# Dynamic interactions between the promoter and terminator regions of the mammalian *BRCA1* gene

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Communicated by Aaron J. Shatkin, Center for Advanced Biotechnology and Medicine, Piscataway, NJ, February 1, 2008 (received for review December 13, 2007)

**The 85-kb breast cancer-associated gene *BRCA1* is an established tumor suppressor gene, but its regulation is poorly understood. We demonstrate by gene conformation analysis in both human cell lines and mouse mammary tissue that gene loops are imposed on *BRCA1* between the promoter, introns, and terminator region. Significantly, association between the *BRCA1* promoter and terminator regions change upon estrogen stimulation and during lactational development. Loop formation is transcription-dependent, suggesting that transcriptional elongation plays an active role in *BRCA1* loop formation. We show that the *BRCA1* terminator region can suppress estrogen-induced transcription and so may regulate *BRCA1* expression. Significantly, *BRCA1* promoter and terminator interactions vary in different breast cancer cell lines, indicating that defects in *BRCA1* chromatin structure may contribute to dysregulated expression of *BRCA1* seen in breast tumors.**

transcriptional regulation | chromatin conformation | gene repression | mammary gland | breast cancer

Expression of the tumor suppressor gene *BRCA1* is reduced in a significant proportion of human breast tumors (1–3). Although up to one-third of these cases can be explained by promoter hypermethylation (4, 5) for most cases the cause is unknown. Understanding the underlying mechanisms of *BRCA1* gene repression is critical for generating effective strategies for re-establishing *BRCA1* expression and thus restoring its tumor suppressor function.

Transcriptional initiation of protein-coding genes depends on a coordinated interplay of protein–DNA and protein–protein interactions (6). In addition to the assembly of RNA polymerase II (Pol II) with basal transcription machinery on the gene promoter, numerous transcription factors are recruited to either activate or repress transcription. As many of these factors associate with DNA sequences distant to the promoter, transcriptional regulation often involves long-range DNA associations, possibly mediated by the formation of chromatin loops (7). Chromatin loops can be detected by the chromosome conformation capture (3C) technique (8), which involves formaldehyde cross-linking of chromatin in live cells, digesting DNA with restriction enzymes, and then religating DNA in dilute solution to favor intramolecular ligation. PCR is then used to detect the presence of such ligation products. 3C has been used to study the normal regulation of genes in multiple eukaryotic species and supports a looping model for gene activation and repression. For example, transcriptional activation of the $\beta$-globin gene in mouse is associated with interactions between multiple hypersensitive sites spanning >50 kb of DNA (9), whereas repression of the maternal *IGF2* gene is linked to a long-range association between *IGF2* and *H19* loci, restricting access to an *IGF2* enhancer (10).

Several human diseases are associated with mutations in long-range control elements (11). Examples include Campomelic dysplasia, which can be caused by deletion of critical regulatory elements ≈50 kb upstream of the *SOX9* gene (12), Aniridia, which is associated with mutations up to 75 kb 3′ of the Aniridia gene *PAX6* (13), and Blepharophimosis syndrome, where deletion of conserved sequences 230 kb upstream of the *FOXL2* gene has been detected in some patients (14).

*BRCA1* transcription is controlled at least in part by a bidirectional promoter (15, 16), the activity of which can be modulated by positive and negative regulatory sequences within *BRCA1* introns (17). A 140-kb P1 artificial chromosome containing human *BRCA1* plus 60 kb of flanking sequence can rescue the embryonic lethal phenotype of *Brca1* null mice (18), suggesting that all of the sequences required for correct temporal and spatial expression are contained within this sequence. The identity of these elements, how they associate with one another, and whether they contribute to breast tumorigenesis is unknown.

We describe the analysis of potential long-distance interactions associated with *BRCA1* and demonstrate the existence of *BRCA1* gene loop structures between the promoter and sequences including the introns and the termination region. Significantly, this latter gene loop structure is altered in response to estrogen stimulation and in several breast cancer cell lines.

## Results and Discussion

**Long-Range Associations Involving the BRCA1 Promoter and Regulatory Regions.** We performed 3C analysis on the human *BRCA1* gene, using primers flanking either BanI and DpnII restriction sites. Initially our studies focused on previously characterized regulatory regions of *BRCA1*: the promoter (16) including 3 kb upstream of transcription; an evolutionarily conserved region in intron 2 (17), and the 3′ end of the gene, including the 3′UTR (19) and 2 kb downstream of exon 24 (Fig. 1*A*). Critical controls are essential for correct interpretation of 3C data (20). We therefore confirmed that all 3C primers amplified *in vitro*-generated 3C products (Fig. 1 *Bi* and *Ci*, labeled 3C positive controls), there was a nonlinear relationship between detection of 3C products and distance between associated restriction fragments (Fig. 1 *Bi* and *Ci Top*), 3C primers did not amplify undigested and ligated, or digested but not ligated, chromatin (data not shown), and the sequence of all 3C products was correct (data not shown).

