1 has a transcriptional activation domain might seem paradoxical with its function in repression, but other lines of evidence suggest that BRCA1 might be able to modulate transcription in a context-dependent way. When a cDNA expression library was probed with a histidine-tagged BRCT domain in a far-western screen, one retinoblastoma (Rb)-binding protein, RbAp46 (a component of histone deacetylase complexes), was obtained[32] (far-western analysis uses a protein probe to detect interactions with

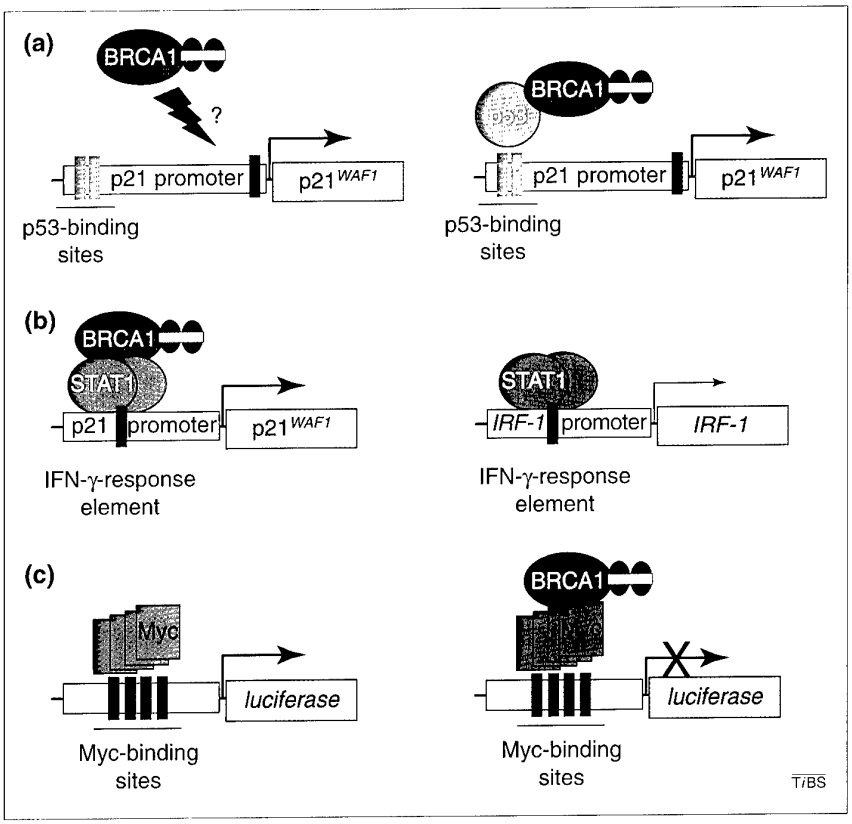

Figure 3

Transcriptional regulation by BRCA1. Functional interaction of BRCA1 with other DNA-binding proteins. **(a)** BRCA1 is able to activate the $p21^{WAF1/CiP1}$ promoter (yellow box) in a p53-dependent (right panel) and -independent (left panel) manner targeting two different regions of the promoter. **(b)** Selectivity of BRCA1 coactivation of STAT1 (signal transducer and activator of transcription 1; green circles). BRCA1 coactivates STAT1-mediated transcription in the $p21^{WAF1/CiP1}$ promoter (left panel, yellow box) but not the *IRF-1* promoter (right panel, yellow box). **(c)** BRCA1 repression of Myc-mediated transcription of a reporter gene.

proteins immobilized on filter membranes). Other proteins in the Rb tumor suppressor pathway, such as RbAp48 and Rb itself, are also capable of interacting with BRCA1. Similarly, the C-terminal region of BRCA1 was shown to bind to the histone deacetylases HDAC1 and HDAC2 *in vitro*[32].

Two independent approaches to identify BRCA1-binding proteins, one using the yeast two-hybrid system and the other using the Sos-recruitment system (a variation of the two-hybrid system commonly used when the proteins to be tested display intrinsic transcriptional activity), resulted in the isolation of CtIP (CtBP-interacting protein), a co-repressor for different cellular transcription factors[33,34]. Cancer-associated mutations in BRCA1 abolished binding to CtIP (Refs 33,34), as did DNA damage[35].

Of particular importance to breast and ovary carcinogenesis are preliminary reports that BRCA1 modulates estrogen receptor α transcription[36], but further studies are needed to determine the physiological relevance of this modulation. It is possible that BRCA1 also regulates promoter selectivity of ER α target genes.

A recent report shows that BRCA1 and its RING-finger-interacting protein BARD1 are present in a complex containing the cleavage-stimulating factor CstF50, which helps specify the site of mRNA processing during polyadenylation[37]. Whereas addition of a monoclonal antibody against BARD1 specifically enhanced the cleavage reaction *in vitro* and suggests that BARD1 antagonizes 3′-end formation, the role of BRCA1 is unclear[37].

It is still too early to judge the significance of the interactions of BRCA1 with different proteins (Table 1), but they provide indirect evidence of its role in transcription and suggest that BRCA1 can switch from a positive to a negative regulator in different contexts. The next step will be to investigate the physiological relevance of these interactions by complementary methods.

Transcription-coupled repair: a unifying theme?

Although it is possible that BRCA1 has two separate functions in DNA repair and in transcription regulation, it is also conceivable that the role of BRCA1 in DNA repair is mediated by transcriptional regulation. In agreement with the latter notion, intact BRCA1 seems to be required for transcription-coupled repair (TCR) of oxidative DNA damage but not for its global removal in embryonic stem cells from *Brca1*$^{-/-}$ mice[38]. Experiments with human BRCA1-deficient cells also suggest that BRCA1 is required for TCR (Ref. 39). TCR, also called preferential repair of the transcribed strand, refers to the fact that genes being actively transcribed are repaired more rapidly than other genes in the genome, and rapid repair is confined to the transcribed strand only. It is believed that one of the components of the RNA pol II-associated general factors, TFII-H, can shift from a transcription to a repair mode in the stalled RNA pol II (Ref. 40). Could BRCA1 also display such a switch? We should keep in mind that the data do not reveal whether BRCA1 is directly required for TCR or rather necessary for the transcription of genes required for TCR.

One way to test the hypothesis that the observed role of BRCA1 in repair is an indirect effect of its transcriptional function is to examine the cancer-predisposing alleles of BRCA1 and their outcome in functional assays for transcriptional activation and for DNA repair activity. With the recent progress on functional assays with BRCA1 for both of these functions, this approach might now be possible[19,41]. If all mutations that affect transcriptional activation also affect its DNA-repair function, this would provide strong evidence that it is the same biochemical pathway. To be informative, these mutations should not lead to instability of the mRNA, or the protein, or lead to drastic disruption of the folding. Although many mutations have been recorded in the C-terminal region of BRCA1, only a few have been characterized as either benign polymorphisms or cancer-associated mutations. It is expected that the lack of genetic data regarding cancer predisposition of BRCA1 alleles will be balanced by knowledge of the crystal structure of the BRCT domain of XRCC1 (a scaffolding protein in the mammalian base excision repair pathway), which allowed modeling of the BRCA1 BRCT domain and prediction of structural changes in the protein

caused by different mutations[42]. Unfortunately, until further structural data emerge for other regions of the protein, the analysis will be restricted to the BRCT and RING-finger domains. Another possible approach to the same problem could be the use of reciprocal mutants on components of the RNA pol II that restore binding and function to a mutant transcription factor. An analogous approach has been used successfully to probe TBP–TFIIB interaction[43]. In this case, it would be possible to design reciprocal mutants in pol II components that restore binding of mutant BRCA1. The prediction would be that if transcription is a necessary and sufficient step in processing repair, then, in this context, a mutant BRCA1 will be able to perform DNA repair. If, however, the reciprocal mutation is then able to sustain transcription but not repair, we will have strong evidence that the two functions assigned for BRCA1 are separate. Certainly, any outcome will reveal exciting new avenues of research.

Future perspectives

Is BRCA1 a transcription factor or a coactivator? As we have seen, BRCA1 contacts the RNA polymerase II holoenzyme components p300/CBP, RNA helicase A, RPB10α and RPB2. The fact that RPB10α is a common component of all three RNA polymerases raises the possibility that BRCA1 might also regulate pol I and pol III transcription, and it will be important to see if BRCA1 is also present in these complexes. However, to date, no one has been able to demonstrate sequence-specific binding to DNA by BRCA1, although several instances where BRCA1 functionally interacts with other DNA-binding proteins have been reported.

What is the role of BRCA1 in the RNA pol II holoenzyme? Crude stoichiometric estimates indicate that BRCA1 is only present in a subpopulation of the holoenzyme[20], suggesting that, instead of a general role in transcription, BRCA1 might be present in only a fraction of pol II possibly involved in transcription of a particular subset of genes. Alternatively, BRCA1 might only interact with holoenzyme complexes engaged in transcription. The *in vitro* transcription reconstitution assays can be used to distinguish between these possibilities. It might be feasible to immunodeplete BRCA1-containing holoenzyme complexes and compare the activities of different holoenzyme preparations.

Are BRCA1-target genes the same in different tissues? Because tissue specificity for tumor formation cannot be explained by expression patterns (as BRCA1 is ubiquitously expressed), it is plausible that BRCA1 might be required for a subset of genes that are highly transcribed in certain tissues such as breast and ovary. Therefore, lack of functional BRCA1 would prime cells in these tissues to transformation. In light of the data on the involvement of BRCA1 in TCR, this idea is particularly appealing. It will be crucial to determine which biochemical step in TCR requires BRCA1.

The evidence for the role of BRCA1 in transcriptional regulation can be summarized as follows: (i) the C terminus of BRCA1 acts as a transcriptional activation domain when fused to a heterologous DNA-binding domain; (ii) BRCA1 can be found in complex with the RNA polymerase II (core and holoenzyme); (iii) ectopic expression of BRCA1 induces transcription from a variety of different promoters; and (iv) several BRCA1-interacting proteins have well-characterized roles in transcription. The strong correlation of cancer-associated mutations and its loss of function phenotype in the experiments described strengthen the idea that the role of BRCA1 in transcription is physiologically relevant during the development of the disease. Many questions remain unanswered, but the biochemical and genetic approaches discussed here form the basis to attribute a definite biological function for BRCA1.

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References

1 Easton, D.F. *et al.* (1993) Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *Am. J. Hum. Genet.* 52, 678–701
2 Smith, S.A. *et al.* (1992) Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nat. Genet.* 2, 128–131
3 Somasundaram, K. *et al.* (1997) Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* 389, 187–190
4 Thompson, M.E. *et al.* (1995) Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Nat. Genet.* 9, 444–450
5 Miki, Y. *et al.* (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266, 66–71
6 Bork, P. *et al.* (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *FASEB J.* 11, 68–76
7 Gowen, L.C. *et al.* (1996) Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. *Nat. Genet.* 12, 191–194
8 Hakem, R. *et al.* (1996) The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. *Cell* 85, 1009–1023
9 Shen, S.X. *et al.* (1998) A targeted disruption of the murine Brca1 gene causes gamma-irradiation hypersensitivity and genetic instability. *Oncogene* 17, 3115–3124
10 Wang, Y. *et al.* (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.* 14, 927–939
11 Zhong, Q. *et al.* (1999) Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science* 285, 747–750
12 Scully, R. *et al.* (1997) Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 88, 265–275
13 Foray, N. *et al.* (1999) Gamma-rays-induced death of human cells carrying mutations of BRCA1 or BRCA2. *Oncogene* 18, 7334–7342
14 Moynahan, M.E. *et al.* (1999) Brca1 controls homology-directed DNA repair. *Mol. Cell* 4, 511–518
15 Ptashne, M. and Gann, A. (1997) Transcriptional activation by recruitment. *Nature* 386, 569–577
16 Monteiro, A.N. *et al.* (1996) Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc. Natl. Acad. Sci. U. S. A.* 93, 13595–13599
17 Chapman, M.S. and Verma, I.M. (1996) Transcriptional activation by BRCA1. *Nature* 382, 678–679
18 Monteiro, A.N. *et al.* (1997) Common BRCA1 variants and transcriptional activation. *Am. J. Hum. Genet.* 61, 761–762
19 Hayes, F. *et al.* (2000) Functional assay for BRCA1: mutagenesis of C-terminal region reveals critical residues for transcriptional activation. *Cancer Res.* 60, 2411–2418
20 Scully, R. *et al.* (1997) BRCA1 is a component of the RNA polymerase II holoenzyme. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5605–5610
21 Anderson, S.F. *et al.* (1998) BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nat. Genet.* 19, 254–256
22 Haile, D.T. and Parvin, J.D. (1999) Activation of transcription *in vitro* by the BRCA1 carboxy-terminal domain. *J. Biol. Chem.* 274, 2113–2117
23 Schlegel, B.P. *et al.* (2000) BRCA1 interaction with RNA polymerase II reveals a role for hRPB2 and hRPBα in activated transcription. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3148–3153
24 Pao, G.M. *et al.* (2000) CBP/p300 interact with and function as transcriptional coactivators of BRCA1. *Proc. Natl. Acad. Sci. U. S. A.* 97, 1020–1025
25 Ouchi, T. *et al.* (1998) BRCA1 regulates p53-dependent gene expression. *Proc. Natl. Acad. Sci. U. S. A.* 95, 2302–2306
26 Zhang, H. *et al.* (1998) BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* 16, 1713–1721
27 Ouchi, T. *et al.* (2000) Collaboration of STAT1 and BRCA1 in differential regulation of IFN-γ target genes. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5208–5213
28 Harkin, D.P. *et al.* (1999) Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. *Cell* 97, 575–586
29 Takekawa, M. and Saito, H. (1998) A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* 95, 521–530
30 MacLachlan, T.K. *et al.* (2000) BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. *J. Biol. Chem.* 275, 2777–2785
31 Wang, Q. *et al.* (1998) BRCA1 binds c-Myc and inhibits its transcriptional and transforming activity in cells. *Oncogene* 17, 1939–1948
32 Yarden, R.I. and Brody, L.C. (1999) BRCA1 interacts with components of the histone deacetylase complex. *Proc. Natl. Acad. Sci. U. S. A.* 96, 4983–4988

33 Yu, X. *et al.* (1998) The C-terminal (BRCT) domains of BRCA1 interact *in vivo* with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J. Biol. Chem.* 273, 25388–25392

34 Wong, A.K. *et al.* (1998) Characterization of a carboxy-terminal BRCA1 interacting protein. *Oncogene* 17, 2279–2285

35 Li, S. *et al.* (1999) Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. *J. Biol. Chem.* 274, 11334–11338

36 Fan, S. *et al.* (1999) BRCA1 inhibition of estrogen receptor signaling in transfected cells. *Science* 284, 1354–1356

37 Kleiman, F.E. and Manley, J.L. (1999) Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50. *Science* 285, 1576–1579

38 Gowen, L.C. *et al.* (1998) BRCA1 required for transcription-coupled repair of oxidative DNA damage. *Science* 281, 1009–1012

39 Abbott, D.W. *et al.* (1999) BRCA1 expression restores radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. *J. Biol. Chem.* 274, 18808–18812

40 Hanawalt, P.C. (1994) Transcription-coupled repair and human disease. *Science* 266, 1957–1958

41 Scully, R. *et al.* (1999) Genetic analysis of BRCA1 function in a defined tumor cell line. *Mol. Cell* 4, 1093–1099

42 Zhang, X. *et al.* (1998) Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. *EMBO J.* 17, 6404–6411

43 Tansey, W.P. and Herr, W. (1997) Selective use of TBP and TFIIB revealed by a TATA-TBP-TFIIB array with altered specificity. *Science* 275, 829–831

44 Wu, L.C. *et al.* (1996) Identification of a RING protein that can interact *in vivo* with the BRCA1 gene product. *Nat. Genet.* 14, 430–440

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Functional analysis of *BRCA1* C-terminal missense mutations identified in breast and ovarian cancer families

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Germline mutations in the breast and ovarian cancer susceptibility gene *BRCA1* are responsible for the majority of cases involving hereditary breast and ovarian cancer. Whereas all truncating mutations are considered as functionally deleterious, most of the missense variants identified to date cannot be readily distinguished as either disease-associated mutations or benign polymorphisms. The C-terminal domain of *BRCA1* displays an intrinsic transactivation activity, and mutations linked to disease predisposition have been shown to confer loss of such activity in yeast and mammalian cells. In an attempt to clarify the functional importance of the *BRCA1* C-terminus as a transcription activator in cancer predisposition, we have characterized the effect of C-terminal germline variants identified in Scandinavian breast and ovarian cancer families. Missense variants A1669S, C1697R, R1699W, R1699Q, A1708E, S1715R and G1738E and a truncating mutation, W1837X, were characterized using yeast- and mammalian-based transcription assays. In addition, four additional missense variants (V1665M, D1692N, S1715N and D1733G) and one in-frame deletion (V1688del) were included in the study. Our findings demonstrate that transactivation activity may reflect a tumor-suppressing function of *BRCA1* and further support the role of *BRCA1* missense mutations in disease predisposition. We also report a discrepancy between results from yeast- and mammalian-based assays, indicating that it may not be possible to unambiguously characterize variants with the yeast assay alone. We show that transcription-based assays can aid in the characterization of deleterious mutations in the C-terminal part of *BRCA1* and may form the basis of a functional assay.

INTRODUCTION

Germline mutations in the breast and ovarian cancer susceptibility gene *BRCA1* (OMIM 113705) predispose carriers to early-onset breast and breast–ovarian cancer (1,2) and it is estimated that ~5% of all breast cancer cases are caused by inherited mutations in dominant disease genes. The majority of familial cases with both breast and ovarian cancer and a substantial part of families with breast cancer alone involve mutations in *BRCA1* (3). The *BRCA1* gene product is an 1863 amino acid phosphoprotein with a RING-finger motif at its N-terminus and two BRCA1 C-terminal (BRCT) domains at its C-terminus (1,4). With the exception of these domains, BRCA1 displays no similarity to other known proteins. The BRCT domains are mainly found in proteins involved in DNA repair, recombination and cell cycle control (5,6). Early findings suggest that BRCA1 is a tumor suppressor because loss of the wild-type allele was observed in familial breast and ovarian cancer cases (7). Although the function of BRCA1 remains unclear, there is increasing support for a role in DNA repair and transcription activation (for reviews see refs 8 and 9).

