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Regional methylation of the 5' end CpG island of *BRCA1* is associated with reduced gene expression in human somatic cells

FRÉDÉRIQUE MAGDINIER, LISE-MARIE BILLARD, GAËLLE WITTMANN, LUCIEN FRAPPART, MEHDI BENCHAIÏB,* GILBERT M. LENOIR, JEAN FRANÇOIS GUÉRIN,* AND ROBERT DANTE¹

Laboratoire de Génétique, UMR 5641 CNRS, UCBL1, and *Laboratoire de Biologie de la Reproduction et du Développement, UCBL1, 69373 Lyon cedex 08, France

ABSTRACT In mammals, demethylation of specific promoter regions often correlates with gene activation; inversely, dense methylation of CpG islands leads to gene silencing, probably mediated by methyl-CpG binding proteins. In cell lines and cancers, inhibition of tissue-specific genes and tumor suppressor genes expression seems to be related to such hypermethylation. The 5' end of the breast cancer predisposition gene *BRCA1* is embedded in a large CpG island of ~2.7 kb in length. In human sporadic breast cancers, the down-regulation of *BRCA1* does not seem to be related to *BRCA1* gene alterations. Southern blot analysis and the bisulfite sequencing method indicate that the *BRCA1* CpG island is regionally methylated in all human tissues analyzed and unmethylated in the gametes, suggesting a role for DNA methylation in the control of gene expression. We have therefore investigated the potential role of methyl-CpG binding proteins in the regulation of *BRCA1* gene expression. *In vitro*, partial methylation of constructs containing this region strongly inhibits gene expression in the presence of MeCP2 protein. Moreover, in the five human cell lines analyzed, chemically induced hypomethylation is associated with *BRCA1* gene activation. These data suggest that methyl-CpG binding proteins might be associated with the control of *BRCA1* gene expression and that methyl-DNA binding proteins may participate in the regulation of gene expression in mammalian cells.—Magdinier, F., Billard, L.-M., Wittmann, G., Frappart, L., Benchaïb, M., Lenoir, G. M., Guérin, J. F., Dante, R. Regional methylation of the 5' end CpG island of *BRCA1* is associated with reduced gene expression in human somatic cells.

Key Words: DNA methylation • oocytes • spermatozoa • breast cancer

GERM-LINE ALTERATIONS OF the *BRCA1* gene confer a lifetime risk of 40% for ovarian cancers and

40–80% for breast cancers (1). It is likely that *BRCA1* acts as a tumor suppressor gene (2). Indeed, in breast cancers linked to *BRCA1*, as expected for a tumor suppressor gene, allelic deletions at this locus invariably involve the wild-type allele (3, 4). *BRCA1* involvement in breast cancers does not seem to be restricted to familial cancers. Despite the absence of somatic mutation in the breast tissues, a down-regulation of *BRCA1* expression is associated with malignancy in human sporadic breast cancers (4–6).

Although its biological function is still unknown, *BRCA1* may have an important role in cellular differentiation and proliferation. In transgenic mice, homozygous disruption of the *Brcal* gene results in embryonic lethality (7). The progressive changes in *Brcal* expression during mouse embryogenesis (8) also imply a role for *Brcal* in the differentiation process. In addition, variations of *Brcal* expression are observed during postnatal mammary gland development (9, 10). More recently, it had been shown that Cre-mediated invalidation of this gene affects final differentiation of the gland during gestation (11). In humans, the up-regulation of *BRCA1* gene expression observed during the first stages of prenatal development of the mammary gland also suggests a role for *BRCA1* in the differentiation of the mammary gland (12). Taken together, these data indicate that variations of *BRCA1* expression may have some physiological consequences in human breast tissue.

Analysis of the genomic region containing this gene indicates that another gene, *NBR2*, lies head to head with the *BRCA1* gene (13). Site-directed deletion mutagenesis experiments led to the identification of a bidirectional promoter region, position –258 to +43 (14), and a minimal positive regulatory region has been mapped at position –198 to –162 (15). Structural studies have shown that a minor part

¹ Correspondence: Laboratoire de Génétique, UMR 5641 CNRS, Domaine Rockefeller, UCBL1, 8 avenue Rockefeller, 69373 Lyon cedex 08, France. E-mail: dante@cismisun.univ-lyon1.fr

of breast cancers (~10%) exhibits hypermethylated sites in the region containing the putative *BRCA1* promoter (16–18). However, a down-regulation of *BRCA1* expression has been observed in most sporadic breast cancers (4–6), indicating that abnormal methylation of this 5' end region of *BRCA1* does not account for the decrease in *BRCA1* expression in most cases of sporadic breast cancers. In addition, in a small series (37 cases) of sporadic breast cancers, the down-regulation of *BRCA1* was not correlated with DNA hypermethylation in the vicinity of the promoter region (5).