Using these validated 3C primers, we initially analyzed the conformation of *BRCA1* in the breast cancer cell line MCF7. 3C analysis of chromatin from cells grown in defined media (serum-free phenol-red free) showed that the *BRCA1* 5′ region (primers B2 and D3) associates with sequences in *BRCA1* intron 2

Author contributions: S.M.T.-W. and J.D.F. contributed equally to this work; S.M.T.-W., N.J.P., and M.A.B. designed research; S.M.T.-W., J.D.F., and M.A.B. performed research; S.M.T.-W., J.D.F., and M.A.B. analyzed data; and S.M.T.-W., N.J.P., and M.A.B. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0801048105/DCSupplemental.

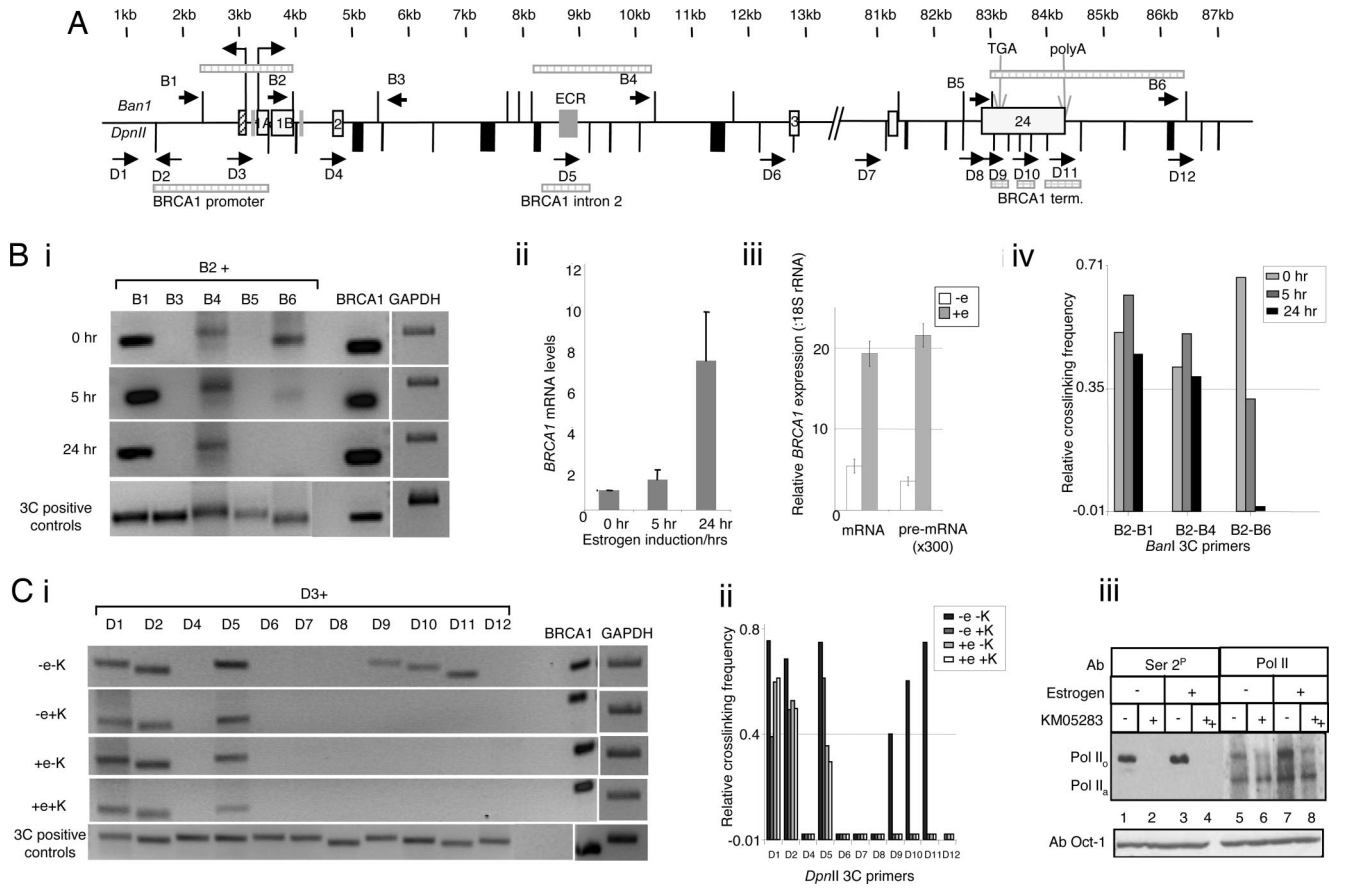


**Fig. 1.** Dynamic long-range association between regulatory regions of *BRCA1*. (*A*) Diagram of *BRCA1*, exons 1–24 (bold lines) flanked by 3 kb upstream of exon 1 and 2 kb downstream of exon 24 (thin lines) with BanI (above sequence) and DpnII (below sequence) sites indicated. Upright tall arrows indicate divergent transcription initiation positions for *NBR2* and *BRCA1*. *BRCA1* exons are indicated by open rectangles, and an *NBR2* exon is indicated by a striped rectangle. The gray rectangles indicate regions known to possess regulatory (promoter, enhancer, or repressor) activity. Vertical lines represent restriction sites. Black labeled arrows indicate primer direction. Checked boxes represent restriction fragments involved in gene looping based on 3C analysis. (*B*) (*i*) BanI 3C analysis of *BRCA1* in the MCF7 breast adenocarcinoma cell line before and after 5 or 24 h of 100-nM estrogen stimulation and artificial 3C products (see *Materials and Methods*) using primer B2 in combination with one of the other BanI primer as indicated. *BRCA1* load represents the product of PCR analysis of the same BanI 3C using *BRCA1*-specific internal primers in intron 2. (*ii*) qRT-PCR analysis of *BRCA1* mRNA, relative to 18S rRNA, after induction with 100 nM estrogen showing a slight increase after 5 h and a significant increase after 24 h. (*iii*) qRT-PCR analysis of *BRCA1* mRNA and pre-mRNA, relative to 18S rRNA, after induction with 100 nM estrogen showing a significant increase in both after 24 h of stimulation. (*iv*) Quantitation of 3C band intensity relative to intensity of 3C control bands (see *Materials and Methods*). (*C*) 3C and Western analysis of MCF7 cells that are either unstimulated or stimulated with 100 nM E2 for 24 h and then untreated or treated with the transcription inhibitor KM05283 for 1 h. (*i*) 3C analysis of MCF7 cells grown in serum-free phenol red free media (−e) or stimulated with 100 nM estrodiol for 24 h (+e), and either untreated (−K) or treated with KM05283 for 1 h (+K). 