BRCA1 interacts with large protein complexes involved in DNA repair such as Rad51/BRCA2 (10,11) and Rad50/Mre11/p95 (12,13). Importantly, BRCA1 becomes hyperphosphorylated and disperses from Rad51-containing nuclear foci in response to DNA damage (14,15). In mice, Brca1 is required for transcription-coupled repair of oxidative DNA damage (16) and *Brca1*$^{-/-}$ embryonic cells accumulate genetic aberrations (17). However, no direct mechanism of action has been described which explains how BRCA1 exerts its functions.

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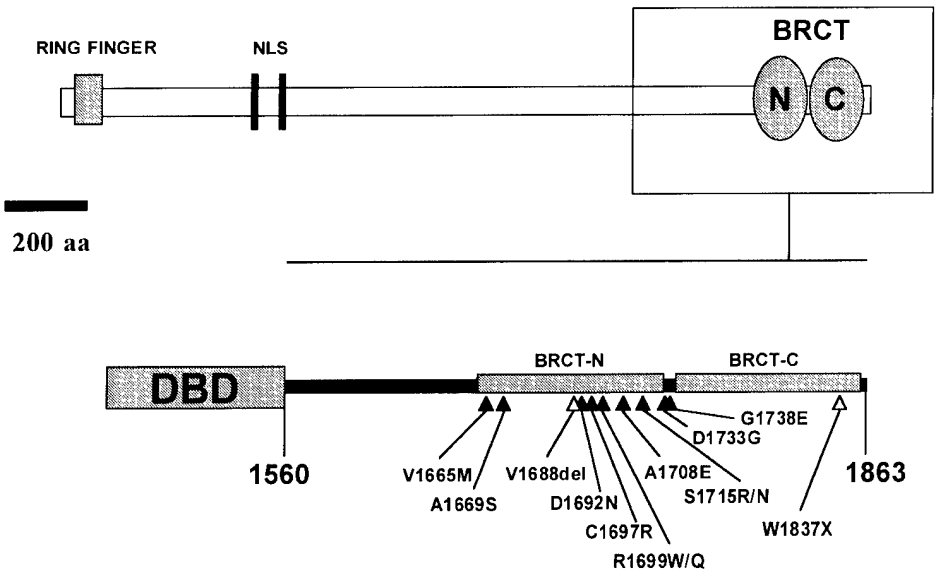

Figure 1. Domain structure of BRCA1. (Top) Schematic representation of full-length BRCA1 protein featuring the RING domain in the N-terminal region and the BRCT domains in the C-terminal region. The region analyzed in this study (amino acids 1560–1863) is contained in the box, which is enlarged and represented (bottom). Gray circles represent the two BRCT domains, BRCT-N (amino acids 1649–1736) and BRCT-C (amino acids 1756–1855). NLS, nuclear localization signals. (Bottom) GAL4- and LexA-DNA binding domain fusions to BRCA1 C-terminal region (amino acids 1560–1863). Mutations analyzed in this study are depicted as black (missense) or open (nonsense and in-frame deletion) triangles.

A transactivation activity was first ascribed to *BRCA1* by demonstrating that, when fused to a heterologous DNA-binding domain, the C-terminus of *BRCA1* acts as a transcription activator (18,19). BRCA1 associates *in vivo* with RNA polymerase II (pol II) holoenzyme as well as with the core pol II (20–22) and modulates transcription mediated by several transcription factors (9 and references therein).

The discovery of a transactivation activity revealed a testable function of BRCA1 and yeast-based assays have been proposed as a means of characterizing missense variants because disease-associated mutations abolish this activity (23,24). Numerous mutations in *BRCA1* have been described and established as disease-associated (Breast Cancer Information Core database, BIC). Such mutations are located throughout the gene and typically result in premature translation termination. Apart from a handful of clearly linked or strongly suspected disease-associated mutations, most amino acid substitutions reported hitherto cannot readily be distinguished as either disease-associated or benign polymorphisms and are classified as variants of uncertain significance (BIC), posing a very relevant problem in genetic counseling. Nevertheless, although the precise biochemical function of the protein remains unknown, increasing knowledge of the structural properties and biological roles of BRCA1 provides support in discriminating these alterations, eventually allowing functional assays to be developed (24,25). Yeast-based assays have been able to discriminate between disease-associated mutations and benign polymorphisms in the C-terminus of *BRCA1* (18,24,26,27). Therefore, it is tempting to suggest that the transactivation activity reflects a tumor-suppressing function of BRCA1 *in vivo*. Here we use a transcription activation assay to characterize the effect of unique germline variants identified in Scandinavian breast and ovarian cancer families. Seven of the included variants are of missense type and one is of nonsense type. In addition, we analyzed five C-terminal BRCA1 variants reported by others (BIC).

RESULTS

Analysis of hereditary breast and ovarian cancer has revealed several novel as well as previously described variants of *BRCA1*. Patients have been screened for mutations in *BRCA1* and *BRCA2* as described by Hakansson *et al.* (28). Here we analyze missense variants and one truncating mutation that localize to the C-terminal region of *BRCA1* (Fig. 1). These variants were not found in 50 healthy Swedish control individuals (no screen has been done for G1738E). Moreover, >450 index cases with familial history of breast–ovarian cancer have been screened for mutations in *BRCA1* and the variants reported here have been found only in their respective families, indicating that they represent rare variants.

We introduced the variants in constructs containing the fusion GAL4 DNA-binding domain (DBD):BRCA1 (amino acids 1560–1863) (Fig. 1) (18,26). In order to assess their transactivation activity these constructs were transformed into two *Saccharomyces cerevisiae* strains, HF7c and SFY526, containing reporter genes under the control of the GAL1 upstream activating sequence (UAS), recognized by GAL4 DBD. Wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and vector without insert was used as a negative control. Results were comparable in both yeast strains in a semi-quantitative assay (Table 1).

Table 1. Transcriptional activity of *BRCA1* variants identified in Scandinavian breast–ovarian cancer families and variants obtained from the BIC database

Family/ source	Exon	Mutation	Dog[a]	Mouse[b]	Rat[c]	Nucleotide[d]	Base change	Probable secondary structure elements[e]	Transcriptional activity		
									HF7c (His)[f]	SFY526 (β-gal)[g]	EGY48 (β-gal)[h]
Lund 321	17	A1669S	A	A	A	5124	G→T	α-helix 1 of BRCT-N	+	+	+
Lund 275	18	C1697R	C	C	C	5208	T→C	α-helix 2 of BRCT-N	–	–	–
Lund 279	18	R1699W	R	R	R	5214	C→T	α-helix 2 of BRCT-N	+	+	+
Lund 488	18	R1699Q	R	R	R	5215	G→A	α-helix 2 of BRCT-N	+	+[i]	+
Lund 20	18	A1708E	A	A	A	5242	C→A	α-helix 2/β-strand 4 loop of BRCT-N	–[j]	–[j]	Not done
Lund 184	18	S1715R	S	S	S	5262	A→C	β-strand 4/α-helix 3 loop of BRCT-N	–	–	–
Lund 32	20	G1738E	G	G	G	5332	G→A	BRCT-N/BRCT-C interval	–	–	–[k]
Lund 190	24	W1837X	W	W	W	5630	G→A	α-helix 3 of BRCT-C; conserved W in BRCT domains	–	–	–
BIC	17	V1665M	V	V	V	5112	G→A	α-helix 1 of BRCT-N	+	+	+
BIC	17	V1688del	I	I	I	5181	delGTT	β-strand 3 of BRCT-N	–	–	–
BIC	17	D1692N	D	D	D	5193	G→A	β-strand 3/α-helix 2 of BRCT-N	+	+	+
BIC	18	S1715N	S	S	S	5263	G→A	β-strand 4/α-helix 3 loop of BRCT-N	–	–	–
BIC	20	D1733G	D	E	E	5317	A→G	BRCT-N/BRCT-C interval	+	+	+[k]

[a]Amino acids correspond to predicted translation from canine *Brca1* cDNA (GenBank accession no. U50709).
[b]Amino acids correspond to predicted translation from murine *Brca1* cDNA (GenBank accession no. U68174).
[c]Amino acids correspond to predicted translation from rat *Brca1* cDNA (GenBank accession no. AF036760).
[d]Nucleotide numbering corresponds to human *BRCA1* cDNA (GenBank accession no. U14680). Alignment was performed using Vector NTI Multiple Sequence Alignment version 1.0.1.1.
[e]According to a BRCA1 BRCT model from Zhang *et al.* (29).
[f]Thirty-six individual colonies were streaked on solid SD medium lacking tryptophan and histidine and scored for growth after 2 days at 30°C. A positive score (+) was noted if growth was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if growth was visually identical to the negative control (vector with no insert).
[g]Thirty-six individual colonies were streaked on filter overlaid on solid SD medium and assayed for β-gal activity after 2 days at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative control (vector with no insert). Clones were scored 6 h after addition of X-gal.
[h]At least six individual colonies were streaked on filter overlaid on solid SD medium lacking tryptophan and uracyl and assayed for β-gal activity the next day at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative controls (M1775R and Y1853X). Clones were scored 2 h after addition of X-gal.
[i]Partly reduced β-gal activity.
[j]Published results, Monteiro *et al.* (18).
[k]Published results, Hayes *et al.* (24).

Analysis of variants identified in Lund families

Variants A1669S, R1699W and R1699Q displayed wild-type activity, suggesting that they represent benign polymorphisms (Table 1). For variant A1669S, the data from functional assays are in agreement with the pedigree analysis (Fig. 2). One of the affected family members did not carry the mutation and cases of uterine and very early-onset ovarian cancer indicate involvement of predisposing genes other than *BRCA1* or *BRCA2*. Additional clinical data should provide insight regarding A1669S and will serve as measurement of the prediction provided by the assay. Interestingly, pedigree analysis seemed to indicate that the R1699W is a cancer-predisposing allele (Fig. 2, Lund 279). Disease association is further emphasized by other findings where R1699W was found in a large pedigree in which several women, through three generations and across four degrees of relatedness, diagnosed with ovarian cancer carried the variant (T.S. Frank and J. Scalia, in preparation). Disease association is less clear for R1699Q, found in a patient diagnosed with breast cancer at the age of 39 but without familial history of disease. Others found this variant in an unaffected individual, whose mother was diagnosed with premenopausal breast cancer and considered to be an obligate carrier of the R1699Q variant and whose grandmother was diagnosed with ovarian cancer at the age of ~60 years but without known mutation status (T.S. Frank, personal communication). The apparent discrepancy between the family and functional data prompted further examination of the R1699 variants.

Variant W1837X results in a truncated protein lacking the last 27 residues and displayed loss of activity. Smaller truncations (11 residues) have been linked to disease (2) and shown to confer loss of function in transcription and in small colony phenotype assays (18,27). Our result is in agreement with pedigree analysis in which the mutation segregates with the disease (Fig. 2, Lund 190).

Variants C1697R, A1708E, S1715R and G1738E displayed loss of activity, suggesting that they represent disease-associated

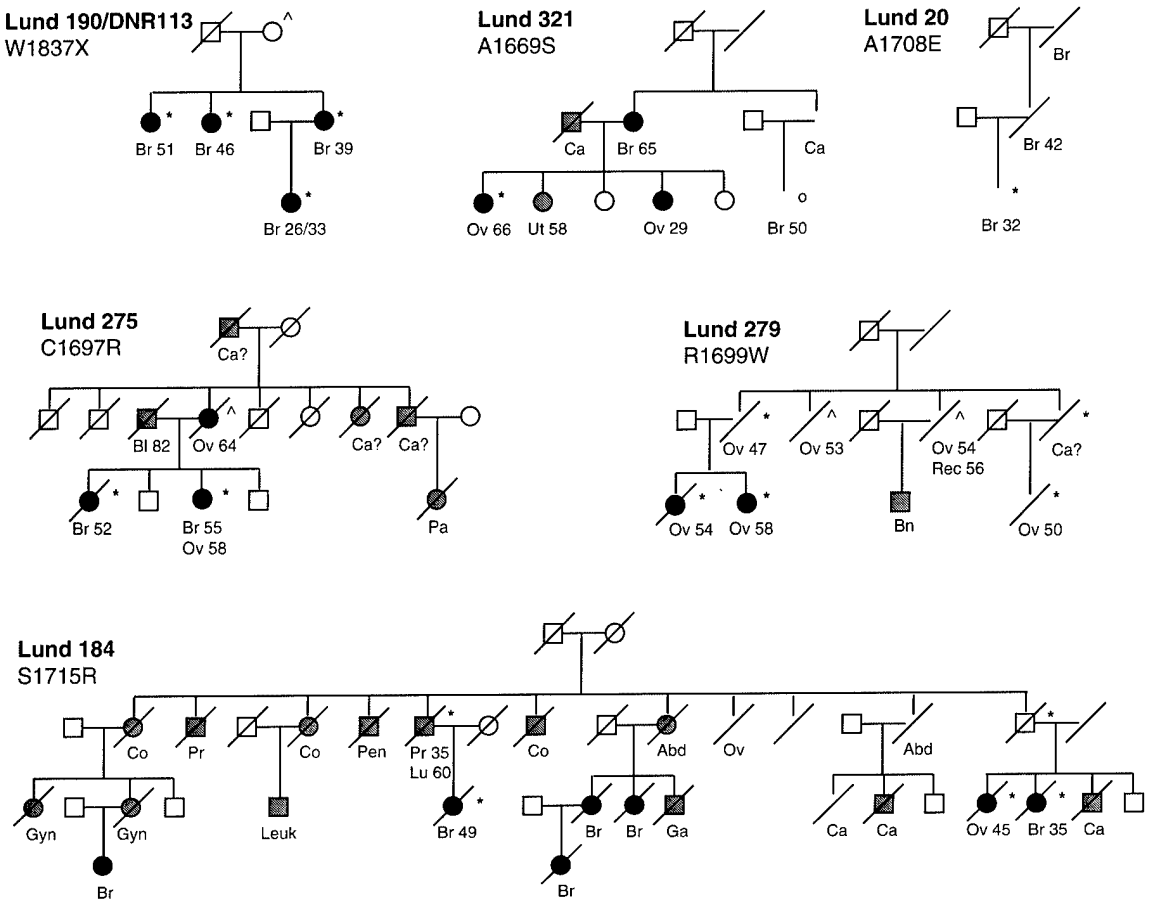

Figure 2. Scandinavian breast and breast–ovarian cancer families with germline *BRCA1* C-terminal missense or truncating mutations. Cancer types, age at diagnosis and mutation status are shown. *, mutation; °, confirmed from blood sample not to carry mutation; ^, determined from paraffin embedded tumor tissue not to carry mutation. Cancer type: Br, breast; Ov, ovary; Ut, uterus; Gyn, gynecological; Pr, prostate; Co, colon; Rec, rectal; Leuk, leukemia; Lu, lung; Bn, brain; Bl, bladder; Pa, pancreas; Ga, gastric; Pen, Penile; Abd, abdominal; Ca, cancer of unknown type; Ca?, possibly affected.

mutations (Table 1), an observation in agreement with pedigree analysis for mutations C1697R, A1708E and S1715R (Fig. 2). The amino acid substitution C1697R is a rather dramatic one, from a non-polar residue capable of forming disulfide linkages to a positively charged residue, located in a critical α-helix based on the structure of XRCC1 BRCT (29). Furthermore, the residue in question is strictly conserved in other *BRCA1* homologs (Table 1) (30,31). In addition to family Lund 275, in which it segregates with the disease, the C1697R variant has been found in three other breast cancer patients. One case had multicentric disease at age 35 and a family history of breast cancer (sister and mother), whereas the other two cases had bilateral disease at ages 41 and 44 and mothers with breast/skin cancer and cancer of unknown origin, respectively (J.T. Bergthorsson *et al.*, unpublished data). Thus, combined clinical data indicate association between the variant and breast cancer. Variant A1708E has been reported to the BIC database 14 times, including our finding in Lund 20. It has been previously shown to cause loss of function in different assays (18,19,27) and the presence of A1708E in Lund 20 further demonstrates the variant as a disease-associated mutation. S1715 is an evolutionarily conserved residue. However, the disease pattern in Lund 184 (harboring an S1715R substitution; variant S1715N was also analyzed) is not satisfactorily explained by a mutation in *BRCA1* alone because it presents an uncharacteristic phenotype. Multiple cases of colon cancer might suggest the involvement of a mismatch repair gene defect. However, co-segregation between the mutation and breast and ovarian cancer is observed and these cancer forms are predominant among women in the pedigree (Fig. 2). We recently found the G1738E variant, which displayed loss of transactivation activity in our assays, in a young patient affected with bilateral breast cancer and a family history of disease. In addition, others found the variant in a family with a strong pattern of hereditary disease in which the patient carrying the alteration suffered from breast cancer at an early age (T.S. Frank, personal communication). These findings strengthen the correlation between disease predisposition and predictions made by the transcription assay (24).

Analysis of variants in the BIC database

Variants V1665M, D1692N and D1733G displayed wild-type activity, suggesting that they represent benign polymorphisms

(Table 1). The V1665M variant affects a residue close to A1669, in the predicted BRCT conformation (29), which also displayed wild-type activity (Table 1 and Fig. 3), suggesting that this small stretch is tolerant to mutations. Variant D1692N affects the residue predicted to form a salt bridge with S1715, thereby stabilizing the interactions between BRCT α2 and α4 regions (29). However, D1692N displayed wild-type transactivation activity, suggesting that the predicted salt bridge is not important for the transactivation ability of BRCA1 (Table 1 and Fig. 3). The D1733G variant is a conserved acidic residue located in the BRCT-N/BRCT-C interval. However, more information is still needed for a reliable characterization of this variant.