Although transient transfection assays indicate that essential regulatory sites are not present in the upstream region (position –1528 to –202; ref 15), it had been shown for other promoters that methylation of the surrounding sequences may repress gene expression (19). The efficiency of the inhibition seems to be dependent on CpG density and promoter strength (19).

We have therefore determined the methylation status of the 5' end of *BRCA1* in a variety of cell lines and tissues, including fetal and cancer breast tissues. This analysis led to an unexpected finding, since the 5' end of *BRCA1*, which is embedded in a large CpG island, appears to be regionally methylated in all somatic tissues analyzed, suggesting that this region may participate in the regulation of *BRCA1* gene expression.

Differences in DNA methylation are associated with differentiation and carcinogenesis, CpG methylation correlating with the silencing of many genes (20, 21). Two types of mechanism could be involved in silencing genes by DNA methylation. CpG methylation can down-regulate gene expression by preventing the binding of transcription factors to their recognition sequences or through repressor molecules that bind to methylated DNA (22, 23). The methyl-CpG binding proteins (MBD), a family of vertebrate proteins, bind to methylated DNA in any sequence context (22); for some members of this family it has been shown that the binding of such proteins represses gene expression at a distance (24).

Among the DNA binding proteins potentially involved in the negative regulation of gene expression, the methyl-CpG binding protein MeCP2 seems to play an important role (25, 26). MeCP2 is a chromosomal protein that binds specifically to methylated CpG and represses densely methylated genes in association with a histone deacetylase complex (27, 28). Although inhibition of histone deacetylase by trichostatin A can relieve the transcriptional repression mediated by MBD (27, 28), for some genes it has been shown that dense CpG island methylation might be a dominant factor in gene silencing (29).

We investigated, therefore, whether methyl-CpG

binding proteins such as MeCP2 might contribute to the control of *BRCA1* expression.

MATERIALS AND METHODS

Preparation of tissues

Human tissues were snap frozen immediately after removal and stored in liquid nitrogen until use. Spermatozoa were purified from semen samples by Percoll gradient centrifugation (Pharmacia, Uppsala, Sweden). One milliliter of semen was loaded at the top of a 50–90% Percoll gradient and centrifuged at 700 g for 15 min. After centrifugation, each fraction was collected by aspiration. The spermatozoa were washed in phosphate buffer 1× to remove the Percoll and resuspended in an appropriate volume of phosphate-buffered saline (PBS) 1×. The purity of the preparation was controlled by microscopy.

Human oocytes that had failed to fertilize 3 days after *in vitro* insemination were collected from the In Vitro Fertilization Laboratory (Hospital E. Herriot, Lyon, France). To remove the follicular cells linked to the zonae pellucida, oocytes were treated by enzymatic digestion with hyaluronidase (150 units, type VIII hyaluronidase, Sigma, France) to discard contaminating somatic cells. Then a digestion by trypsin was performed to remove the zonae pellucida and the remaining somatic cells. Oocytes were rinsed several times in PBS 1× and stored in liquid nitrogen until use. The cumulus cells were collected after hyaluronidase digestion for further studies.

Cell culture

Human breast cell lines (MCF7, BT20, and HBL 100), cervix cell line (HeLa), or kidney cell line (Bosc 23) were obtained from ATCC (Rockville, Md.) and grown in Dulbecco's modified Eagle's medium (Sigma, L'isle d'Abeau, France) supplemented with FCS 5% and 0.5 µg/ml insulin for MCF7 and BT 20 cell lines. All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