3C-positive controls and *BRCA1* loading control were as for *Bi*. (*ii*) Quantitation of 3C band intensity relative to intensity of 3C control bands (see *Materials and Methods*). (*iii*) Western analysis with antibodies against Pol II Ser-2 phosphoylated CTD (Ser 2P) or the N terminus of the large subunit of Pol II showing that the transcription inhibitor has worked. Anti-Oct antibody provides loading control.

(primers B4 and D5) and sequences at the 3′ end of the gene (primers B6 and D9 and primers D10 and D11; Fig. 1 *B* and *C*). No evidence for interactions between the *BRCA1* promoter and sequences elsewhere in intron 2 (primers D4 and D6), intron 22 (primer D7), intron 23 (primer B5), or 2 kb downstream of exon 24 (primer D12) was found.

**The Association Between 5′ and 3′ Ends of BRCA1 Is Lost upon Estrogen Stimulation.** To investigate whether induction of *BRCA1* expression was associated with changes in the 3C profile, we examined the effect of stimulating MCF7 cells with estrogen ($\beta$-estradiol; E2). This treatment induces *BRCA1* mRNA levels (21, 22), indirectly through associated changes in cell proliferation (23). We therefore analyzed *BRCA1* transcription levels in MCF7 cells either without E2 (defined media as above) or after 5 and 24 h of E2 stimulation. Quantitative RT-PCR (qRT-PCR) showed that *BRCA1* mRNA levels increased slightly after 5 h and 5- to 7-fold after 24 h (Fig. 1*Bii* and ref. 24). Using RT-PCR primers that discriminate between pre-mRNA and mature mRNA (19), we also showed that increased *BRCA1* expression occurred largely at the pre-mRNA level, indicating that E2 treatment activates transcription rather than increases mRNA stability (Fig. 1*Biii*). 3C analysis was performed by using the BanI 3C primers described above and showed a significant decrease in the 3C product after 5 h of estrogen stimulation between the 5′ and 3′ ends of *BRCA1* (Fig. 1 *Bi* and *Biv*). Significantly, after 24 h *BRCA1* 5′ to 3′ end association was no longer detectable. Consistent with the results of BanI 3C, the association between the *BRCA1* promoter and terminator region detected by DpnII 3C was also lost after estrogen stimulation (Fig. 1 *Ci* and *Cii*). In contrast, association between the 5′ end and intron 2 of *BRCA1* was unchanged upon E2 stimulation. Overall, these data indicate that the 5′ and 3′ ends of *BRCA1* are juxtaposed when gene expression is repressed and released upon transcriptional induction. The timing of the change in *BRCA1* chromatin structure further suggests that it is likely to be an indirect consequence of