Variants V1688del and S1715N displayed loss of activity, suggesting that they represent cancer-associated mutations (Table 1). Alteration V1688del is an in-frame deletion of a conserved hydrophobic residue predicted to be part of β3 in the BRCT-N domain (29). Previous mutation analysis has underscored the importance of hydrophobic residues for the function of BRCA1 (24). Similar to S1715R (Lund 184), the substitution S1715N (BIC) resulted in loss of activity in the assay.

Fusion protein and promoter stringency do not influence assay outcome

To rule out the possibility that the results obtained with the GAL4 DBD fusions were dependent on the DBD, we also performed the experiments using fusions to LexA DBD in the *S.cerevisiae* strain EGY48 (24). A fusion of wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and two mutants defined by genetic linkage as disease-associated, M1775R and Y1853X, were used as negative controls (1,2). Results from the LexA-based and GAL4-based assays were comparable (Table 1).

The reporter genes used in the yeast experiments contain multiple binding sites in their promoters (eight for LexA; four for GAL4), raising the possibility that variants with partial loss of function could score as wild-type in the semi-quantitative filter β-galactosidase assay. This could be particularly important in the case of the R1699W variant for which we found a contradiction between the family data and transcription activity. Therefore, EGY48 experiments with the R1699W variant were performed with the *LacZ* reporter under the control of one, two or eight LexA operators (32). In all cases, R1699W was indistinguishable from the wild-type allele (data not shown).

Quantitative assessment of transcription activation

Despite the fact that we saw no difference that could be attributed to promoter stringency, it was still possible that variants with partial loss of activity could be differentiated only using quantitative liquid β-galactosidase assay. However, results were comparable to the semi-quantitative assays (Fig. 3A and Table 1). Interestingly, R1699W was ~2-fold more active than the wild-type control. In conclusion, the contradiction found for variant R1699W was not due to a partial loss of function indistinguishable from the wild-type in semi-quantitative assays.

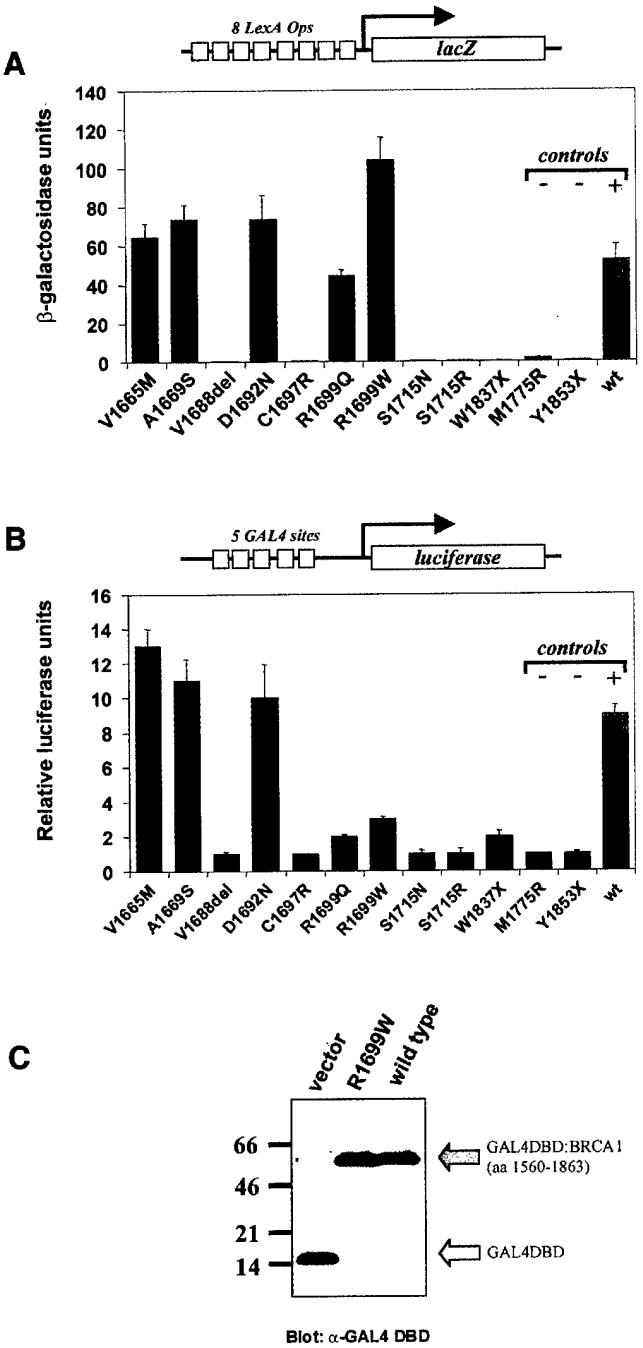

Figure 3. Transcriptional activity of BRCA1 variants. (**A**) Activity in yeast cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (**B**) Activity in human cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (**C**) Mutant R1699W is expressed at the same level as wild-type (gray arrow). The white arrow indicates expression of the GAL4 DBD moiety in the absence of any fusion fragment.

Analysis in mammalian cells

To further examine the transcription activity of the variants we performed assays in mammalian cells. With the exception of variants R1699W and R1699Q, transcription activation was comparable between yeast and mammalian cells (Fig. 3A and B). In 293T cells, variants R1699W and R1699Q displayed loss of function phenotype in accordance with pedigree analysis, suggesting that these variants are indeed cancer-associated mutations. Protein levels of R1699W and wild-type were similar, ruling out increased instability of the protein as the cause for the loss-of-function phenotype (Fig. 3C).

DISCUSSION

The notion that cancer-predisposing mutations in tumor-suppressor genes cause a loss-of-function phenotype is a key concept in cancer genetics. Here we utilized a functional assay to characterize clinically relevant *BRCA1* variants. Our rationale was that transactivation activity of BRCA1 might mirror a functionally important feature of the protein *in vivo* and form the basis for a functional assay. Several lines of evidence have called attention to BRCA1 as a transcription regulator and it has been demonstrated that disease-associated mutations abolish the transactivation by BRCA1 in different experimental systems (for a review see ref. 9). Importantly, BRCA1 alleles carrying benign polymorphisms retain wild-type activity (24,26). Thus, relevant functional information might be gained from characterizing the effect of *BRCA1* mutations on transcription activation. In addition, development of a functional assay for BRCA1 will fill a gap within the field directed at providing risk assessment information for counseling. The main difference between the present and past studies (24,25) is that this study is combined with pedigree and segregation analysis, providing a background to validate the results.

As demonstrated by our results in Table 1, the effect of an introduced *BRCA1* mutation on transcription activation in the yeast-based assay is not affected by the DBD of the fusion protein or the promoter context of the reporter gene. Problems in interpreting results might nevertheless arise when characterizing variants that do not affect protein function in yeast. This is exemplified by the R1699 variants (Table 1). Although the clinical data indicate that R1699W is likely to predispose carriers to ovarian cancer (Fig. 2), our yeast-based tests revealed a wild-type activity, an apparent divergence between disease predisposition *in vivo* and the transcription activation assay (Table 1). This disagreement could not be explained by vector background or by differences in promoter stringency. However, we found that in the mammalian cell-based assay, transactivation activity of the R1699 variants was reduced in a fashion comparable to the negative controls. In fact, all variants presented here, with the exception of R1699W and R1699Q, behave similarly in the yeast- and mammalian-based assays. Thus, it is possible that specific protein alterations that have an effect on *in vivo* phenotype remain undetected in the simplified yeast model. We are currently investigating the reasons for this difference. Consequently, at this time we cannot unambiguously characterize variants that do not disrupt transcription activation in yeast as benign polymorphisms. Using a mammalian-based assay to supplement results from the yeast assay might provide the scrutiny necessary to exclude or confirm disease predisposition of a certain variant. Similarly, mutations that affect mRNA processing *in vivo* might also be erroneously scored as a benign polymorphism because our assay is based on expression from an artificial cDNA. This could be the case for variant D1692N because the alteration affects a conserved guanine at a splice donor site and its potential effects on mRNA have not been examined. Conceivably, false negative results (i.e. benign polymorphisms that behave as loss-of-function mutants) can also occur when a particular variant causes message or protein instability in yeast. By extending our analysis using mammalian cells we should be able to distinguish those variants.

Considering the excellent correspondence between genetic alterations associated with breast and ovarian cancer in families and those that abolish transactivation, we tentatively characterized several additional *BRCA1* unclassified variants. We propose that variants V1665M, D1692N and D1733G represent benign polymorphisms and variants V1668del and S1715N represent disease-associated mutations. Final characterization of these variants must await independent confirmation.

Our findings, taken together with previously published data (18,24,26), demonstrate a correlation between loss of transactivation activity and disease predisposition and it will be interesting to see whether future data will corroborate the predictions made here. Our results indicate that yeast-based assays can aid in the characterization of deleterious mutations in the C-terminal part of *BRCA1* but it may be unable to unambiguously characterize benign polymorphisms. This is exemplified by mutations at residue R1699, for which we report a discrepancy in effect on transcription between yeast and mammalian cells. Thus, our study underlines the importance of analyzing the effect of putative disease-causing mutations in mammalian-based assays and taking into account data from population-based studies. In summary, we show that transcription activation may reflect the tumor-suppressing function of BRCA1 and provide further support for the role of missense mutations in disease predisposition.

MATERIALS AND METHODS

Yeast strains

Three *S.cerevisiae* strains were used: HF7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)3-CYC1-lacZ) (33); SFY526 (MATa, ura3-52, his 3-200, ade 2-101, lys 2-801, trp 1-901, leu 2-3, 112, canr, gal4-542, gal80-538, URA3::GAL1-lacZ (34); and EGY48 (MATa, ura3, trp1, his3, 6 lexA operator-LEU2) (35). HF7c and SFY526 contain reporter genes under the control of *GAL1* UAS, which is recognized by GAL4 DBD. When activated, the reporter gene in SFY526 will produce β-galactosidase and HF7c will grow in minimal medium lacking histidine. EGY48 cells were transformed with plasmid reporters under control of LexA operators (pSH18-34, pJK103 or pRB1840) that produce β-galactosidase when activated (35).

Yeast expression constructs

A fusion construct containing GAL4 DBD:BRCA1 (amino acids 1560–1863) in pGBT9 (Clontech) used as a wild-type control and as a backbone to introduce mutations was described by Monteiro *et al.* (18). Specific alterations in *BRCA1* were introduced by Quick-change site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. In short, primers containing the alteration were used in a PCR reaction to copy wild-type constructs produced in a methylation-competent bacterial strain and amplification was performed using *Pfu* polymerase. *Dpn*I was subsequently added to digest the parental plasmid, leaving only cDNAs with introduced mutations to be transformed into bacteria. Confirmation of the introduced mutations was obtained by direct sequencing of the *BRCA1* (amino acids 1560–1863) insert using two primers: GAL4 DNA-BD, 5′-TCATCGGAA-GAGAGTAG-3′ (17-mer) (Clontech), and pGBT9 M13 REV, 5′-TGTAAAACGACGGCCCGTTTTAAAACCTAAGAGT-CAC-3′. For experiments in EGY48, BRCA1 inserts with mutations were subcloned into pLex9 (35) in-frame with the DBD of LexA. Both pGBT9 and pLex9 have *TRP1* as a selectable marker, allowing growth in medium lacking tryptophan.

Yeast transformation

Transformations were performed using the yeast transformation system based on lithium acetate (Clontech). Briefly, a single colony was inoculated in YPD medium for 16–18 h to produce a saturated culture. Cells were transferred to fresh medium and grown for 3 h, centrifuged, washed, resuspended in TE/LiAc (10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution and used immediately for transformation. Competent cells were incubated in polyethylene glycol (PEG)/LiAc (40% PEG 4000, 10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution at 30°C for 30 min with appropriate vector and carrier DNA. DMSO was added to 10% final concentration and the mix was heat shocked at 42°C for 15 min. Cells were subsequently chilled, centrifuged and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Cells were plated on synthetic dropout medium (SDM) and incubated at 30°C to select for transformants.

Yeast growth assay

Thirty-six individual HF7c clones for each variant were streaked on solid SDM lacking tryptophan and on SDM lacking both tryptophan and histidine and growth was scored after 2 days. A positive (+) or a negative (–) score was noted if growth was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively.

β-galactosidase assays

Thirty-six individual SFY526 clones and at least six individual EGY48 clones for every variant were streaked on filter paper overlaid on solid SDM lacking tryptophan (or tryptophan and uracil for EGY48). Plates were incubated for 2 days (SFY526) or 24 h (EGY48) and cells growing on the filter paper were lysed by freeze–thawing in liquid nitrogen and assayed for β-galactosidase activity in 2.5 ml of Z buffer (16 g/l $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/l $NaH_2PO_4 \cdot H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4 \cdot 7H_2O$, pH 7.0) containing 40 µl of X-gal solution (20 mg/ml in *N,N*-dimethylformamide) and 6.6 µl of β-mercapto-ethanol. For SFY526, a positive (+) or a negative (–) score was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively. For EGY48 a positive score (+) was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was identical to the negative controls (M1775R and Y1853X). Clones were scored 6 h (SFY526) or 2 h (EGY48) after addition of X-gal. Liquid assays were performed as described by Brent and Ptashne (36). At least three separate transformants were assayed and each was performed in triplicate.

Transcription assay in mammalian cells

GAL4 DBD:BRCA1 fusions were subcloned into pCDNA3 (Invitrogen). We used pG5Luc, which contains a firefly luciferase gene under the control of five GAL4 binding sites (37). Transfections were normalized with an internal control, pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter using a dual luciferase system (Promega). Human 293T cells were cultured in DMEM supplemented with 10% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were performed in triplicates using Fugene 6 (Roche) and harvested 24 h post-transfection.

Western blot

Cells were lysed in RIPA (150 mM NaCl, 10 mM Tris–Cl pH 7.4, 5mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% sodium deoxycholate), boiled in sample buffer and separated on a 10% SDS–PAGE. Gels were electroblotted on a wet apparatus to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked overnight with 5% skim milk using TBS–Tween, and incubated with α-GAL4 DBD monoclonal antibody (Clontech) using 0.5% bovine serum albumin in TBS–Tween. The blots were subsequently incubated with the α-mouse IgG–horseradish peroxidase conjugate in 1% skim milk in TBS–Tween and developed using an enhanced chemiluminescent reagent (NEN).

Electronic database information

Online Mendelian Inheritance in Man (OMIM) is available at http://www.ncbi.nlm.nih.gov/omim. The Breast Cancer Information Core (BIC) is an online database of mutations in breast cancer susceptibility genes hosted by the National Human Genome Research Institute and can be accessed at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