PCR-based methylation assay

DNA extracted from tissue samples, cell lines, and cells treated with 5azadC were digested with a fivefold excess of *RsaI* or *RsaI* plus *HpaII* or *CfoI* and incubated overnight at 37°C in the appropriate buffer (Roche Diagnostics, Meylan, France). Control experiments were performed using the methyl-insensitive enzyme *MspI*. Enzymes were inactivated by heating at 65°C for 1 h and an aliquot of the reaction was used for polymerase chain reaction (PCR) amplification. The PCR amplification was performed in the following conditions: 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 4% DMSO, 100 µM of each of the four deoxyribonucleoside triphosphates, 0.25 µM of the primers (forward: 5' TTG GGA GGG GGC TCG GGC AT 3'; reverse: 5' CAG AGC TGG CAG CGG ACG GT), and 0.6 units of *Taq* DNA polymerase (Roche Diagnostics) after 35 cycles in an Eppendorf thermocycler (1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C). In each experiment, the sample digested with *RsaI*, which does not cleave the sequence between the two primers, was amplified to verify the efficiency of the amplification. The sequence analyzed contains 9 *HpaII* sites and 10 *CfoI* sites and PCR amplification occurs only when the sites are methylated and uncut.

5-aza-2'-deoxycytidine treatments

For 5-aza-2'-deoxycytidine (5azadC, Sigma, France) treatments, cell lines were seeded at low density ($3\text{--}4 \times 10^5$ cells/100 mm dish) 16 h before treatment with a final concentration of $1 \mu\text{M}$ 5azadC. The medium was changed 24 h after drug addition and every subsequent day. RNA and DNA were isolated after 72 h. DNAs were extracted and quantitated (Hoechst staining method) from treated cells and control cells. Different aliquots were digested by *RsaI* (in order to normalize the size of the DNA molecules) plus: *HpaII*, *CfoI* (methylation-sensitive enzymes), *MspI* (methylation-insensitive), and no enzyme. The 5' end of the *BRCA1* gene was then amplified by PCR from these samples and the signals obtained were quantitated by densitometry. No signal was observed after *MspI* digestion. The extent of demethylation was evaluated by the ratio between the signal obtained after digestion by *HpaII*, *CfoI*, or both and the signal was obtained from DNAs digested with *RsaI* alone. The data indicated that $> 90\%$ of the DNA molecules contained unmethylated *CfoI* and *HpaII* sites after 5azadC treatment. Exon 1, which was found unmethylated in normal and tumoral tissues, was amplified as a control experiment of complete digestion with methylation-sensitive enzymes.

Transfections

pCMV-MeCP2-HA (kindly provided by Dr. A. Bird), pCMV- β gal plasmids, or pGL3-control plasmid (Promega, Lyon, France) were transfected using the calcium phosphate precipitation technique (30). Cells were collected 48 h after transfection.

For the immunofluorescence assay, Bosc 23 cells were grown on Lab-tek Permanox (Nunc) 2-well chambered coverslips at 1×10^5 cells per well and transfected as described previously. After 48 h, the cells were washed twice in PBS $1 \times$, fixed in paraformaldehyde 4% for 15 min at room temperature, and rinsed several times with PBS $1 \times$. Then cells were permeabilized in 0.1 M glycine-PBS $1 \times$ buffer, followed by incubation in 0.5% Triton X-100-PBS $1 \times$ buffer, and blocked in 0.2% gelatin-PBS $1 \times$ buffer for 15 min at room temperature. The anti-tag HA monoclonal antibody (12 CA5, Roche Diagnostics) diluted 1:40 in 0.5% Triton X-100-PBS $1 \times$ buffer was incubated with the cells for 1 h. The cells were rinsed several times with PBS $1 \times$ and incubated for 1 h with a 1:200 dilution of the FITC-conjugated secondary antibody (goat anti-mouse IgG, Dako, France). Fluorescence was visualized with a $25 \times$ or $40 \times$ immersion lens on a Leica microscope. In these experiments, MeCP2 expression was consistently observed in more than 50% of the cells and β -galactosidase activity (30) was observed in more than 60% of the cells; five adjacent fields, at magnification $40 \times$, were counted.

pGL3 constructs, methylation, and luciferase assay

A 1757 bp fragment of the *BRCA1* gene containing a part of the 5' CpG island and 43 bp of exon 1a was isolated from genomic DNA by PCR using primers containing *KpnI* site at the 5' end and *BglII* site at the 3' end (primer forward-*KpnI*: 5' CTG GTA CCT TGG GAG GGG GCT CGG GCA A3'; primer reverse-*BglII*: 5' GAA GAT CTT CCA GGA AGT CTC AGC GAG C 3'). The PCR products were ligated between *KpnI* and *BglII* sites into the pGL3 basic vector (Promega) and transformed into competent JM-109 *Escherichia coli* cells (Promega).