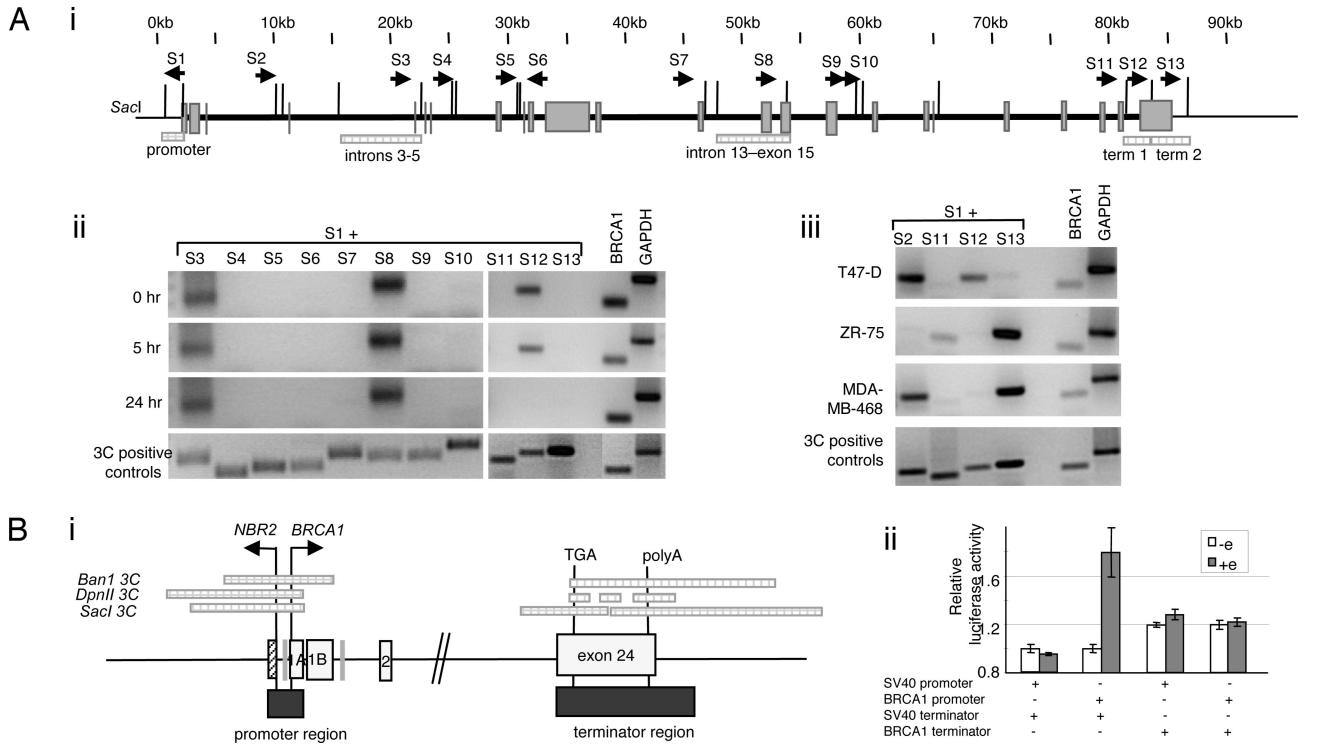


**Fig. 2.** Estrogen-responsive promoter-terminator gene loop of *BRCA1*. (*A*) SacI 3C analysis across *BRCA1*, before and after estrogen stimulation. (*i*) Map of *BRCA1* showing SacI restriction sites with relevant exons and primers. 3C-positive fragments are indicated by checked boxes. (*ii*) SacI 3C analysis of MCF7 cells grown in serum-free phenol-red free media (0 h) or treated with 100 nM E2 for 5 or 24 h. 3C-positive controls and loading controls are as for Fig. 1. (*iii*) SacI 3C analysis of three different human breast cancer cell lines grown in complete media (T47-D, ZR-75, MDA-MB-468) show differential chromatin loops. (*B*) (*i*) Map showing the overlap between interacting 3C fragments identified by using three different restriction fragments and the corresponding sequences used to examine the regulatory activity of *BRCA1* promoter and terminator sequences in reporter assays (dark shaded boxes). (*ii*) Luciferase activity in cells transfected with reporter constructs containing combinations of the SV40 or *BRCA1* promoter and the SV40 and *BRCA1* terminator region and either grown in serum-free phenol-red free media (empty bars) or stimulated with 100 nM E2 (filled bars). Data shows that the significant induction of the *BRCA1* promoter by E2 ($P = 0.008$) is suppressed in the presence of the *BRCA1* terminator ($P = 0.67$).

E2 stimulation, as is E2-mediated induction of *BRCA1* (23). Interestingly, for *FMP27* and *SEN1* in yeast, a similar promoter and terminator association was detected. This gene loop conformation was associated with the promoter in a transcriptionally poised state but not under conditions of complete repression (24, 25).

**Long-Range Associations Between 5′ and 3′ Ends of BRCA1 Depend on Basal Transcription.** To further investigate the mechanism of promoter and terminator interaction in *BRCA1*, we examined the effect of transiently inhibiting transcription after E2 induction. We used KM05283, which specifically inhibits transcriptional elongation by blocking phosphorylation of Pol II on the carboxyl-terminal domain (CTD) Ser-2 (26) (Fig. 1*Ciii*). DpnII 3C analysis of cells treated with KM05283 revealed that association between *BRCA1* promoter and terminator does not occur if transcription is inhibited. This finding suggests that although the observed promoter-terminator loop is associated with gene repression, transcription is required for this loop to form, which raises the possibility that either transcription of a regulatory molecule is required for loop formation, or transcriptional elongation plays an active role. It is also possible that *BRCA1* is autoregulated so that when threshold levels of *BRCA1* mRNA are reached, loop formation is initiated to repress future transcription and so maintain low levels required for certain physiological situations (see Fig. 4). Precedents exist for negative transcriptional autoregulatory mechanisms in genes encoding Mashi, a neural-specific basic helix–loop–helix transcription factor (27) and Hairy-related transcription factors (28).