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REFERENCES

1. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W. *et al.* (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, **266**, 66–71.
2. Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E. and King, M.C. (1994) Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genet.*, **8**, 399–404.
3. Ford, D., Easton, D.F. and Peto, J. (1995) Estimates of the gene frequency of *BRCA1* and its contribution to breast and ovarian cancer incidence. *Am. J. Hum. Genet.*, **57**, 1457–1462.
4. Koonin, E.V., Altschul, S.F. and Bork, P. (1996) BRCA1 protein products. Functional motifs. *Nature Genet.*, **13**, 266–268.
5. Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F. and Koonin, E.V. (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *FASEB J.*, **11**, 68–76.
6. Callebaut, I. and Mornon, J.P. (1997) From *BRCA1* to *RAP1*: a widespread BRCT module closely associated with DNA repair. *FEBS Lett.*, **400**, 25–30.
7. Smith, S.A., Easton, D.F., Evans, D.G. and Ponder, B.A. (1992) Allele losses in the region 17q12–21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nature Genet.*, **2**, 128–131.
8. Welcsh, P.L., Owens, K.N. and King, M.C. (2000) Insights into the functions of *BRCA1* and *BRCA2*. *Trends Genet.*, **16**, 69–74.
9. Monteiro, A.N. (2000) *BRCA1*: exploring the links to transcription. *Trends Biochem. Sci.*, **25**, 469–474.
10. Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M. and Scully, R. (1998) Stable interaction between the products of the *BRCA1* and *BRCA2* tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell*, **2**, 317–328.
11. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T. and Livingston, D.M. (1997) Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell*, **88**, 265–275.
12. Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P.L., Sharp, Z.D. and Lee, W.H. (1999) Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science*, **285**, 747–750.
13. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J. and Qin, J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.*, **14**, 927–939.
14. Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. (1997) Dynamic changes of *BRCA1* subnuclear location and phosphorylation state are initiated by DNA damage. *Cell*, **90**, 425–435.
15. Thomas, J.E., Smith, M., Tonkinson, J.L., Rubinfeld, B. and Polakis, P. (1997) Induction of phosphorylation on *BRCA1* during the cell cycle and after DNA damage. *Cell Growth Differ.*, **8**, 801–809.
16. Gowen, L.C., Avrutskaya, A.V., Latour, A.M., Koller, B.H. and Leadon, S.A. (1998) BRCA1 required for transcription-coupled repair of oxidative DNA damage. *Science*, **281**, 1009–1012.
17. Shen, S.X., Weaver, Z., Xu, X., Li, C., Weinstein, M., Chen, L., Guan, X.Y., Ried, T. and Deng, C.X. (1998) A targeted disruption of the murine *Brca1* gene causes gamma-irradiation hypersensitivity and genetic instability. *Oncogene*, **17**, 3115–3124.
18. Monteiro, A.N., August, A. and Hanafusa, H. (1996) Evidence for a transcriptional activation function of *BRCA1* C-terminal region. *Proc. Natl Acad. Sci. USA*, **93**, 13595–13599.
19. Chapman, M.S. and Verma, I.M. (1996) Transcriptional activation by *BRCA1*. *Nature*, **382**, 678–679.
20. Scully, R., Anderson, S.F., Chao, D.M., Wei, W., Ye, L., Young, R.A., Livingston, D.M. and Parvin, J.D. (1997) *BRCA1* is a component of the RNA polymerase II holoenzyme. *Proc. Natl Acad. Sci. USA*, **94**, 5605–5610.
21. Anderson, S.F., Schlegel, B.P., Nakajima, T., Wolpin, E.S. and Parvin, J.D. (1998) BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nature Genet.*, **19**, 254–256.
22. Schlegel, B.P., Green, V.J., Ladias, J.A. and Parvin, J.D. (2000) BRCA1 interaction with RNA polymerase II reveals a role for hRPB2 and hRPB10alpha in activated transcription. *Proc. Natl Acad. Sci. USA*, **97**, 3148–3153.
23. Monteiro, A.N. and Humphrey, J.S. (1998) Yeast-based assays for detection and characterization of mutations in *BRCA1*. *Breast Dis.*, **10**, 61–70.
24. Hayes, F., Cayanan, C., Barilla, D. and Monteiro, A.N. (2000) Functional assay for *BRCA1*: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation. *Cancer Res.*, **60**, 2411–2418.
25. Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M. and Livingston, D.M. (1999) Genetic analysis of *BRCA1* function in a defined tumor cell line. *Mol. Cell*, **4**, 1093–1099.
26. Monteiro, A.N., August, A. and Hanafusa, H. (1997) Common *BRCA1* variants and transcriptional activation. *Am. J. Hum. Genet.*, **61**, 761–762.
27. Humphrey, J.S., Salim, A., Erdos, M.R., Collins, F.S., Brody, L.C. and Klausner, R.D. (1997) Human BRCA1 inhibits growth in yeast: potential use in diagnostic testing. *Proc. Natl Acad. Sci. USA*, **94**, 5820–5825.
28. Hakansson, S., Johannsson, O., Johansson, U., Sellberg, G., Loman, N., Gerdes, A.M., Holmberg, E., Dahl, N., Pandis, N., Kristoffersson, U. *et al.* (1997) Moderate frequency of *BRCA1* and *BRCA2* germ-line mutations in Scandinavian familial breast cancer. *Am. J. Hum. Genet.*, **60**, 1068–1078.
29. Zhang, X., Morera, S., Bates, P.A., Whitehead, P.C., Coffer, A.I., Hainbucher, K., Nash, R.A., Sternberg, M.J., Lindahl, T. and Freemont, P.S. (1998) Structure of an XRCC1 BRCT domain: a new protein–protein interaction module. *EMBO J.*, **17**, 6404–6411.
30. Bennett, L.M., Brownlee, H.A., Hagavik, S. and Wiseman, R.W. (1999) Sequence analysis of the rat *Brca1* homolog and its promoter region. *Mamm. Genome*, **10**, 19–25.
31. Szabo, C.I., Wagner, L.A., Francisco, L.V., Roach, J.C., Argonza, R., King, M.C. and Ostrander, E.A. (1996) Human, canine and murine *BRCA1* genes: sequence comparison among species. *Hum. Mol. Genet.*, **5**, 1289–1298.
32. Estojak, J., Brent, R. and Golemis, E.A. (1995) Correlation of two-hybrid affinity data with *in vitro* measurements. *Mol. Cell. Biol.*, **15**, 5820–5829.
33. Feilotter, H.E., Hannon, G.J., Ruddell, C.J. and Beach, D. (1994) Construction of an improved host strain for two hybrid screening. *Nucleic Acids Res.*, **22**, 1502–1503.
34. Bartel, P., Chien, C.T., Sternglanz, R. and Fields, S. (1993) Elimination of false positives that arise in using the two-hybrid system. *Biotechniques*, **14**, 920–924.
35. Golemis, E.A., Gyuris, J. and Brent, R. (1994) Two-hybrid system/interaction traps. In Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J.A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, NY, pp. 13.14.1–13.14.17.
36. Brent, R. and Ptashne, M. (1985) A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell*, **43**, 729–736.
37. Seth, A., Gonzalez, F.A., Gupta, S., Raden, D.L. and Davis, R.J. (1992) Signal transduction within the nucleus by mitogen-activated protein kinase. *J. Biol. Chem.*, **267**, 24796–24804.

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BRCA1 can stimulate gene transcription by a unique mechanism

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Most familial breast and ovarian cancers have been linked to mutations in the *BRCA1* gene. BRCA1 has been shown to affect gene transcription but how it does so remains elusive. Here we show that BRCA1 can stimulate transcription without the requirement for a DNA-tethering function in mammalian and yeast cells. Furthermore, the BRCA1 C-terminal region can stimulate transcription of the p53-responsive promoter, *MDM2*. Unlike many enhancer-specific activators, non-tethered BRCA1 does not require a functional TATA element to stimulate transcription. Our results suggest that BRCA1 can enhance transcription by a function additional to recruiting the transcriptional machinery to a targeted gene.

INTRODUCTION

BRCA1 mutations are thought to account for ~45% of families with high breast cancer risk and >80% of families with high risk of early-onset breast and ovarian cancer (Easton *et al.*, 1993). The human *BRCA1* gene encodes a 1863 amino acid nuclear protein that has been implicated in DNA repair and transcription activation (see Monteiro, 2000; Welcsh *et al.*, 2000). A role for BRCA1 in gene transcription has been proposed mainly for the following reasons: (i) the C-terminal portion of BRCA1, which bears an excess of negatively charged residues, can activate transcription of a target gene when attached to a DNA-binding domain (DBD) *in vivo* (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Anderson *et al.*, 1998) and *in vitro* using a highly purified system (Haile and Parvin, 1999); (ii) it associates with a form of RNA polymerase II holoenzyme (Scully *et al.*, 1997); and (iii) it modulates the activity of certain transcriptional activators (Somasundaram *et al.*, 1997; Ouchi *et al.*, 1998, 2000; Wang *et al.*, 1998). It is noteworthy that the above mentioned work implies BRCA1 as a physiological regulator of p53-dependent genes such as $p21^{WAF}$ and *MDM2*.

Here we show that BRCA1 can stimulate gene transcription of a variety of reporter gene constructs without the requirement for a specific DBD in human and in yeast cells both *in vivo* and *in vitro*. We further show that the BRCA1 C-terminal region can increase transcription of a reporter gene independently of a functional TATA element.

RESULTS

BRCA1 stimulates transcription without requiring a specific DBD in human cells

While studying the effect of the BRCA1 C-terminal region on gene transcription, we noticed that expression of a protein fragment (aa 1528–1863) could stimulate gene transcription in transiently transfected human cells. We thus hypothesized that perhaps the activation elicited by a DNA-tethered BRCA1 chimera (e.g. Gal4–BRCA1) might not be solely dependent on a heterologous DBD. To test this hypothesis, we transfected human HCC1937 $BRCA1^{-/-}$ breast cancer cells (Tomlinson *et al.*, 1998) with a vector overexpressing Gal4–BRCA1 (aa 1528–1863) or the BRCA1 fragment without the Gal4 DBD. As previously reported (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Anderson *et al.*, 1998), the Gal4–BRCA1 fusion activated transcription from a reporter template bearing four Gal4-binding sites upstream of the c-*fos* minimal promoter fused to the luciferase gene (Figure 1A). Interestingly, the BRCA1 fragment, without a Gal4 DBD, was also able to enhance the level of transcription several-fold in a dose-dependent fashion. To determine

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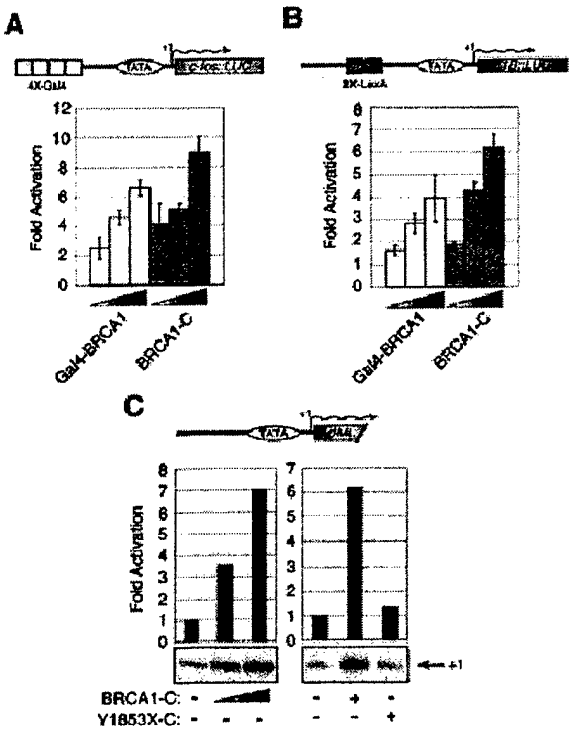

Fig. 1. BRCA1 does not require specific DNA binding to enhance gene transcription. (**A**) Activation by Gal4–BRCA1(1528–1863) and BRCA1(1528–1863) at a reporter template bearing Gal4 sites in HCC1937 cells. Transfected cells were assayed for luciferase activity using a reporter template (0.1 μg) bearing four Gal4-binding sites upstream of the c-*fos* TATA element. Increasing amounts (up to 1.0 μg DNA) of either Gal4–BRCA1(1528–1863) or BRCA1-C(1528–1863) constructs were co-transfected along with the reporter. Activation for BRCA1 and Gal4–BRCA1 is represented as fold increase over activity obtained with the pcDNA3 vector and the Gal4 DBD, respectively. (**B**) Activation by Gal4–BRCA1 and BRCA1-C at a reporter template lacking Gal4 sites. Transfected HCC1937 cells were assayed for luciferase activity using 0.1 μg of a reporter bearing two LexA sites upstream of the *E1B* TATA element. The BRCA1 plasmid constructs were transiently transfected as in (A) and activation is represented as the fold increase over activity obtained with the pcDNA3 vector for both constructs. (**C**) BRCA1 can stimulate gene transcription *in vitro*. *In vitro* transcription reactions were carried out with a HeLa nuclear extract and an *AdML* template. Primer extensions were carried out to measure the extent of activation. Recombinant BRCA1(1528–1863) was added at 100 and 400 ng (left panel, lanes 2 and 3, respectively). The right panel shows that a recombinant Y1853X-bearing mutant (200 ng, lane 3) does not significantly stimulate transcription as compared with the wild-type protein fragment (200 ng, lane 2).

whether the effect could be specific to the promoter context of the template, we made use of a promoter template bearing two LexA-binding sites in place of the Gal4 sites, and the *E1B* core promoter in place of the c-*fos* promoter. The results show that even though both the Gal4–BRCA1 fusion and BRCA1 are not expected to bind the promoter template, they are both able to stimulate transcription when overexpressed in the cell (Figure 1B). These results would be expected for an activator that does not require DNA binding to exert its function.

Next, we tested whether the BRCA1 C-terminal fragment could stimulate transcription *in vitro* without the requirement for a DBD. Using a HeLa nuclear extract, we tested the activation potential of recombinant BRCA1(1528–1863) *in vitro* using a DNA template bearing the *AdML* core promoter. We found that the recombinant BRCA1 fragment could stimulate transcription up to 7-fold (Figure 1C), a result consistent with those of our transient transfection experiments. A similar BRCA1 fragment bearing the cancer-associated Y1853X mutation, which results in deletion of the last 11 amino acids (Friedman *et al.*, 1994), had no significant stimulatory effect on transcription (Figure 1C, right panel, lane 3).

Transcription stimulation of *MDM2* by BRCA1 derivatives

Previous reports have suggested that p53-responsive genes were targets of BRCA1-mediated transcription enhancement. Moreover, p53 has been shown to interact physically with residues 224–500 of BRCA1 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). Although physical interaction between these two molecules surely contributes in recruiting BRCA1 to p53-responsive genes, it is conceivable that proper overexpression of the BRCA1 activation-effector region would also result in stimulation of the target gene. We thus wanted to test whether the BRCA1 C-terminal region was sufficient, as compared with the full-length molecule, to stimulate transcription of an *MDM2-luciferase* reporter template (Ouchi *et al.*, 1998). To test this, we transfected HCC1937 cells with vectors expressing C-terminal and full-length BRCA1 derivatives (wild-type and Y1853X mutants), and luciferase activity was analyzed. The results shown in Figure 2 demonstrate that the C-terminal BRCA1 fragment, as well as full-length BRCA1, can efficiently induce transcription elicited at the *MDM2* promoter while the Y1853X mutants did not stimulate the reporter as efficiently. These results suggest that overexpression of BRCA1 can bypass the requirement for a p53–BRCA1 interaction in order to stimulate a responsive gene.

The BRCA1 C-terminal region stimulates transcription of a TATA-mutated promoter

In an effort to discriminate further the mechanism of action of BRCA1, as compared with enhancer-binding activators, we set out to test whether BRCA1 required a functional TATA element to stimulate transcription. Hence, we determined the ability of BRCA1 to activate transcription at a reporter template that has a mutation in the TATA element (TGTA) (Figure 3A). This mutation prevents or severely reduces TBP binding to the core promoter, nearly abolishing the ability of a classical activator to activate transcription in certain contexts (Bryant *et al.*, 1996). Thus, the reporter templates used in this experiment have either a wild-type c-*fos* TATA or a mutated TGTA element both with Gal4-binding sites upstream and the *luciferase* reporter gene downstream of the transcription initiation site (Bryant *et al.*, 1996). While the acidic activators Gal4–VP16 and Gal4–E2F1 are able to activate transcription efficiently at the TATA template, both activators are severely crippled in their ability to stimulate transcription at the TGTA template. Surprisingly, BRCA1(1528–1863) was able to stimulate transcription even more efficiently at

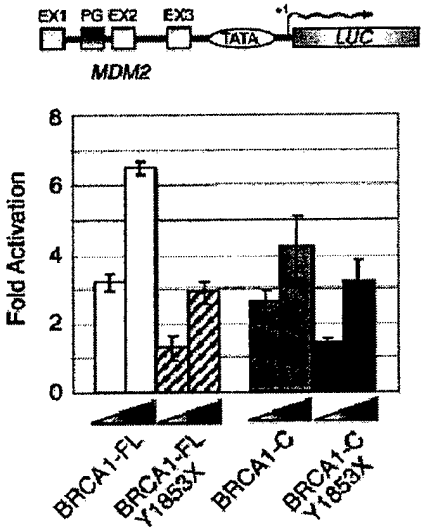

Fig. 2. Transcription stimulation of the *MDM2* promoter by BRCA1 derivatives. HCC1937 cells were transfected with various BRCA1 derivatives (using either 0.5 or 1.0 μg of DNA) and 25 ng of an *MDM2-luciferase* template (Ouchi *et al.*, 1998). Exons 1–3 (EX1–EX3) and p53-responsive elements (PG) are indicated. The vector control denotes pcDNA3; BRCA1-C denotes BRCA1(1528–1863); BRCA1-C Y1853X is the BRCA1 C-terminal region with the Y1853X mutation; and BRCA1-FL are the full-length molecules. Activation was measured as in Figure 1A.

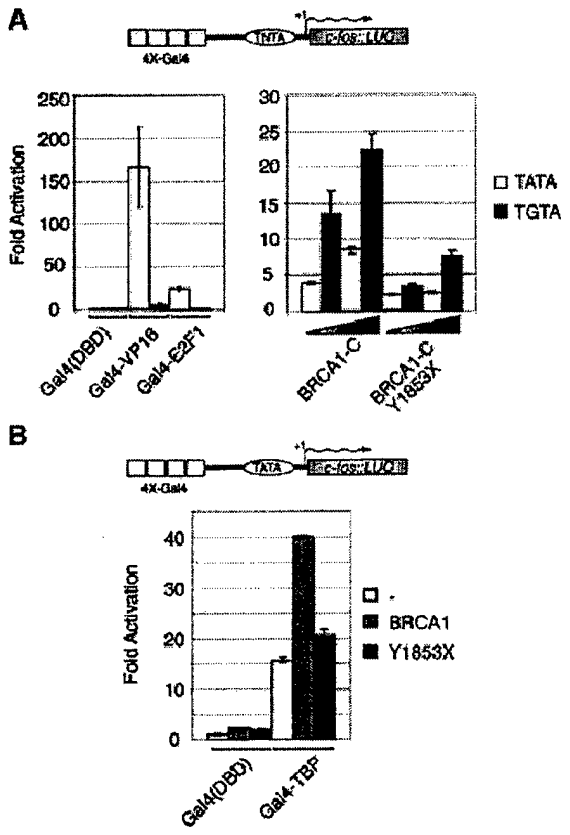

Fig. 3. BRCA1 stimulates transcription independently of TBP binding. (**A**) BRCA1 can efficiently stimulate transcription in the absence of a functional TATA element. HCC1937 cells were transfected with either a reporter template bearing a functional TATA element or one with a mutated TATA (TGTA) but otherwise identical. The left part of the figure measures the ability of the Gal4 DBD, Gal4–VP16 and Gal4–E2F1 to activate transcription at both templates. The right part of the figure measures the ability of various BRCA1 derivatives (using either 0.5 or 1.0 μg of DNA) to stimulate both reporters. The vector control denotes pcDNA3; BRCA1-C denotes BRCA1(1528–1863); BRCA1-C Y1853X is the BRCA1 C-terminal region with the Y1853X mutation. Activation was measured as in Figure 1A and B. (**B**) The BRCA1 C-terminal region stimulates activation elicited by Gal4–TBP. Transiently transfected HEK-293 cells were assayed for luciferase activity using the reporter template of Figure 1A. Either the Gal4 DBD (1–147) (0.2 μg) or Gal4–TBP (0.2 μg) was used in the experiment with or without 0.4 μg of either BRCA1-C derivative (wild-type or Y1853X mutant).

the TGTA template as compared with the TATA template (Figure 3A, right panel). Introduction of a Y1853X mutation markedly impaired stimulation of transcription.