**Other Long-Range Associations Across the BRCA1 Gene Exist, but Do Not Change in Response to Estrogen.** To address the possibility that other long-range associations occur between the 5′ end of *BRCA1* and other regions of the gene, we performed 3C analysis with primers mapping to 10 SacI restriction fragments spanning ≈100 kb, including *BRCA1* and ≈2 kb of 5′ and 3′ flanking region (Fig. 2*Ai*). This analysis revealed two additional gene loops, one between sequences in the 5′ end of *BRCA1* (primer S1) and sequences in a region spanning introns 3–5 (primer S3) and another region spanning intron 13 to exon 15 (primer S8; Fig. 2*Aii*). No 3C products were detected in MCF7 cells by using validated combinations of primers mapping to the 5′ end and sequences intron 7 (primer S4), intron 8 (primers S5 and S6), intron 13 (primer S7), intron 16 (primer S9), intron 23 (primer S11), or sequences 2 kb downstream of exon 24 (primer S13). In contrast to the association between *BRCA1* 5′ end and 3′ end, associations between the 5′ end of *BRCA1* and sequences detected by primers S3 and S8 were unchanged upon E2 stimulation (Fig. 2*Aii*).

**Altered BRCA1 Chromatin Loops in Breast Cancer Cell Lines.** *BRCA1* mRNA levels are lower in most breast tumors than in normal tissue (1). To begin to address the possibility that this observation may be caused by differential gene loop associations across the *BRCA1* gene, resulting in abnormal regulation of *BRCA1* gene expression, we performed 3C analysis on three additional cell lines derived from breast tumors: T47-D, ZR-75, and MDA-MB-468. 3C products were detected in the three cell lines (Fig. 2*Aiii*). In T47-D cells, we observed the same association

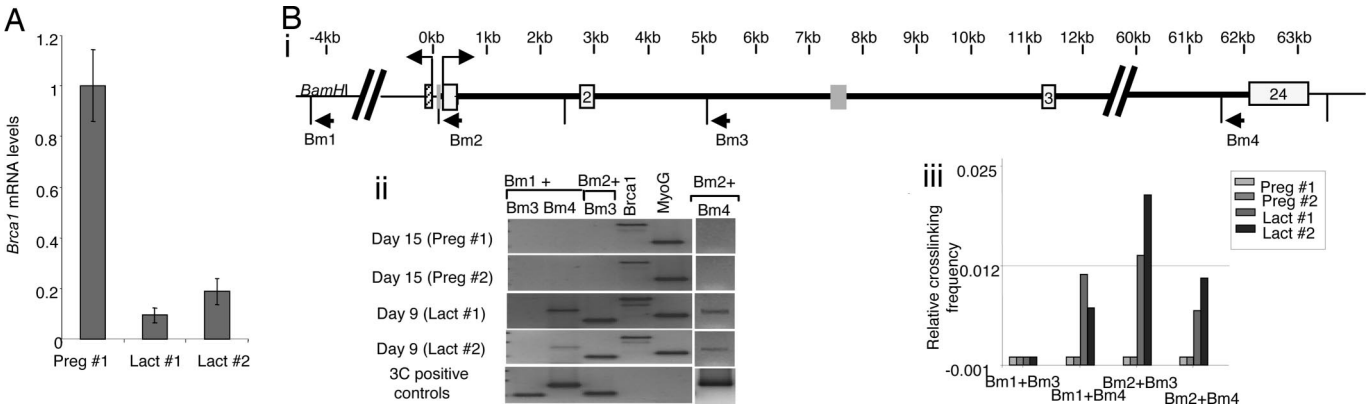


**Fig. 3.** Repression of mouse *Brca1* is also associated with long-range associations. (*A*) qRT-PCR of *Brca1* mRNA levels relative to 18S rRNA in mammary glands from mice at day 15 of pregnancy (Preg #1) or day 9 of lactation (Lact #1 and Lact #2). Quantitation was performed by using the comparative ΔCt method as described in *Materials and Methods*. (*B*) BamHI 3C analysis of *Brca1* in pregnant and lactating mammary glands. (*i*) Map of mouse *Brca1* showing BamHI restriction sites and relevant exons and primers as above. (*ii*) 3C analysis of chromatin extracted from pregnant and lactating mammary glands from two independent mice (nos. 1 and 2). 3C-positive controls and *Brca1* and MyoG loading controls are as described above and in *Materials and Methods*. (*iii*) Quantitation of 3C band intensity relative to intensity of 3C control bands (see *Materials and Methods*).

between *BRCA1* promoter and terminator (primers S1 and S12), as seen with MCF7 cells (Fig. 2*Aii*). However, this association was not observed in ZR-75 and MDA-MB-468 cells where instead another promoter–terminator association was observed involving the adjacent 3′ SacI fragment (primers S1 and S13). This interesting difference between promoter–terminator region associations in the different breast tumor cell lines may relate to their different properties. Significantly, *BRCA1* has been shown to have altered promoter DNA–protein interactions in a cell line in which *BRCA1* expression is undetectable (30). Although BRCA1 repression may be caused by the associated promoter hypermethylation, an alternate explanation could be the formation of an aberrant chromatin loop between the 5′ and 3′ ends of the gene.

**Estrogen-Mediated Induction of the BRCA1 Promoter *in Vitro* Is Repressed by Sequences in the 3′ End of BRCA1.** Our results suggest that sequences at the 5′ and 3′ ends of *BRCA1* colocalize and that this event is associated with transcriptional repression. To investigate the possibility that this association contributes to the regulation of *BRCA1*, we generated luciferase reporter constructs driven by either the simian virus 40 (SV40) or the *BRCA1* promoters and followed by sequences from the 3′ end of either SV40 or *BRCA1*, including both polyadenylation and terminator sequences. A map showing how the fragments used in these assays correspond to the 3C-positive restriction fragments is shown in Fig. 2*Bi*. We then examined the luciferase activity of cells transfected with each of these four constructs, before and after E2 induction. The *BRCA1*, but not the SV40 promoter, responds to E2 stimulation when followed by 3′ sequences from SV40 (Fig. 2*Bii*). This finding is consistent with previous reports (16) and with our data showing that induction of *BRCA1* occurs at a transcriptional level (Fig. 1*Biii*). Interestingly, when sequences from the 3′ end of *BRCA1* replace the SV40 3′ sequences, this induction is no longer evident. Possibly, in the context of this reporter system, the 3′ end of *BRCA1* represses estrogen-mediated induction of the *BRCA1* promoter. Transcriptional repressor elements mapping to the 3′ end of other genes have been described, including one in the 3′ UTR of the cyclin-dependent kinase inhibitor p21 (29) and three in the 3′ UTR of the serine protease inhibitor 2.3 gene (26). The existence of a transcriptional repressor element in the 3′ region of *BRCA1* raises the possibility that the promoter–terminator interaction participates in this transcriptional repression mechanism.

**BRCA1 Chromatin Loops in Mouse Mammary Tissue.** If chromatin looping is an important means of regulating *BRCA1* expression, we predict that it may be an evolutionarily conserved process. We therefore performed 3C analysis on chromatin-extracted epithelial cells isolated from mouse mammary glands during lactational development. *Brca1* mRNA levels are known to be induced during pregnancy and decrease during lactation (refs. 31 and 32 and confirmed by qRT-PCR in Fig. 3*A*).

A map of the mouse *Brca1* gene and the primers used in 3C experiments is shown in Fig. 3*Bi*. Again, primers were selected for their suitability in 3C analysis, and the 3C products obtained were verified by sequence analysis. 3C analysis indicated the presence of *Brca1* chromatin loops in mammary epithelial cells during lactation (Fig. 3*Bii*), when *Brca1* expression was repressed. As with our findings for human *BRCA1* (Figs. 1 and 2), we observed associations between the 5′ region of *Brca1* contained within a 4.5-kb BamH1 fragment detected by primer Bm1 or the adjacent 2-kb nucleotide BamHI fragment (detected by primer Bm2) and 3′ region of *Brca1* (1.9-kb BamHI fragment containing exon 24, detected by primer Bm4). Significantly, this association was not detectable in mammary epithelia from pregnant mice, when *Brca1* expression is induced (Fig. 3*A*). This result supports the hypothesis that the *BRCA1* promoter and terminator regions are juxtaposed when *BRCA1* expression is repressed and that this association is released when gene expression is induced.

A 3C product consistent with an association between the promoter region and intron 2 of *Brca1* was also observed (detected by primers Bm2 and Bm3). Interestingly, the intron 2 fragment contains the mouse orthologue of the conserved transcriptional regulatory sequence identified in human *BRCA1* (17). If this represents the same *BRCA1* promoter to intron 2 association observed in human MCF7 cells, it is interesting that, whereas the 3C pattern is unchanged upon estrogen induction in human cells, it is only present in mouse cells when *Brca1* is repressed. This temporal difference may reflect the more complex regulatory pathways controlling *BRCA1* expression *in vivo* in mouse mammary glands versus effects of estrogen on cultured human breast cancer cell lines.

**A Possible Model for BRCA1 Gene Looping.** We propose a model for transcriptional regulation of the human *BRCA1* by gene looping (Fig. 4). We depict the uninduced *BRCA1* in a "four-leaf clover" conformation, where the promoter associates with sequences in