We next wanted to test whether the BRCA1 C-terminal region could further enhance transcription of a gene activated by artificially recruited TBP. Figure 3B shows that TBP, when fused to the Gal4 DBD, can activate transcription some 15-fold in human HEK-293 cells. When BRCA1(1528–1863) is transfected along with Gal4–TBP, we see an ~3-fold increase in activation by Gal4–TBP. Transfection of the Y1853X BRCA1 mutant did not enhance transcription of Gal4–TBP nearly as efficiently as its wild-type counterpart. These results suggest that BRCA1 can stimulate transcription above levels elicited by artificial recruitment of TBP to the TATA box. We cannot, however, exclude the possibility that BRCA1 further enhances TBP binding to the promoter beyond the recruiting effect of the Gal4 DBD.

BRCA1 stimulates transcription without requiring a specific DBD in yeast cells

To determine whether the effect of BRCA1 on transcription was limited to mammalian cells, we performed experiments in yeast. Figure 4A shows that, when expressed in yeast cells, full-length BRCA1 is able to stimulate transcription (bar 2) of a *GAL1::lacZ* reporter template efficiently. Expression of the Y1853X mutant (Figure 4A, bar 4) in yeast failed to activate transcription, while the C61G mutant (bar 3), also a clinically relevant mutation but located at the N-terminus of BRCA1 (Friedman *et al.*, 1994), could elicit levels of activation comparable to the wild-type protein. The N-terminal region of BRCA1 seems dispensable for this function, confirming our data from mammalian cells where the C-terminal alone is sufficient for activation. Figure 4B shows that recombinant BRCA1(1528–1863) can stimulate transcription of the *GAL1* promoter *in vitro* using a yeast nuclear extract preparation (Wu *et al.*, 1996). The figure also shows that, as in Figure 1C, BRCA1 can stimulate transcription in a dose-dependent fashion. The Y1853X mutant had no stimulatory effect (Figure 4B, right panel, lane 3).

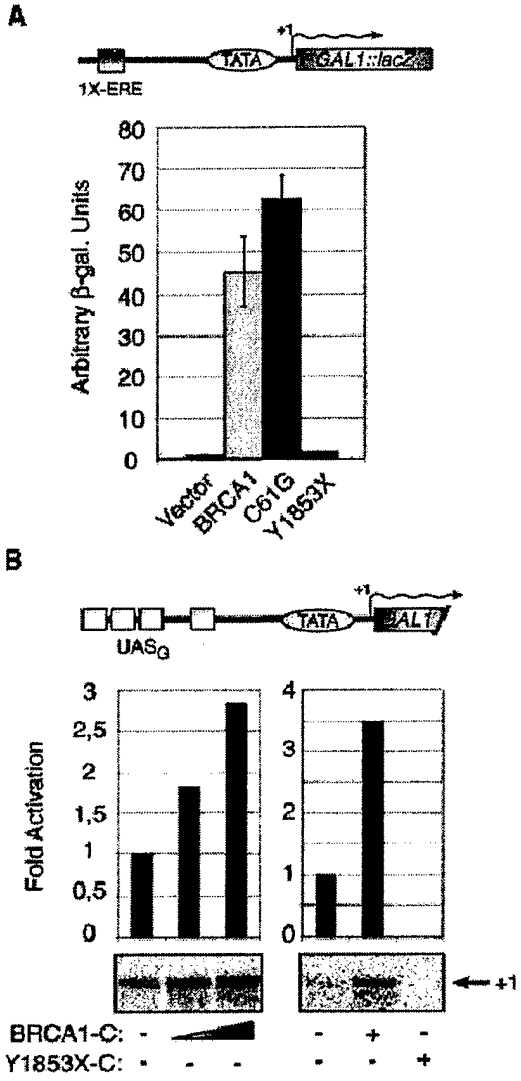

Fig. 4. BRCA1 can stimulate gene transcription in yeast without the requirement for a DBD. **(A)** *In vivo*. The ability of BRCA1(1–1863) to stimulate transcription in yeast without a DBD was assessed in a yeast strain harboring a *lacZ* reporter template with an ER binding site upstream of the *GAL1* TATA. Activation was measured as arbitrary β-galactosidase units. **(B)** *In vitro*. *In vitro* transcription reactions were carried out with a yeast nuclear extract and *GAL1* DNA template. Primer extensions were carried out as in Figure 1C. Recombinant BRCA1(1528–1863) was added at 200 and 400 ng (lanes 2 and 3, respectively). The right panel depicts a Y1853X control (200 ng, lane 3) along with BRCA1 (200 ng, lane 2) as in Figure 1C.

DISCUSSION

Our results show that the BRCA1 C-terminal region has the surprising ability to stimulate transcription without the need for a DNA-binding function and, in so doing, exploits a mechanism different from one typified by many enhancer-binding activators. Importantly, these activities were crippled upon introduction of a cancer-associated mutation, Y1853X, which destabilizes the BRCT region leaving one of the two BRCT motifs intact. This intact BRCT could perhaps explain the transcriptional activity of that mutant when it is well overexpressed in some experiments. We have also shown that the C-terminal region of BRCA1, as well as the full-length molecule, could stimulate transcription of a p53-responsive promoter. The experiments described here rely on overexpression of BRCA1 derivatives and could imply a global effect on gene transcription, considering that BRCA1 can activate genes with a variety of promoter contexts. However, we propose that our experimental conditions represent increased local concentrations of BRCA1 under physiological conditions, a condition that we believe bypasses specific targeting of BRCA1 to our reporters, a situation well exemplified by our experiment with the *MDM2* reporter.

Our results showing that BRCA1 does not require a functional TATA element to stimulate transcription could imply, for example, that either BRCA1 stabilizes binding of the transcription machinery (e.g. TFIID) to the mutated promoter or, alternatively, BRCA1 could act independently of TBP binding to DNA to stimulate transcription. Recently, oligonucleotide array-based expression profiling experiments have revealed that BRCA1 expression could efficiently induce transcription of the DNA damage-responsive gene *GADD45* in a p53-independent fashion (Harkin *et al.*, 1999). Interestingly, examination of the *GADD45* proximal promoter region did not reveal the presence of any consensus TATA element (not shown). Thus, the fact that BRCA1 does not require a functional TATA element to stimulate transcription would be relevant in this particular case.

In prokaryotes, there are at least three examples of activators that do not require sequence-specific DNA-binding activity in order to stimulate transcription: (i) DNA-tracking proteins exemplified by the phage T4 Gp54 one-dimensional sliding clamp; (ii) the phage N4 single-stranded binding protein (N4SSB), which interacts with the β′ subunit of RNA polymerase and activates σ^{70}-type promoters; and (iii) σ^{54}-specific activators, which, even if being enhancer-binding proteins, can activate transcription at high concentrations without being DNA tethered (see Ptashne and Gann, 1997; Hochschild and Dove, 1998). It is conceivable that BRCA1 could stimulate transcription by a mechanism similar to any of the bacterial examples listed above. Recent experiments carried out with the TATA-binding protein-interacting protein TIP120 and ABT1 have shown that they too can enhance transcription without requiring a DNA-tethering function (Makino *et al.*, 1999; Oda *et al.*, 2000). Interestingly, our results could explain why BRCA1 activates the *p21WAF1* gene independently of p53 (Somasundaram *et al.*, 1997; Ouchi *et al.*, 1998) and also IFN-γ target genes in the absence of interferon stimulation (Ouchi *et al.*, 2000).

We consider that the mechanism by which BRCA1 stimulates transcription could constitute an advantage in cases where the transcriptional machinery might be paused, for example, at sites of DNA damage in a particular gene, located at a distance from its enhancer sequences. In the latter case, BRCA1 would be able to counteract a transcriptional pause without any interaction(s) with the upstream enhancer. Consistent with this idea is a recent report which suggests that BRCA1 BRCT domains can bind DNA

strand breaks and termini (Yamane and Tsuruo, 1999). Thus, it is conceivable that BRCA1 could recognize damaged DNA at paused transcription sites, and in so doing, increase favorable interactions with the transcriptional machinery thereby further enhancing transcription and perhaps, the transcription-coupled DNA repair process itself. In an alternative mode not mutually exclusive with the previous one, BRCA1 could be targeted to specific genes by interaction with enhancer-binding activators and in so doing, further enhance the transcription process.

METHODS

Transient transfections. Human HCC1937 and HEK-293 cells were used for the transfection experiments and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated in six-well tissue culture plates for 24 h before transfection at a density of $1–2 \times 10^5$ cells/well. Cells were then transfected using either Fugene 6 or Lipofectamine 2000 reagents. After 18–24 h, cells were processed for luciferase assays. Transfection efficiencies, when appropriate, were normalized using the pβgal plasmid; in some experiments, we noticed that BRCA1 increased the activity of our *lacZ* internal control, thus, in many experiments we did not normalize our transfection efficiencies; nonetheless we always measured *lacZ* values to make sure no gross deviations would be observed. Details of plasmid constructions are available upon request.

Recombinant BRCA1 derivatives. Recombinant BRCA1(1528–1863) was expressed in *Escherichia coli* using the pET30a expression vector. The recombinant protein was first chromatographed on Ni-NTA agarose and then further fractionated on an FPLC mono-Q ion exchange column. The Y1853X mutant fragment was also expressed in *E. coli*, affinity purified on Ni-NTA agarose and then subjected to chromatography on a Superdex 200 HR 10/30 column.

Nuclear extracts and *in vitro* transcription. HeLa and HCC1937 nuclear extract preparation and transcription reactions were as previously described (Shapiro *et al.*, 1988). Yeast nuclear extract preparation and transcription reactions were as described in Wu *et al.* (1996) and supplemented with recombinant TBP and TFIIE. For human *in vitro* transcription experiments, the pGML4 template was used at 100 ng/reaction; pGML4 bears the *AdML* promoter without any enhancer sequences. For yeast *in vitro* transcription experiments, the pGDC01 template was used at 100 ng/reaction; pGDC01 bears the *GAL1* promoter with its native UAS_G. All data were quantified by phosphorimaging.

Yeast manipulations. The yeast strain TGY14 (*MATa, ura3-251-373-328, leu2, pep4.3*) was used and contained a *GAL1::lacZ* reporter gene inserted downstream of an ERE. Competent yeast cells were obtained using the yeast transformation system (Clontech) based on lithium acetate, and cells were transformed according to the manufacturer's instructions. Activity of the reporter was measured by liquid β-galactosidase assays.

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REFERENCES

Anderson, S.F., Schlegel, B.P., Nakajima, T., Wolpin, E.S. and Parvin, J.D. (1998) BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nature Genet.*, **19**, 254–256.

Bryant, G.O., Martel, L.S., Burley, S.K. and Berk, A.J. (1996) Radical mutations reveal TATA-box binding protein surfaces required for activated transcription *in vivo*. *Genes Dev.*, **10**, 2491–2504.

Chapman, M.S. and Verma, I.M. (1996) Transcriptional activation by BRCA1. *Nature*, **382**, 678–679.

Easton, D.F., Bishop, D.T., Ford, D. and Crockford, G.P. (1993) Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. Breast cancer linkage consortium. *Am. J. Hum. Genet.*, **52**, 678–701.

Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E. and King, M.C. (1994) Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nature Genet.*, **8**, 399–404.

Haile, D.T. and Parvin, J.D. (1999) Activation of transcription *in vitro* by the BRCA1 carboxyl-terminal domain. *J. Biol. Chem.*, **274**, 2113–2117.

Harkin, D.P., Bean, J.M., Miklos, D., Song, Y.-H., Truong, V.B., Englert, C., Maheswaran, S., Oliner, J.D. and Haber, D.A. (1999) Induction of *GADD45* and JNK/SAPK-dependent apoptosis following inducible expression of *BRCA1*. *Cell*, **97**, 575–586.

Hochschild, A. and Dove, S.L. (1998) Protein–protein contacts that activate and repress prokaryotic transcription. *Cell*, **92**, 597–600.

Makino, Y. *et al.* (1999) TATA-binding protein-interacting protein 120, TIP120, stimulates three classes of eukaryotic transcription via a unique mechanism. *Mol. Cell. Biol.*, **19**, 7951–7960.

Monteiro, A.N.A. (2000) BRCA1: exploring the links to transcription. *Trends Biochem. Sci.*, in press.

Monteiro, A.N.A., August, A. and Hanafusa, H. (1996) Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc. Natl Acad. Sci. USA*, **93**, 13595–13599.

Oda, T., Kayukawa, K., Hagiwara, H., Yudate, H.T., Masuho, Y., Murakami, Y., Tamura, T.A. and Muramatsu, M.A. (2000) A novel TATA-binding protein-binding protein, ABT1, activates basal transcription and has a yeast homologue that is essential for growth. *Mol. Cell. Biol.*, **20**, 1407–1418.

Ouchi, T., Monteiro, A.N.A., August, A., Aaronson, S.A. and Hanafusa, H. (1998) BRCA1 regulates p53-dependent gene expression. *Proc. Natl Acad. Sci. USA*, **95**, 2302–2306.

Ouchi, T., Lee, S.W., Ouchi, M., Aaronson, S.A. and Horvath, C.M. (2000) Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-γ target genes. *Proc. Natl Acad. Sci. USA*, **97**, 5208–5213.

Ptashne, M. and Gann, A. (1997) Transcriptional activation by recruitment. *Nature*, **386**, 569–577.

Scully, R., Anderson, S.F., Chao, D.M., Wei, W., Ye, L., Young, R.A., Livingston, D.M. and Parvin, J.D. (1997) BRCA1 is a component of the RNA polymerase II holoenzyme. *Proc. Natl Acad. Sci. USA*, **94**, 5605–5610.

Shapiro, D.J., Sharp, P.A., Wahli, W.W. and Keller, M.J. (1988) A high-efficiency HeLa cell nuclear transcription extract. *DNA*, **7**, 47–55.

Somasundaram, K. *et al.* (1997) Arrest of the cell cycle by the tumour suppressor BRCA1 requires the CDK-inhibitor $p21^{WAF/Cip1}$. *Nature*, **389**, 187–190.

Tomlinson, G.E. *et al.* (1998) Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. *Cancer Res.*, **58**, 3237–3242.

Wang, Q., Zhang, H., Kajino, K. and Greene, M.I. (1998) BRCA1 binds c-Myc and inhibits its transcriptional and transforming activity in cells. *Oncogene*, **17**, 1939–1948.

Welcsh, P.L., Owens, K.N. and King, M.C. (2000) Insights into the functions of BRCA1 and BRCA2. *Trends Genet.*, **16**, 69–74.

Wu, Y., Reece, R. and Ptashne, M. (1996) Quantitation of putative activator-target affinities predicts transcriptional activating potentials. *EMBO J.*, **15**, 3951–3963.

Yamane, K. and Tsuruo, T. (1999) Conserved BRCT regions of TopBP1 and of the tumor suppressor BRCA1 bind strand breaks and termini of DNA. *Oncogene*, **18**, 5194–5203.

Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B.L. and El-Deiry, W.S. (1998) BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene*, **16**, 1713–1721.

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Functional Assay for BRCA1: Mutagenesis of the COOH-Terminal Region Reveals Critical Residues for Transcription Activation[1]

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ABSTRACT

The breast and ovarian cancer susceptibility gene product BRCA1 is a tumor suppressor, but its precise biochemical function remains unknown. The BRCA1 COOH terminus acts as a transcription activation domain, and germ-line cancer- predisposing mutations in this region abolish transcription activation, whereas benign polymorphisms do not. These results raise the possibility that loss of transcription activation by BRCA1 is crucial for oncogenesis. Therefore, identification of residues involved in transcription activation by BRCA1 will help understand why particular germ-line missense mutations are deleterious and may provide more reliable presymptomatic risk assessment.

The BRCA1 COOH terminus (amino acids 1560–1863) consists of two BRCTs preceded by a region likely to be nonglobular. We combined site-directed and random mutagenesis, followed by a functional transcription assay in yeast: (*a*) error-prone PCR-induced random mutagenesis generated eight unique missense mutations causing loss of function, six of which targeted hydrophobic residues conserved in canine, mouse, rat, and human BRCA1; (*b*) random insertion of a variable pentapeptide cassette generated 21 insertion mutants. All pentapeptide insertions NH_2-terminal to the BRCTs retained wild-type activity, whereas insertions in the BRCTs were, with few exceptions, deleterious; and (*c*) site-directed mutagenesis was used to characterize five known germ-line mutations and to perform deletion analysis of the COOH terminus. Deletion analysis revealed that the integrity of the most COOH-terminal hydrophobic cluster (I1855, L1854, and Y1853) is necessary for activity. We conclude that the integrity of the BRCT domains is crucial for transcription activation and that hydrophobic residues may be important for BRCT function. Therefore, the yeast-based assay for transcription activation can be used successfully to provide tools for structure-function analysis of BRCA1 and may form the basis of a BRCA1 functional assay.

INTRODUCTION

Individuals carrying mutations in the *BRCA1* gene have an increased risk of developing breast and ovarian cancer (1). Mutations in *BRCA1* alone account for ~45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (2). After an extensive search, *BRCA1* was mapped to the long arm of chromosome 17 by linkage analysis (3) and was cloned by positional cloning techniques (4). Human *BRCA1* codes for a 1863-amino acid protein with no detectable similarity to known proteins, with the exception of a zinc-binding RING finger domain located in the NH_2-terminal region (4), and two BRCT[4] domains found in a variety of proteins involved in cell cycle control and DNA repair (5–7).

Recent evidence points to the involvement of BRCA1 in two basic cellular processes: DNA repair and transcriptional regulation. BRCA1 is present in a complex containing Rad51 (8) and BRCA2 (9), and DNA damage may control BRCA1 phosphorylation and subnuclear location (10, 11), strongly suggesting its involvement in the maintenance of genome integrity. Additional evidence for the role of BRCA1 in maintenance of genome integrity is provided by targeted disruption of *Brca1* in the mouse. Mouse embryos lacking *Brca1* are hypersensitive to γ-irradiation, and cells display numerical and structural chromosomal aberrations (12).