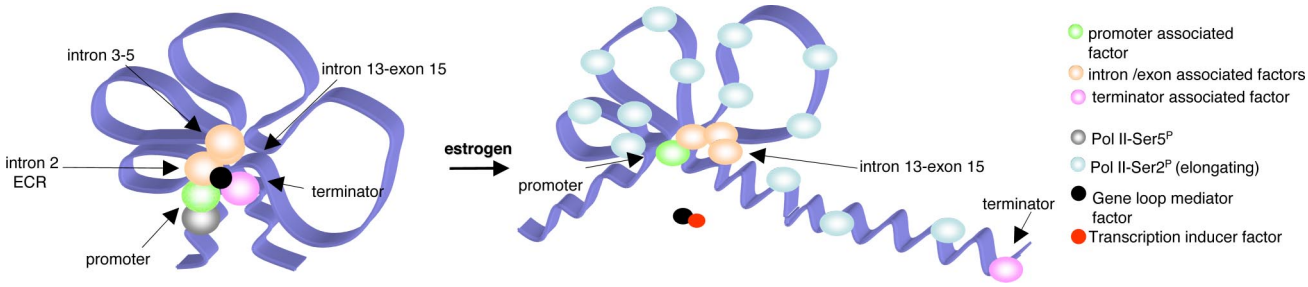


**Fig. 4.** Proposed model of *BRCA1* gene looping. Diagram shows two possible states of the human *BRCA1* gene during transcriptional regulation. (*Left*) A factor (black) mediates a long-range association between the *BRCA1* promoter and terminator, which in turn drives formation of a chromatin loop preventing further transcription. We refer to this as regulated repression of *BRCA1*, based on our finding that loop formation is transcription-dependent. (*Right*) When transcription of *BRCA1* is induced (e.g., upon estrogen stimulation), we propose that expression of another factor (red) is induced that interferes with the function of the black factor, causing chromatin to become relaxed and promote transcription.

the *BRCA1* introns and with the terminator region (Figs. 1 and 2). We propose that a "gene loop mediator" factor, which could be protein or RNA, recruits proteins and/or RNAs that associate with the promoter and terminator regions of *BRCA1*, juxtaposing them and the DNA sequences to which they interact. In this looped conformation transcription of *BRCA1* is disabled, maintaining *BRCA1* expression in a "regulated repressed" state. We suggest that the repression is regulated because the associated gene loop is transcription-dependent (Fig. 1*C*). Possibly new transcription is required to synthesize the mediator factor. The stimulus for such transcription could be any physiological situation necessitating down-regulation of *BRCA1* and could even be regulated by *BRCA1* itself, potentially representing an autoregulatory loop. It is known that overexpression of *BRCA1* can be toxic, so the existence of such a regulatory mechanism is a reasonable, albeit unproven, possibility.

When *BRCA1* transcription is induced, for example as a consequence of estrogen stimulation, we predict that the expression of another regulatory factor is induced. We hypothesize that this factor is able to sequester the mediator protein proposed above, thus allowing the chromatin conformation to relax into a "three-leaf clover" conformation. In this conformation the *BRCA1* promoter is sufficiently exposed to allow RNA Pol II to perform its role in transcriptional elongation.

## Conclusions

These studies demonstrate an interaction between the promoter and terminator region of *BRCA1* that is associated with gene silencing. Further investigations into the molecular mechanisms and consequences of such *BRCA1* gene loops and whether they contribute to breast tumorigenesis are clear priorities for the future.

## Materials and Methods

**Cell Culture.** MCF7, T47-D, ZR-75, and MDA-MB-468 cells were obtained from Cancer Research UK and grown according to their recommendations. MCF7 cells were grown in serum and phenol red free RPMI medium 1640 (Invitrogen) for 24 or 48 h, then washed twice and grown in the same media, with or without $10^{-8}$ M estrogen (Sigma) for 5 or 24 h.

**Animals.** Female C57BL/6 mice were obtained from the University of Queensland Central Animal Breeding House at 6 weeks of age. At 10 weeks they were estrous-matched and mated, with conception being diagnosed by vaginal plugs. Two independent pools of at least two pregnant mice were killed 15 days later, and gestation date was confirmed by morphological examination of fetuses. Two independent pools of two lactating mice were killed 9 days after parturition. Inguinal, abdominal, and thoracic mammary glands were collected, microdissected from any associated lymph nodes, fat, tendons, or musculature, and pooled. Mammary epithelial cells were isolated (33) by mincing tissue with scalpel blades, digesting with 12 mg/g collagenase (Worthington), filtering through 100-$\mu$m mesh and separating from stromal cells by differential adhesion to tissue culture plastic after 1 h. Unattached cells were then divided and used immediately for either 3C or expression analysis.

**3C Analysis.** 3C analysis was performed as described (34). Briefly, chromatin was cross-linked with 1% formaldehyde and nuclei were isolated by using Nonidet P-40. DNA was digested with 800 units of restriction enzyme and ligated in 8 ml of 1× ligation buffer. 3C products were phenol/chloroform-extracted, ethanol-precipitated, and quantitated by spectroscopy before PCR analysis as follows: initial denaturation of 95°C/5 min, 35 cycles of 95°C/45s, 60°C/45s, 72°C/60s, final extension of 72°C/5 min. Restriction enzymes were chosen based on their proximity to regions of interest, size of restriction sites flanking these regions, and their lack of colocalization with repetitive elements in *BRCA1* using the sequence information available from the National Center for Biotechnology Information database [human *BRCA1* accession no. L78833; mouse *BRCA1* accession nos. NT_033681 (exons 1–24 and intervening introns) and AL590996 (flanking sequences)]. 3C primers were designed by using the Primer 3 program (http://frodo.wi.mit.edu) under the following criteria: optimal length, 26 nt (range 24–28); optimal melting temperature, 62°C (range 60–64); optimal size, 200 nt with restriction site midway between the two primers. Primers for human and mouse *BRCA1* were obtained from Sigma-Aldrich and Geneworks. Artificial 3C products used in controls were generated by digesting and ligating PCR products spanning 100 nt 5′ and 3′ of the restriction sites. Two types of loading controls were also included to control for the efficiency of the 3C method and for the total amount of DNA used in the 3C analysis. First, a nonestrogen responsive housekeeping gene (*hGAPDH* and *mMyoG*) was analyzed by SacI, BanI, and DpnII 3C PCR in human-derived samples and BamHI 3C PCR in the case of mouse. Second, a *BRCA1* loading control was generated by amplifying the same 3C chromatin with primers mapping to *BRCA1* intron 2. Primer sequences are provided in supporting information (SI) Table S1 and Table S2.

***BRCA1* mRNA Analysis (qRT-PCR).** Total RNA was extracted from either cultured cell lines or primary mammary epithelial cells by using TRIzol (Invitrogen) and DNase-treated with DNA-free (Ambion). cDNA was synthesized from 5 $\mu$g of RNA by using *BRCA1* and 18S-specific reverse transcriptase primers and SuperScript III (Invitrogen). *BRCA1* pre-mRNA, *BRCA1* mRNA, and 18S rRNA transcripts were quantitated by using qPCR with SYBR green reaction mix (Qiagen) or SYBR green PCR master mix (Applied Biosystems), primer mix (200 nM for *BRCA1* mRNA; 50 nM for *BRCA1* pre-mRNA and 18S rRNA). All RT-PCR primers flanked large intronic sequences, thus preventing amplification of genomic DNA. Cycling conditions were: 10 min (95°C), followed by 45 cycles of 15 s (95°C) and 1 min (58°C). PCR products were quantitated by using the ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS software (v2.2.2; Applied Biosystems). No reverse transcriptase controls were conducted for genomic DNA contamination. The comparative ΔCt method (Applied Biosystems) was used to determine relative *BRCA1* mRNA and pre-mRNA expression, taking into account the primer set efficiencies (*E* values): target/18S = $(E_{18S})^{Ct18S}/(E_{target})^{Cttarget}$ (35). Mean *E* values calculated from two independent experiments were: *BRCA1* pre-mRNA, 1.9544; *BRCA1* mRNA, 1.7364, 18S rRNA, 1.9998.

**Luciferase Assays.** Luciferase reporter plasmids were generated by using sequences from either promoter or terminator regions of SV40 (from pGL3; Promega) or the *BRCA1* promoter (499 bp) fragment containing *NBR2* exon 1, *BRCA1* exon 1A, and the intervening promoter sequences (17) or the *BRCA1*

terminator region (2,222 bp) fragment containing the *BRCA1* exon 24 plus 500 bp of sequence after the polyadenylation signal. MCF7s were plated on 24-well plates in phenol red free RPMI medium 1640 containing 10% FBS. The next day, the serum was removed, and the cells were serum-starved for 24 h before transfection. Cells were transfected under serum-free conditions with 0.8 $\mu$g of the luciferase reporter plasmid and 20 ng of pRL-TK by using Lipofectamine 2000 (Invitrogen) for 24 h. Cells were induced with 100 nM E2, and relative luciferase activity was determined 24 h after induction by using a dual luciferase reporter assay kit (Promega) and a Microbeta Trilux Luminometer counter (Wallac) according to the manufacturer's instructions. To correct for any differences in transfection efficiency or cell lysate preparation, *Firefly* luciferase activity was normalized to *Renilla* luciferase. The activity of each test construct was calculated relative to no E2 treatment, the activity of which was arbitrarily defined as 1.

**Transient Inhibition of Transcriptional Elongation.** MCF7 was grown in 10-cm$^2$ dishes in phenol red free RPMI medium 1640 serum-free media for 24 h. Cells were either stimulated with 100 nM E2 for 24 h or left unstimulated. Transcription was inhibited in one of the E2-stimulated and one of the unstimulated dishes by treating cells with 100 nM KM05283 (a kind gift from S. Murphy, University of Oxford, Oxford) for 1 h. Dishes proceeded to either 3C analysis or expression analysis. For the latter, cells were washed and scraped in ice-cold PBS and extracted with either Trizol (for RNA) or Laemmli buffer (for protein). RNA was then analyzed by qRT-PCR. Protein was analyzed by Western blotting, using standard procedures. Antibodies against the N terminus of the large subunit of Pol II (H-224) (1:500; Santa Cruz biotechnology) or Ser-2 phosphorylated CTD (H5) (1:500; Covance) were used to determine the relative levels of elongating versus total Pol II. Antibody against Oct-1 (Santa Cruz) was used as a loading control.

**ACKNOWLEDGMENTS.** We thank Kelly Perkins for useful advice; Chanel Smart for expertise and help extracting primary mammary epithelial cells; and John Moore and Adrian Harris (John Radcliffe Hospital, Oxford, United Kingdom) for human cell lines. This work was supported by grants from the Wellcome Trust and the Medical Research Council (to N.J.P.) and a National Health and Medical Research Council grant (to M.A.B.). M.A.B. received a International Union Against Cancer International Cancer Technology Transfer Fellowship for sabbatical leave to Oxford. J.D.F. is supported by a National Breast Cancer Foundation Postdoctoral Fellowship.

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