We and others have shown that the BRCA1 COOH terminus has the ability to activate transcription in mammalian and yeast cells and that the introduction of germ-line disease-associated mutations, but not benign polymorphisms, abolishes this activity (13–15). BRCA1 can be copurified with the RNA polymerase II holoenzyme, supporting the idea that BRCA1 is involved in transcription regulation (16, 17). In addition, BRCA1 causes cell cycle arrest via transactivation of $p21^{WAF1/CiP1}$ (18) and regulates p53-dependent gene expression, acting as a coactivator for p53 (19, 20). In all of these studies, the COOH-terminal region was necessary for activity. It is still not clear whether BRCA1 is a multifunctional protein with repair and transcription regulation functions or whether the role of BRCA1 in repair is mediated through transcription activation. In either case, these functions are not necessarily mutually exclusive.

The dearth of knowledge concerning the precise biochemical function of BRCA1 is a major hurdle in developing a functional test to provide reliable presymptomatic assessment of risk for breast and ovarian cancer. The available data derived from linkage analysis indicate that all mutations that cause premature termination (even relatively subtle mutations such as the deletion of 11 amino acids from the COOH terminus) will confer high risk (21). However, a considerable number of mutations result in amino acid substitutions that, in the absence of extensive population-based studies or a functional assay, do not allow assessment of risk. Two related yeast-based assays designed to characterize mutations in the BRCA1 COOH terminal region have generated results that provide an excellent correlation with genetic linkage analysis (13, 14, 22). This led us to propose the general use of a yeast-based assay to provide functional information and a more reliable risk assessment (23).

In this report, we use site-directed and random mutagenesis to generate mutations in the BRCA1 COOH terminal region that disrupt transcription activation with the intention of both defining critical residues for BRCA1 function and deriving general rules to predict the impact of a particular mutation.

MATERIALS AND METHODS

Yeast Strains. Three *Saccharomyces cerevisiae* strains were used in this study: HF7c [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4–542, gal80–538, LYS::GAL1-HIS3, URA3::(GAL4 17mers)$_3$-CYC1-lacZ*]; SFY526 [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3,112,* can^r, *gal4–542, gal80–538, URA3::GAL1-lacZ*]

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[4] The abbreviations used are: BRCT, BRCA1 COOH terminal domain; DBD, DNA binding domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MTD, minimal transactivation domain.

(24); and EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] (25). HF7c has an *HIS3* reporter gene under the control of the *GAL1* upstream activating sequence, responsive to GAL4 transcription activation. The vectors used for expression confer growth in the absence of tryptophan (see below). The SFY526 strain has a *lacZ* reporter under the control of *GAL1* upstream activating sequence and was transformed with the GAL4 DBD fusion. EGY48 cells were cotransformed with the LexA fusion vectors and plasmid reporters of *lacZ* under the control of LexA operators (see below). If the fusion proteins activate transcription, EGY48 and SFY526 yeast transformants will produce β-galactosidase, and HF7c transformants will grow in medium lacking histidine.

Yeast Expression Constructs. The GAL4 DBD fusion of the wild-type human BRCA1 COOH terminal region (amino acids 1560–1863) was described previously (13). Alternatively, this fragment was subcloned into the yeast expression vector pLex9 (25) in-frame with the DBD of LexA. Both plasmids have *TRP1* as a selectable marker, allowing growth in the absence of tryptophan. We noticed that our previously described BRCA1 (amino acids 1560–1863) construct (13) was made with a 3′ primer lacking a termination codon. This introduces 16 exogenous amino acids to the COOH-terminal region of BRCA1. We have corrected this by using primer 24ENDT (5′-GCGGATCCTCAGTAGTGGCTGTGGGGGAT-3′). We compared both constructs and ascertained that qualitatively and quantitatively, they have the same activity (not shown).

BRCA1 deletion mutants were generated by PCR on a BRCA1 (amino acids 1560–1863) context using pcBRCA1–385 (a gift from Michael Erdos, National Human Genome Research Institute) as a template and the following primers: H1860X (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S970074, 5′-GCGGATCCTCAGGGGATCTGGGG-3′); P1856X (S9503101, S970073, 5′-GCGGATCCTCATATCAGGTAGGTGTCC-3′); I1855X (S9503101, 1855STOP, 5′-GCGGATCCTCACAGGTAGGTGTCC-3′); and L1854X (S9503101, 1854STOP, 5′-GCGGATCCTCAGTAGGTGTCCAGC-3′). Mutant Y1853X corresponds to a germ-line mutation and has been described previously (13). The constructs were sequenced to verify the mutations. The PCR products were digested with *Eco*RI and *Bam*HI and subcloned into similarly digested pGBT9 vectors. Alternatively, the PCR fragments were subcloned into a vector, pAS2–1 (Clontech), with higher expression levels. Introduction of additional mutations was made using the Quick-Change method. Briefly, a pair of primers encoding each mutation flanked by homologous sequence on each side was added to the wild-type plasmid pLex9 BRCA1 (amino acids 1560–1863) prepared in a methylation-competent strain. The plasmid was amplified using *Pfu* polymerase (one cycle at: 96°C for 30 s; 12 cycles at: 96°C for 30 s; 50°C for 1 min; and 68°C for 12 min), and *Dpn*I was added at the end of the reaction to digest the parental plasmid. The mixture was then transformed into bacteria. The following oligonucleotide primers were used: T1561I (T1561IF, 5′-CTGGAATTCGAGGGAATCCCTTACCTCGAGTCTGG-3′; T1561IR, 5′-CCAGACTCGAGGTAAGGGATTCCCTCGAATTCCAG-3′); L1564P (L1564PF, 5′-GGGTACCCCTTACCCGGAATCTGGAATCAG-3′;L1564PR,5′-CTGATTCCAGATTCCGGGTAAGGGGTACCCTC-3′); D1733G (D1733GF, 5′-GAAAAATGCTCAATGAGCATGGTTTTGAAGTCCGCGGAG-3′; D1733GR, 5′-CTCCGCGGACTTCAAAACCATGCTCATTCAGCATTTTTC-3′); G1738E (G1738EF, 5′-GAGCATGATTTTGAAGTCAGAGAAGATGTGGTTAACGGAAG-3′; G1738ER, 5′-CTTCCGTTAACCACATCTTCTCTGACTTCAAAATCATGCTC-3′); P1806A (P1806AF, 5′-GGTACCGGTGTCCACGCAATTGTGGTTGTGCAGC-3′; and P1806AR, 5′-GCTGCACAACCACAATTGCGTGGACACCGGTACC-3′).

Yeast Plasmid Reporters. Plasmid pSH18-34 (25), a kind gift of Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA), was used as a reporter in the LexA fusion assays. This vector has *lacZ* under the control of eight LexA operators, conferring low stringency of gene expression (26).

Yeast Transformation. Competent yeast cells were obtained using the yeast transformation system (Clontech) based on lithium acetate, and cells were transformed according to the manufacturer's instructions.

Filter β-Galactosidase Assay. SFY526 and EGY48 transformants (several clones for each construct) were streaked on a filter overlaid on solid medium lacking tryptophan (SFY526) or tryptophan and uracil (EGY48) and allowed to grow overnight. Cells growing on the filter were lysed by freeze/thawing in liquid nitrogen, and each filter was incubated in 2.5 ml of Z buffer (16.1 g/liter $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, and 0.246 g/l $MgSO_4{\cdot}7H_2O$, pH 7.2) containing 40 μl of X-gal solution (20 mg/ml of X-gal in *N,N*-dimethylformamide) at 30°C for up to 16 h.

Liquid β-Galactosidase Assay. Liquid assays were performed as described previously (27). At least three separate transformants were assayed, and each was performed at least in duplicate.

Growth Curves. HF7c transformants (several clones) containing different pGBT9 or pAS2 constructs were grown overnight in synthetic medium plus 2% dextrose (SD medium) lacking tryptophan. The saturated cultures were used to inoculate fresh medium lacking tryptophan or tryptophan and histidine to an initial A_{600} of 0.0002. Cultures were grown at 30°C in the shaker, and the absorbance was measured at different time intervals starting at 12 h, then every 4 h up to 36 h after inoculation.

Plasmid Recovery from Yeast Cells. EGY48 transformants were grown to saturation in liquid medium lacking uracil (but in the presence of tryptophan). Cells were harvested and treated with yeast lysis solution [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA], phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 g of acid-washed beads. The sample was vortexed for 2 min and centrifuged, and the supernatant precipitated with one-tenth volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of ethanol. Alternatively, plasmid rescue was performed as suggested by Strathern and Higgins (28).

Screening in X-gal Plates. To allow direct screening of the clones with loss of activity, EGY48 cells transformed with the mutagenized cDNAs were plated on X-gal-containing plates: 2% galactose, 1% raffinose, 80 mg/l X-gal, and 1× BU salts (1 liter of 10× BU salts: 70 g $Na_2HPO_4{\cdot}0.7H_2O$, 30 g NaH_2PO_4).

Error-prone PCR Mutagenesis. A 60-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using Taq polymerase and p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). The PCR product was gel purified and cotransformed in an equimolar ratio with a *Nco*I-linearized wild-type pLex9 BRCA1 (amino acids 1560–1863) plasmid and pSH18-34. After transformation, cells were plated on X-gal plates and incubated for 5 days. Eighty-one white and four control blue clones were recovered and restreaked on master plates. White clones were screened again on a filter assay, and the 62 clones that were consistently white were analyzed further. Plasmid DNA was recovered from the yeast cells and transformed into *Escherichia coli*. Miniprep DNAs from each of two bacterial transformants from the 62 candidates were retransformed into yeast cells and tested again for β-galactosidase production. The BRCA1 inserts in plasmid DNAs generating white clones were subjected to direct sequencing using dye terminators.

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a technique whereby 5-amino acid insertions are introduced at random in a target protein (29). Briefly, an *E. coli* donor strain containing the target plasmid and pHT385, a conjugative delivery vector for transposon Tn*4430*, is mated with a plasmid-free *E. coli* recipient strain. By plating the mating mix simultaneously on antibiotics selecting for the recipient, the target plasmid, and Tn*4430*, transconjugants containing pHT385::target plasmid cointegrates are isolated. This cointegrate resolves rapidly *in vivo*, regenerating pHT385 and the target plasmid into which a copy of Tn*4430* has been inserted. Tn*4430* contains *Kpn*I restriction enzyme sites located 5-bp from both ends of the transposon and duplicates 5-bp of target site sequence during transposition. By digesting the target plasmid::Tn*4430* hybrid with *Kpn*I and religating the digested DNA, the bulk of the transposon is deleted to generate a target plasmid derivative containing a 15-bp insertion. If the insertion is in a protein-encoding sequence, this will result in a 5-amino acid insertion in the target protein.

Tn*4430* insertions in the COOH-terminal region of BRCA1 were identified either by genetic or physical means. In the former case, 30 separate matings were performed as detailed previously (30) using appropriate antibiotic selections and in which the target plasmid was pLex9 containing the BRCA1 COOH terminal region fused to LexA DBD. Transconjugant colonies were harvested by washing from the mating plates, and plasmid DNA was isolated from the pooled colonies. The plasmid preparations were pooled further and transformed into *Saccharomyces cerevisiae* EGY48 harboring the pSH18-34 reporter plasmid. Transformants were tested for transcription activation by replica-plating to plates containing X-gal. Plasmid DNA was recovered from white colonies and transformed into *E. coli* XL1-Blue selecting on X-gal-

Table 1 *Missense mutations leading to loss of function (PCR-mediated mutagenesis screen)*

Exon	Mutation	Dog[a]	Mouse[b]	Rat[c]	Nucleotide[d]	Base change	Comments and probable secondary structure elements[e]
16	M1652K	M	M	M	5074	T to A	Residue mutated in germ line (M1652T, M1652I).
18	K1702E	K	K	K	5223	A to G	α-Helix 2 of BRCT-N.
18	Y1703H	Y	Y	Y	5226	T to C	α-Helix 2 of BRCT-N.
18	L1705P	L	L	L	5233	T to C	Found in two independent clones. Located just after α-helix 2 of BRCT-N.
21	F1761S	F	F	F	5401	T to C	BRCT-N/BRCT-C interval.
21	F1761I	F	F	F	5400	T to A	BRCT-N/BRCT-C interval.
22	L1780P	L	L	L	5458	T to C	α-Helix 1 of BRCT-C. Hydrophobic residue conserved in BRCT superfamily. Mediates interaction between α-helix 1 and α-helix 3.
24	V1833E	V	V	V	5617	T to A	β-Strand 4 of BRCT-C. Residue mutated in germ line (V1833M) and found in two independent clones. Hydrophobic residue conserved in BRCT superfamily.

[a] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[b] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[c] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[d] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[e] According to a BRCA1 BRCT model from Zhang *et al.* (36).

containing plates. Plasmid DNA was isolated from white colonies (which contain only pLex9::BRCA1 COOH-terminal::Tn*4430*), and the insertion of Tn*4430* into the BRCA1 COOH terminal region was confirmed by restriction enzyme mapping. For the identification of Tn*4430* insertions by physical means, pooled plasmid DNA from *E. coli* consisting of the target plasmid into which Tn*4430* was inserted was digested with *Eco*RI and *Bam*HI, enzymes which liberate the BRCA1 insert but do not cut Tn*4430*. This digestion of pooled plasmid DNA generates four fragments: the pLex9 vector backbone, the pLex9 vector containing Tn*4330* insertions, the BRCA1 COOH-terminal fragment, and the BRCA1 COOH terminal fragment containing Tn*4430* insertions. The latter fragment was recovered from an agarose gel and recloned in *Eco*RI-*Bam*HI-digested pLex9 to produce a library of pLex9::BRCA1 COOH terminal domain plasmids containing Tn*4430* insertions in the BRCA1 COOH terminal region. In the case of Tn*4430* insertions identified by either genetic or physical means, following further restriction mapping the bulk of Tn*4430* was deleted from selected clones by digestion with *Kpn*I and religation. The positions of the 15-bp insertions were determined by sequence analysis. Twenty-one plasmids harboring the BRCA1 COOH terminal region with 15-bp insertions were analyzed for transcription activation in *S. cerevisiae* EGY48 containing pSH18–34.

Western Blots. Yeast cells were grown in selective media to saturation, and A_{600} was measured. Cells were harvested and lysed in cracking buffer [8 M urea, 5% SDS, 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, and 0.4 mg/ml bromphenol blue; used 100 μl/7.5 total A_{600}] containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a polyvinylidene difluoride membrane. The blots were blocked overnight with 5% skim milk using TBS-Tween and incubated with the α-pLexA (for LexA constructs) or α-GAL4 DBD (for GAL4 constructs) monoclonal antibodies (Clontech) using 0.5% BSA in TBS-Tween. After four washes, the blot was incubated with the α-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-Tween. The blots were developed using an enhanced chemiluminescent reagent (DuPont NEN, Boston, MA).

RESULTS

Germ-Line Mutations. We analyzed missense mutations occurring in the region from amino acid 1560 to amino acid 1863 described in the Breast Cancer Information Core[5] database. To date, 63 missense variants representing mutations in 55 different residues have been documented, most of which have not been characterized either as disease-associated or as benign polymorphisms. Only four missense mutations have been either confirmed or considered very likely to be associated with disease: A1708E (31–33), P1749R (34), R1751Q (33), and M1775R (4, 31, 35). Three of these four mutations target hydrophobic residues that are conserved in canine, mouse, and rat Brca1. Amino acid composition analysis of this region reveals that only 39% of the residues are hydrophobic. Thus, although the number of characterized mutations is limited, it suggests a preference for loss-of-function mutations to target hydrophobic residues.

Mutagenesis Strategies. To shed light on the critical residues and regions necessary for function, we used four complementary strategies: (*a*) error-prone PCR mutagenesis followed by a screen for loss of function; (*b*) pentapeptide insertion mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus.

Error-prone PCR Mutagenesis Reveals Critical Residues for Activation. Approximately 10^5 yeast clones were screened for loss of transcription activation function. Sixty-two clones were isolated that had lost activity, most of which contained small insertions or deletions causing frameshift mutations and premature termination of the BRCA1 protein, as subsequently confirmed by SDS-PAGE and Western blot analysis (not shown). Two independent clones displayed the same nonsense mutation (Y1769X). Four clones had two mutations (E1660G/M1689K, K1727R/L1786P, S1722P/N1774Y, and S1715N/Q1811L), limiting their further characterization. The 10 remaining clones each had a single missense mutation (one clone also had a silent mutation) and corresponded to eight distinct mutations (Table 1). Interestingly, the screen revealed that hydrophobic residues were the major targets of mutation (six of eight). Furthermore, all of the targeted residues are perfectly conserved in canine, mouse, and rat Brca1 (Table 1). Even conservative mutations may not be well accepted in residues that are perfectly conserved in all species. This is illustrated by mutation F1761I, where a smaller hydrophobic residue is not tolerated in place of a bulkier one. Loss-of-function mutations were located primarily in the BRCT domains. In particular, mutations that occur in BRCT-C [the most COOH-terminal BRCT (amino acids 1756–1855); BRCT-N (amino acids 1649–1736) is located NH_2-terminally to BRCT-C] are in residues that constitute the hydrophobic clusters conserved in the BRCT superfamily. Western blot analysis of the mutant clones (three independent clones of each) revealed that all of the mutants were expressed at levels comparable with the wild type, ruling out the possibility that loss of function was attributable to instability of the protein (Fig. 1). It is important to stress, however, that protein levels are relatively variable in different yeast clones carrying the same constructs and should only be taken as a rough estimate.

Pentapeptide Scanning Mutagenesis Reveals Buried Regions Necessary for Activity. The BRCA1 COOH terminal region was subjected to pentapeptide scanning mutagenesis in which a variable, 5-amino acid cassette was introduced at random. The resulting set of mutated proteins included mutants that displayed complete loss of activity, mutants with reduced activity, and mutants with similar or

[5] Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

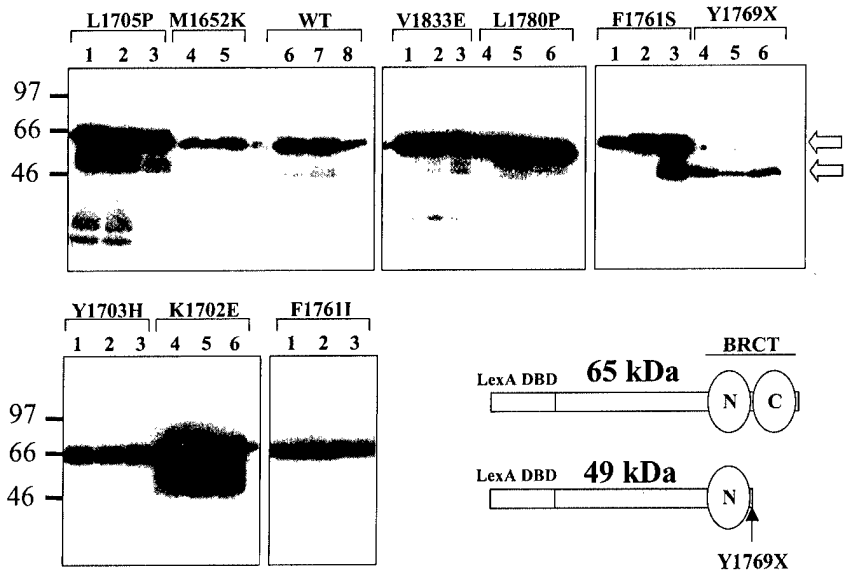

Fig. 1. Expression levels of loss-of-function BRCA1 mutants. Cell lysates containing comparable cell numbers were separated on SDS-PAGE. At least two independent transformants were assayed for each mutant to control for clonal variation. LexA fusion mutant proteins expressed in yeast were detected by Western blot using a monoclonal α-LexA antibody. A schematic representation of the fusion proteins bearing missense mutations [*white arrow*, M_r 65,000 (*65 kDa*)] and a nonsense mutation [*gray arrow*, M_r 49,000 (*49 kDa*)] is shown. Note that mutation Y1769X disrupts the BRCT-C but retains BRCT-N.

higher activity than wild type. Table 2 groups the insertions by location: the first group includes mutations in the region NH_2-terminal to the BRCT domains (amino acids 1560–1649); the second group contains mutations in BRCT-N; and the third group includes mutations in the intervening region between BRCT-N and BRCT-C. The last group includes mutations in BRCT-C. None of the insertions NH_2-terminal to the BRCT domains had a negative effect on transcription activation. Also, insertions in the interval between the BRCT domains or at its boundary (1723RGTPI) had generally less drastic effects. In contrast, all insertions within BRCT-N and several within BRCT-C had a more severe effect. It is clear that BRCT-C tolerates insertions better (only three of five showed loss of activity) than BRCT-N (all mutations reduced activity with six of seven showing drastic impairment). The difficulty in predicting the outcome of mutations can be well exemplified by mutations 1824GGTPI and 1822GVPLH. Both of these mutations target residues at the end of BRCT-C α-helix 2, do not change the net charge of the protein, and are only two residues apart. However, 1822GVPLH has ~6% of the wild-type activity, whereas 1824GGTPI has an activity ~80% higher than wild type. Interestingly, the 1793GVPLK insertion increased transcriptional activation ~4-fold, suggesting that this region of BRCA1 may directly contact a component of the transcription machinery. The pentapeptide mutagenesis results demonstrated that, in addition to substitution mutations, insertion mutagenesis in the COOH-terminal region, particularly in the BRCT domains, can profoundly alter transcriptional activity by BRCA1.

Table 2 *Transcriptional activity of insertion mutants*

Pentapeptide insertion	Miller units[a]	Probable secondary structure element[b]
Empty vector	4.1 ± 3.0	
Wild-type	99.9 ± 14.1	
1571SEGYP	**98.2 ± 98.2**	**Unknown**
1578PSGVP	**120.1 ± 52.6**	**Unknown**
1602PQGVP	**99.3 ± 15.9**	**Unknown**
1620DRGTP	**127.1 ± 11.0**	**Unknown**
1625NGVPH	**81.7 ± 8.2**	**Unknown**
1627MGVPP	**94.4 ± 6.8**	**Unknown**
1665stop	1.6 ± 0.1	α-Helix 1 of BRCT-N
1676RGTPL	2.5 ± 0.2	β-Strand 2 of BRCT-N
1678RGTPN	0.7 ± 0.2	β-Strand 2 boundary of BRCT-N
1695GVPQF	4.3 ± 1.1	β-Strand 3/α-helix 2 loop of BRCT-N
1709GGTPG	1.0 ± 0.7	α-Helix 2/β-strand 4 loop of BRCT-N
1717WGTPF	2.1 ± 0.4	α-Helix 3 of BRCT-N
1723RGTPI	36.5 ± 15.0	α-Helix 3 boundary of BRCT-N
1724GVPLK	10.4 ± 2.5	BRCT-N/BRCT-C interval
1730GVPLN	57.7 ± 7.2	BRCT-N/BRCT-C interval
1737GVPLR	1.0 ± 0.5	BRCT-N/BRCT-C interval
1769GGYPY	11.7 ± 11.1	β-Strand 1/α-helix 1 loop of BRCT-C
1780GVPQL	0.8 ± 0.3	α-Helix 1 of BRCT-C
1793GVPLK	**372.9 ± 113.8**	**β-Strand 2/β-strand 3 turn of BRCT-C**
1822GVPLH	5.7 ± 0.5	α-Helix 2 of BRCT-C
1824GGTPI	**178.0 ± 34.4**	**α-Helix 2 boundary of BRCT-C**

[a] Mutants in bold displayed activity equal to or higher than wild type.
[b] According to a BRCA1 BRCT model from Zhang *et al.* (36).

Characterization of Germ-Line Mutations. To assess the activity of variants that have already been documented but not characterized, we decided to introduce a set of mutations and assay for transcription activation in yeast (Table 3). Mutations T1561I and L1564P are both located in the region preceding the BRCT domains and displayed wild-type activity. L1564P was expected to be a polymorphism because proline is the residue found in the rat Brca1 sequence. The three remaining variants are localized to the BRCT domains. Two variants, D1733G and P1806A, displayed wild-type activity and are suggested to be benign polymorphisms. D1733G introduces a glycine that probably does not affect BRCT structure. P1806A involves a conservative change, and it is important to note that the rat Brca1 sequence has leucine in that position. Only one of the variants tested, G1738E, displayed a loss of function phenotype. Thus, we propose that G1738E is a disease-predisposing variant.

Deletion Mutants of COOH-Terminal Residues Define the Minimal Transactivation Domain (MTD). A construct carrying the germ-line mutation Y1853X does not have detectable transcriptional activity in the context of a GAL4 DBD fusion of the BRCA1 COOH terminus (amino acids 1560–1863; Refs. 13 and 15). A construct containing amino acids 1760–1863 can be considered the MTD, defining I1760 as a 5′ border of this domain (13, 15). Thus, the NH_2-terminal border of the MTD coincides closely with the NH_2-

Table 3 *Transcriptional activity of human BRCA1 unclassified variants (amino acids 1560–1863)*

Exon	Mutation	Activity[a]	Dog[b]	Mouse[c]	Rat[d]	Nucleotide[e]	Base change	Probable secondary structure element[f]	Reference
16	T1561I	+	A	T	T	4801	C to T	Unknown	Durocher *et al.* (41)
16	L1564P	+	L	L	P	4810	T to C	Unknown	BIC[g]
20	D1733G	+	D	E	E	5317	A to G	BRCT-N/BRCT-C interval	BIC
20	G1738E	−	G	G	G	5332	G to A	BRCT-N/BRCT-C interval	BIC
23	P1806A	+	P	P	L	5535	C to G	β-Strand 2/β-strand 3 loop of BRCT-C	BIC

[a] At least 10 independent clones were assayed and scored 8 h after the addition of X-gal. +, blue with same intensity as wild-type control. −, white, similar to two (F1761S and Y1769X) loss-of-function controls.
[b] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[c] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[d] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[e] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[f] According to a BRCA1 BRCT model from Zhang *et al.* (36).
[g] Breast Cancer Information Core.

terminal border of BRCT-C (I1760 is the first conserved hydrophobic residue in the BRCT superfamily). To identify the COOH-terminal border of the MTD, several deletion mutants were made in the amino acids 1560–1863 context and assayed for their ability to activate transcription in yeast. Fig. 2 shows the several deletion mutants analyzed aligned to mouse, rat, dog, and human BRCA1 wild-type sequences. Mutant H1860X introduces a stop codon but maintains all of the conserved amino acids in canine and human BRCA1. P1856X maintains the hydrophobic residues, which are conserved in all of the BRCT domains described in several species. I1855X and L1854X delete one and two conserved hydrophobic residues, respectively. Y1853X is a mutation found in the germ-line of breast and ovarian cancer patients in high-risk families (21). These constructs were transformed into SFY526 and HF7c and analyzed for their ability to activate different reporters (Fig. 2*b*). Activity comparable with the wild-type was obtained with mutants H1860X and P1856X. However, mutations that disrupted the conserved hydrophobic residues (I1855X and L1854X) at the end of the BRCT domain abolished activity. Therefore, we define the MTD in BRCA1 as amino acids 1760–1855. To determine whether the loss of activity by the mutants correlated with the stability of the protein, yeast cells were transformed with the same mutated alleles in a vector conferring high expression (pAS2-1). Transcriptional activity using these constructs (in pAS2-1 backbone) was measured, and results were similar with I1855X showing some residual activity. Expression was highly variable, and mutants were in general expressed at lower levels than wild type (Fig. 2*c*). There was no correlation between loss of activity and lower levels of expression because the transcriptionally active mutant H1860X was expressed at levels lower or comparable with transcriptionally inactive mutants I1855X and Y1853X (Fig. 2*c*).

DISCUSSION

In this report, we describe an extensive mutagenesis analysis of the BRCA1 COOH terminal region and partly define the critical requirements for transcriptional activity by BRCA1. Four complementary strategies were used: (*a*) error-prone PCR mutagenesis, followed by a screen for loss of function; (*b*) pentapeptide scanning mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus. Our results support the notion that there are no particular hot spots for loss-of-function mutations, but rather that these mutations are scattered throughout the coding sequence. Nevertheless, we were able to identify preferential sites critical for activation. An overview of the mutations and their effects is presented in Fig. 3. We discuss the general conclusion of each strategy and then we analyze the possible structural outcome of the mutations based on the crystal structure of XRCC1 BRCT (36).

Error-prone PCR Mutagenesis. Eight distinct BRCA1 mutations were recovered that resulted in loss of transcription activation function. In the course of the screening procedure, many additional clones that displayed a light blue color were noted and were probably mutants with reduced function, but only clones with complete loss of function were analyzed further. No PCR-generated mutations were found in the region external to the BRCT domains, although this constitutes approximately one-third of the tested sequence, indicating a preference for mutations that affect transcription activation to occur in the BRCT domains (Fig. 3).

Six of eight unique PCR-generated mutations were in hydrophobic residues conserved in human, canine, mouse, and rat Brca1 (6, 7), supporting the notion that hydrophobic residues are important for the stability of the BRCT domains and BRCA1 function *in vivo*.

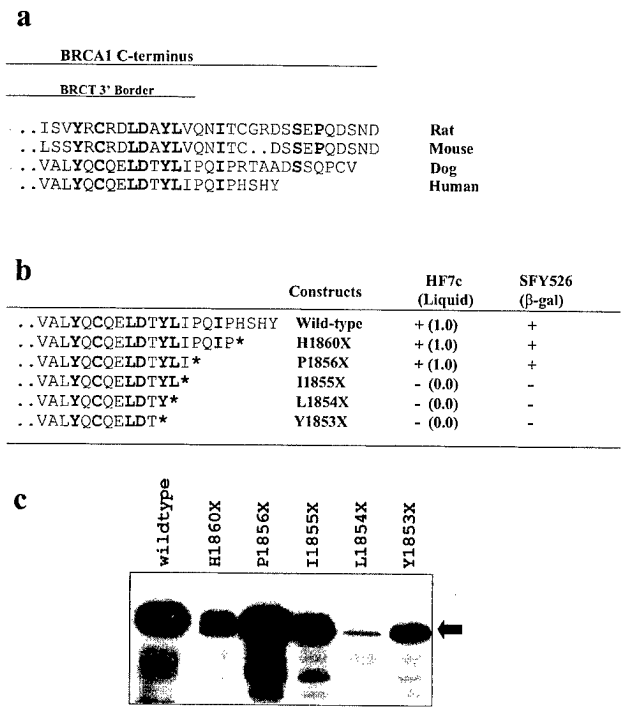

Fig. 2. Deletion analysis of the COOH-terminal region. *a*, alignment of the wild-type sequences of the COOH terminus of rat, mouse, dog, and human BRCA1. Amino acids in *bold* represent conserved residues. *Shaded area*, residues at the 3′ border of the BRCT-C domain. *b*, transcriptional activity of GAL4 DBD fusion deletion constructs, made in the context of BRCA1 amino acids 1560–1863. *S. cerevisiae* (HF7c) carrying the indicated fusion proteins were assayed for growth in the absence of tryptophan and histidine in liquid medium. Activity relative to cells growing in medium lacking tryptophan alone after 36 h is shown in *parentheses*. Filter β-galactosidase assays for SFY526 were scored at 12 h after X-gal addition. At least four independent clones were assayed for each construct. *c*, Western blot showing levels of protein expression of the different constructs (*black arrow*) detected by a α-GAL4-DBD monoclonal antibody.

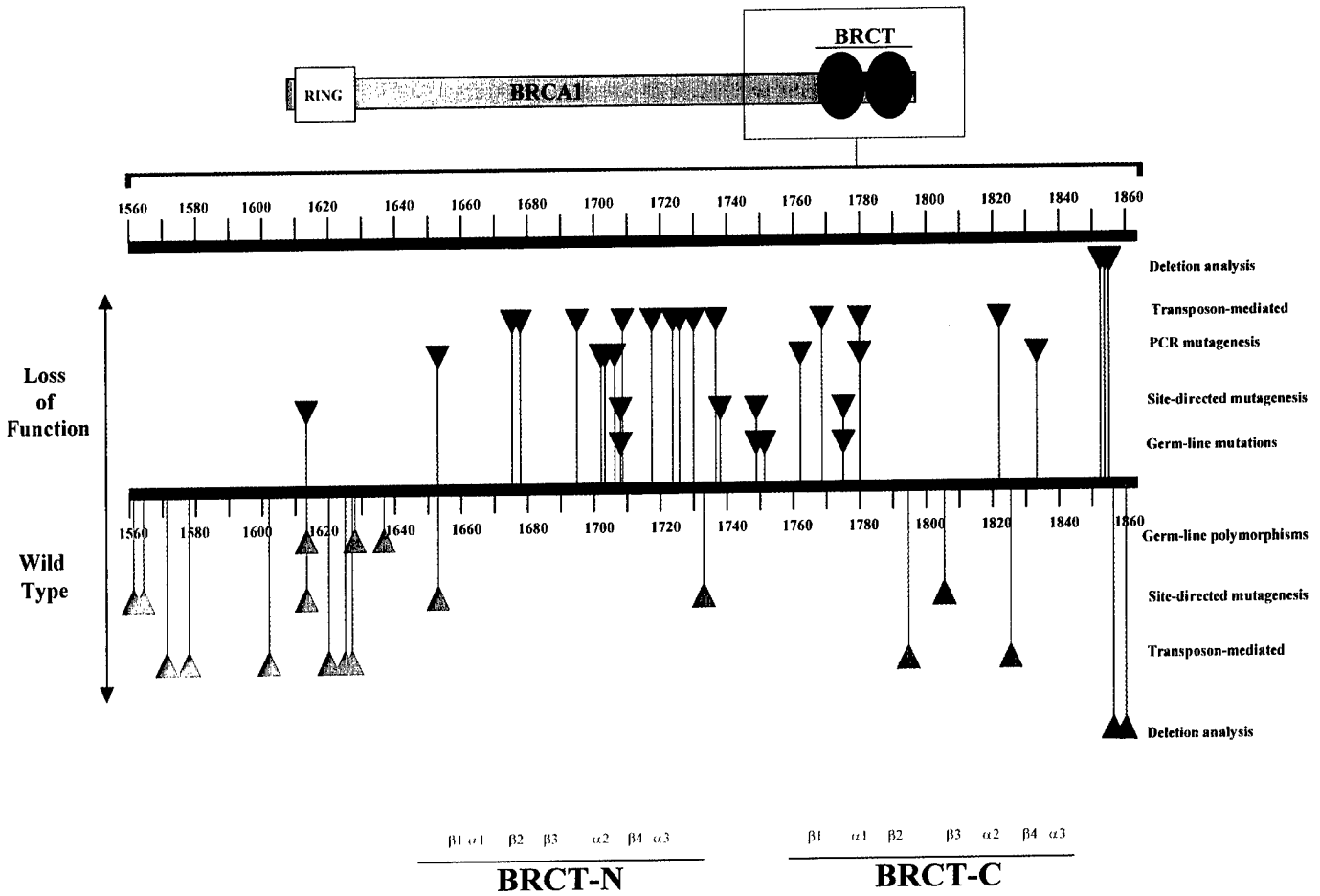

Fig. 3. Domain structure of the BRCA1 COOH terminal region (amino acids 1560–1863) and characterized mutations. *Top panel,* a schematic representation of full-length BRCA1 protein featuring the RING domain (*yellow box*) in the NH_2-terminal region and the BRCT domains (*red circles*) in the COOH-terminal region. The region analyzed in this study is contained in the *red box,* which is enlarged and represented in the *bottom panel. Purple* and *pink bars,* predicted β-strands and α-helices, respectively. Secondary structure predictions were made by Zhang *et al.* (36) based on the crystal structure of the XRCC1 BRCT domain. Mutations represented in the *upper part* (*red triangles*) result in loss of function, whereas mutations in the *lower part* (*green triangles*) result in activity equal or higher than wild type. Germ-line mutations and polymorphisms are variants defined by genetic linkage to be disease-associated and benign polymorphisms, respectively. Site-directed mutagenesis, PCR mutagenesis, transposon-mediated mutagenesis, and deletion analysis represent mutations that have been characterized by transcription activation assay in yeast to be either loss of function (*upper part*) or wild type (*lower part*).

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a method by which a variable 5-amino acid cassette is introduced at random into a target protein (29, 30, 37). This approach differs from error-prone mutagenesis because clones are not selected for loss of activity but rather mutations are analyzed only after they have been generated. Therefore, mutants with gain of function, loss of function, and novel activities can be produced (30, 37). Moreover, it has been shown that insertion is essentially random (29). The results obtained are in agreement with the PCR-mediated mutagenesis in that the region NH_2-terminal to the BRCT domains (amino acids 1560–1649) seems to be more tolerant of mutation; none of six different pentapeptide insertions in this region affected transcription activation. The fact that derivatives containing insertion mutations in this region retained wild-type activity suggests that this region is nonglobular and is probably a flexible part of the COOH-terminal region without many critical secondary structure elements. In fact, the region encompassing amino acids 1524–1661 is predicted to be nonglobular (5). The pentapeptide mutagenesis results also suggest that changing the net charge of the protein does not necessarily correlate with an alteration in transcription activity, as would be expected for classical acidic activators (38), because 1793GVPLK (which adds a positive charge) shows a 4-fold increase in activity. Interestingly, only 4 of the 63 COOH-terminal germ-line variants involve nonconservative substitutions in acidic residues, thought to be important for activation, suggesting that, contrary to initial predictions, BRCA1 may not be a classical acidic activator (4). The 1793GVPLK mutation, which is hyperactive for transcription activation, may define a point of contact between the BRCA1 COOH-terminal region and the transcription machinery.

Deletion Analysis. Our analysis demonstrates that residues COOH-terminal to amino acids 1855 are dispensable for activation, consistent with the extreme evolutionary divergence of those residues (Fig. 2; Refs. 39 and 40). The results also underscore the importance of the last hydrophobic cluster in the sequence (YLI for human and canine; YLV for mouse and rat) and provide a plausible explanation for the complete loss of function (*in vitro* and *in vivo*) of Y1853X alleles.

Site-directed Mutagenesis. Only one of five germ-line mutations analyzed displayed loss of function, suggesting that a large part of variants in the COOH-terminal region will probably be benign polymorphisms, including some variants found in the BRCT domains. Very little data are available at this moment to confirm or contradict the results obtained. In particular, T1561I illustrates the difficulties involved in predicting outcome from population data. T1561I was found in one affected individual but not in control individuals (41). This could suggest that T1561I is a disease-predisposing variant. However, although found as a germ-line mutation, it was absent from

the tumor from the same patient (41), indicating that this mutation is a benign polymorphism.

Structural Basis for Effects of BRCT Domain Mutations. The COOH-terminal BRCT domain of XRCC1 consists of a four-stranded parallel β-sheet (β1–β4) surrounded by three α-helices (α1–α3; Ref. 36). The β-sheet forms the core of the structure with a pair of α-helices (α1 and α3) on one side of the β-sheet and the remaining α-helix (α2) on the other side. A model of the more COOH-terminal BRCT domain of BRCA1 has been constructed based on the crystal structure of the BRCT domain of XRCC1 (36). This model allows an interpretation of the effect of some of the mutations described in this study (Tables 1–3) on BRCT domain structure (Fig. 3).

The position of the M1652K mutation corresponds to a position (Asp4) in the XRCC1 structure that is thought to form a salt bridge at the BRCT dimer interface (36). Although M1652 would not be expected to be involved in salt bridge formation at neutral pH, residues in this region nevertheless may also be involved in homo- or heterodimer formation in BRCA1.

Missense mutations at positions 1702, 1703, and 1705 of the BRCT-N domain and a pentapeptide insertion at position 1822 of the BRCT-C domain abolish transcription activation by the BRCA1 COOH terminus (Tables 1 and 2). These mutations are predicted to occur in a region of highly variable length and composition that encompasses helix α2 in BRCT domains (36). It was suggested that this variability indicated that this region was not involved in formation of the core fold of the BRCT domain (36). Nevertheless, the mutations isolated here reveal that this region of the BRCT domain is critical for the transcription activation function of the BRCA1 COOH terminus.

Residue F6 forms part of a highly conserved hydrophobic pocket centered on residue W74 in helix α3 in the COOH-terminal BRCT domain of XRCC1 (36). Mutations at the corresponding position (F1761) in the BRCT-C domain of BRCA1 abolish transcription activation (Table 1). By analogy with XRCC1, residue F1761 of BRCA1 is also predicted to form part of a hydrophobic pocket, the disruption of which by mutation may compromise correct BRCT domain folding. In contrast, residue L25 is implicated in the interactions between helices α1 and α3, which form a paired helical bundle in the three-dimensional structure of the BRCT domain of XRCC1 (36). A missense mutation of the corresponding residue (L1780) or a pentapeptide insertion at this position in the BRCT-C domain of BRCA1 abolishes transcription activation by the BRCA1 COOH terminus region (Tables 1 and 2). These mutations are likely to affect the interactions between helices α1 and α3, thereby destabilizing the BRCT domain structure. Two other missense mutations in the BRCT-C domain, P1806A and V1833E, were shown, respectively, to display wild-type activity and to abolish transcription activation (Tables 1 and 3). Interestingly, P1806A is predicted to have no obvious effect on the structure, whereas a less drastic mutation at position V1833 (to methionine) has been predicted to destabilize the fold of the domain (36), suggesting that V1833E will behave similarly.

Pentapeptide insertions in many of the predicted secondary structure elements in the COOH-terminal region of BRCA1 abolish transcription activation (Table 1 and Fig. 3). Some of these insertions are likely to disrupt formation of the correct BRCT domain core fold, *e.g.,* insertions in strand β2 (1676RGTPL) and in helices α2 (1822GVPLH) and α3 (1717WGTPF). In contrast, the 1780GVPQL insertion in helix α1 is predicted to be at the BRCT dimer interface and thereby may affect the association of this domain with another protein, *e.g.,* RNA helicase A, which interacts with BRCA1 through residues in helix α1 (17).

Different Roles of BRCT-N and BRCT-C. Our insertion mutagenesis results suggest that BRCT-C can tolerate insertions better than BRCT-N without affecting transcription activation function. In addition, BRCT-N is more highly conserved in other species than is BRCT-C (39, 40), suggesting a higher constraint for function. The BRCT-N seems to be very important for binding to RNA helicase A (17), although it seems to lack an independent activation domain (mutant Y1769X is inactive). The borders of BRCT-C coincide well with the limits of the MTD, but only in combination with BRCT-N are high levels of activation achieved (13). It is tempting to speculate that BRCT-N is involved in the interaction of BRCA1 with RNA helicase A and is responsible for presenting BRCT-C in a correct way to obtain a transcriptionally competent activator.

Functional Assay. We have performed an extensive analysis of the BRCA1 COOH terminal region (amino acids 1560–1863) and have found that there is a correlation between loss of transcription activation function and the human genetic data, suggesting that the assay could be used to predict the effect of missense mutations in this region. Although the effects of mutations on transcriptional activity have been found to be comparable in yeast and mammalian cells (13, 15), it is possible that the effect of some mutations may be evident only in mammalian cells, *e.g.,* because of an interaction with mammalian-specific regulators, raising the possibility of a misinterpretation of the data obtained in yeast.

In the results presented here for substitution mutations, we have used a reporter gene with relatively low stringency (eight Lex operators; Ref. 26). The rationale for this choice was to recover only mutants that cause dramatic reduction or complete loss of activity. Mutations that partially disrupt the function would still activate the reporter. In the absence of knowledge of the minimum *in vivo* threshold of transcription activity needed for tumor suppression, it would be inappropriate to make decisions on whether a particular mutation would represent a wild-type or a cancer predisposing allele. For example, a particular mutation that shows 50% loss of activity in yeast could still be perfectly functional in breast and ovarian cells.

In conclusion, the data presented here suggest that the yeast assay for monitoring transcription activation by BRCA1 will provide a wealth of functional information in a research setting. That includes identifying protein-protein interaction regions, defining critical residues for activity, and providing tools to identify possible regulators. A general use of the assay to help in risk assessment and providing information for clinical decisions must await further confirmation from population-based studies.

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REFERENCES

1. Smith, S. A., Easton, D. G., Evans, D. G. R., and Ponder, B. A. J. Allele losses in the region 17q12–21 in familial breast cancer involve the wild type chromosome. Nat. Genet., *2:* 128–131, 1992.
2. Easton, D. F., Bishop, D. T., Ford, D., Crockford, G. P., and Breast Cancer Linkage Consortium. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. Am. J. Hum. Genet., *52:* 678–701, 1993.
3. Hall, J. M., Lee, M. K., Newman, B., Morrow, J. E., Anderson, L. A., Huey, B., and King, M. C. Linkage of early-onset familial breast cancer to chromosome 17q21. Science (Washington DC), *250:* 1684–1689, 1990.
4. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F. H., Helvering, L., Morrisson, P., Rostek, P., Lai, M., Barret, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., and Skolnick, M. H. A

strong candidate for the breast and ovarian cancer susceptibility gene, *BRCA1*. Science (Washington DC), *266:* 66–71, 1994.
5. Koonin, E. V., Altschul, S. F., and Bork, P. Functional Motifs. Nat. Genet., *13:* 266–267, 1996.
6. Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J., *11:* 68–76, 1997.
7. Callebaut, I., and Mornon, J. P. From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett., *400:* 25–30, 1997.
8. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell, *88:* 265–275, 1997.
9. Chen, J., Silver, D. P., Walpita, D., Cantor, S. B., Gazdar, A. F., Tomlinsom, G., Couch, F., *et al.* Stable interaction between the products of the *BRCA1* and *BRCA2* tumor suppressor genes in mitotic and meiotic cells. Mol. Cell, *2:* 317–328, 1998.
10. Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M, Feunteun, J., and Livingston, D. M. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell, *90:* 425–435, 1997.
11. Thomas, J. E., Smith, M., Tonkinson, J. L., Rubinfeld, B., and Polakis, P. Induction of phosphorylation on BRCA1 during the cell cycle and after DNA damage. Cell Growth Differ., *8:* 801–809, 1997.
12. Shen, S. X., Weaver, Z., Xu, X., Li, C., Weinstein, M., Chen, L., Guan, X. Y., Ried, T., and Deng, C. X. A targeted disruption of the murine *Brca1* gene causes γ-irradiation hypersensitivity and genetic instability. Oncogene, *17:* 3115–3124, 1998.
13. Monteiro, A. N. A., August, A., and Hanafusa, H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc. Natl. Acad. Sci. USA, *93:* 13595–13599, 1996.
14. Monteiro, A. N. A., August, A., and Hanafusa, H. Common variants of *BRCA1* and transcription activation. Am. J. Hum. Genet., *61:* 761–762, 1997.
15. Chapman, M. S., and Verma, I. M. Transcription activation by BRCA1. Nature (Lond.), *382:* 678–679, 1996.
16. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. BRCA1 is a component of the RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. USA, *94:* 5605–5610, 1997.
17. Anderson, S. F., Schlegel, B. P., Nakajima, T., Wolpin, E. S., and Parvin, J. D. BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. Nat. Genet., *19:* 254–256, 1998.
18. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. Arrest of the cell-cycle by the tumor suppressor BRCA1 requires the CDK-inhibitor $p21^{Waf1/CiP1}$. Nature (Lond.), *389:* 187–190, 1997.
19. Ouchi, T., Monteiro, A. N. A., August, A., Aaronson, S. A., and Hanafusa, H. BRCA1 regulates p53-dependent gene expression. Proc. Natl. Acad. Sci. USA, *95:* 2302–2306, 1998.
20. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., and El-Deiry, W. S. BRCA1 physically associates with p53 and stimulates its transcriptional activity. Oncogene, *16:* 1713–1721, 1998.
21. Friedman, L. S., Ostermeyer, E. A., Szabo, C., Dowd, P., Lynch, E. D., Rowell, S. E., and King, M. C. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. Nat. Genet., *8:* 399–404, 1994.
22. Humphrey, J. S., Salim, A., Erdos, M. R., Collins, F. S., Brody, L. C., and Klausner, R. D. Human *BRCA1* inhibits growth in yeast: potential use in diagnostic testing. Proc. Natl. Acad. Sci. USA, *94:* 5820–5825, 1997.
23. Monteiro, A. N. A., and Humphrey, J. S. Yeast-based assays for detection and characterization of mutations in BRCA1. Breast Disease, *10:* 61–70, 1998.
24. Bartel, P. L., Chien, C. T., Sternglanz, R., and Fields, S. Elimination of false positives that arise in using the two-hybrid system. BioTechniques, *14:* 920–924, 1993.
25. Golemis, E. A., Gyuris, J., and Brent, R. Two-hybrid system/interaction traps. *In:* F. M. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. A. Smith, and K. Struhl (ed.), Current Protocols in Molecular Biology, pp. 13.14.1–13.14.17. New York: John Wiley & Sons, 1994.
26. Estojak, J., Bren, R., and Golemis, E. A. Correlation of two-hybrid affinity data with *in vitro* measurements. Mol. Cell. Biol., *15:* 5820–5829, 1995.
27. Brent, R., and Ptashne, M. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell, *43:* 729–736, 1985.
28. Strathern, J. N., and Higgins, D. R. Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors. Methods Enzymol., *194:* 319–329, 1991.
29. Hallet, B., Sherratt, D. J., and Hayes, F. Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. Nucleic Acids Res., *25:* 1866–1867, 1997.
30. Hayes, F., Hallet, B., and Cao, Y. Insertion mutagenesis as a tool in the modification of protein function: extended substrate specificity conferred by pentapeptide insertions in the Ω-loop of TEM-1 β-lactamase. J. Biol. Chem., *272:* 28833–28836, 1997.
31. Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z., Soderkvist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J. D., Marks, J., Ballinger, D. G., Barret, J. C., Skolnick, M. H., Kamb, A., and Wiseman, R. *BRCA1* mutations in primary breast and ovarian carcinomas. Science (Washington DC), *266:* 120–122, 1994.
32. Shattuck-Eidens, D., McClure, M., Simard, J., Labrie, F., Narod, S., Couch, F., Weber, B., Castilla, L., Erdos, M., Brody, L., Friedman, L., Ostermeyer, E., Szabo, C., King, M. C., Jhanwar, S., Offit, K., Norton, L., Gilewski, T., Lubin, M., Osborne, M., Black, D., Boyd, M., Steel, M., Ingles, S., Haile, R., Lindblom, A., Olsson, H., Borg, A., Bishop, D. T., Solomon, E., Radice, P., Spatti, G., Gayther, S., Ponder, B., Warren, W., Stratton, M., Liu, Q., Fujimura, F., Lewis, C., Skolnick, M., and Goldgar, D. A collaborative survey of 80 mutations in *BRCA1* breast and ovarian cancer susceptibility gene: implications for presymptomatic testing and screening. J. Am. Med. Assoc., *273:* 535–541, 1995.
33. Stoppa-Lyonet, D., Laurent-Puig, P., Essioux, L., Pagès, S., Ithier, G., Ligot, L., Fourquet, A., Salmon, R. J., Clough, K. B., Pouillart, P., Bonaiti-Pellie, C., and Thomas, G. BRCA1 sequence variations in 160 individuals referred to a breast/ovarian family cancer clinic. Am. J. Hum. Genet., *60:* 1021–1030, 1997.
34. Gayther, S. A., Harrington, P. A., Russell, P. A., Kharkevich, G., Gargavtseva, R. F., Ponder, B. A. J., and the UKCCCR Familial Ovarian Cancer Study group. Rapid detection of regionally clustered germ-line *BRCA1* mutations by multiplex heteroduplex analysis. Am. J. Hum. Genet., *58:* 451–456, 1996.
35. Szabo, C. I., and King, M. C. Inherited breast and ovarian cancer. Hum. Mol. Genet., *4:* 1811–1817, 1995.
36. Zhang, X., Moréra, S., Bates, P. A., Whitehead, P. C., Coffer, A. I., Hainbucher, K., Nash, R. A., Sternberg, M. J., Lindahl, T., and Freemont, P. S. Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. EMBO J., *17:* 6404–6411, 1998.
37. Cao, Y., Hallet, B., Sherratt, D. J., and Hayes, F. Structure-function correlations in the XerD site-specific recombinase revealed by pentapeptide scanning mutagenesis. J. Mol. Biol., *274:* 39–53, 1997.
38. Gill, G., and Ptashne, M. Mutants of GAL4 protein altered in an activation function. Cell, *51:* 121–126, 1987.
39. Szabo, C. I., Wagner, L. A., Francisco, L. V., Roach, J. C., Argonza, R., King, M. C., and Ostrander, E. A. Human, canine and murine *BRCA1* genes: sequence comparison among species. Hum. Mol. Genet., *5:* 1289–1298, 1996.
40. Bennett, L. M., Brownlee, H. A., Hagavik, S., and Wiseman, R. W. Sequence analysis of the rat brca1 homolog and its promoter region. Mamm. Genome, *10:* 19–25, 1999.
41. Durocher, F., Shattuck-Eidens, D., McClure, M., Labrie, F., Skolnick, M. H., Goldgar, D. E., and Simard, J. Comparison of *BRCA1* polymorphisms, rare sequence variants and/or missense mutations in unaffected and breast/ovarian cancer populations. Hum. Mol. Genet., *5:* 835–842, 1996.