In vitro methylation was performed by incubating the p5'-*BRCA1*-Luc vector with one unit of either *HpaII* (CCGG sites), *HhaI* (GCGC sites), or *SssI* (CG sites) methylases per

microgram of plasmid DNA in the conditions recommended by the manufacturer (Biolabs, Beverly, Mass.); completeness of the modification was checked with the corresponding restriction enzyme. p5'-*BRCA1*-Luc constructs were transfected into Bosc 23 cells as described previously. Transfections were optimized for 12-well plates. The cells were lysed and assayed for luciferase expression (Luciferase assay, Promega) after 48 h and the light emissions were measured in a scintillation counter (Packard, Downers Grove, Ill.). A plasmid (pGL3-control; Promega) containing a promoter and an enhancer derived from SV40 was also transfected in the same plates; values obtained for this control vector were comparable between each experiments.

DNA extraction

High molecular weight DNA was extracted from frozen pulverized tissue samples and cells by standard procedures (30). Briefly, samples were resuspended in 10 mM Tris-0.1M EDTA buffer and digested with proteinase K (300 $\mu\text{g}/\text{ml}$ final concentration) in the presence of SDS. A similar method with the addition of 0.001% (V/V) β -2 mercaptoethanol was used to prepare decondensed DNA from spermatozoa. When DNA was extracted from a small number of cells (6 to 10 oocytes), 2 μg of pGEM-T plasmid (Promega) was added as carrier. The mixture was incubated at 37°C , phenol/chloroform extracted, and DNA was ethanol-precipitated.

RNA extraction

RNA was isolated in a single step procedure by acid-guanidinium-thiocyanate-phenol-chloroform extraction as described previously (31). After extraction, total RNA was precipitated in isopropanol and resuspended in an appropriate volume of sterile water. The integrity and quantity of RNA were examined by gel electrophoresis. Total RNA was quantified by densitometry on a 1.2% agarose gel containing 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide in comparison with serial dilutions of known amount of standard RNA (Roche Diagnostics).

Quantitative reverse transcription PCR (RT-PCR)

The RT-PCR assay was performed as described previously (32) by coamplification of 0.3 μg of total RNA and a known amount of competitor RNA. Primers used were designed to amplify the cDNA fragment from exon 6 to 8 of the *BRCA1* gene (C3F: 5' TGT GCT TTT CAG CTT GAC ACA GG 3' and C3R: 5' CGT CTT TTG AGG TTG TAT CCG CTG 3'). Reactions were performed in 100 μl containing 10 mM Tris-HCl (pH 8.3), 3 mM MgCl_2 , 50 mM KCl, 0.1 mg/ml gelatin, 200 μM of each of the 4 deoxyribonucleoside triphosphates, and 0.25 μM of the C3 primers.

After initial denaturation at 92°C for 2 min, six units of Expand Reverse Transcriptase (Roche Diagnostics) were added to the reaction mixture and incubated for 35 min at $42[\text{de}]\text{C}$. Reverse transcriptase was then inactivated by heating and after cooling to 0°C , PCR amplification was accomplished by adding 0.6 units of *Taq* DNA polymerase (Roche Diagnostics) after 35 cycles in an Eppendorf thermocycler (1 min denaturation at 94°C , 2 min annealing at 55°C , and 3 min extension at 72°C). Aliquots were analyzed on a 2% agarose gel containing 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide and the intensity of the bands corresponding to the wild-type *BRCA1* PCR products and to the competitor PCR products was determined using Image Analyzer Software (Wayne Rasband, National Institutes of Health).

Southern blot analysis

Methylation patterns were determined by Southern blot experiments using methylation-sensitive restriction endonuclease *CfoI* (GCGC site) and *HpaII* (CCGG site) or methylation-insensitive isoschizomer *MspI* (CCGG site). In a typical experiment, 30 µg of total DNA was cleaved with a 10-fold excess of *TaqI* or *PstI* for 10 to 12 h in the appropriate conditions (Roche Diagnostics). Two-thirds of the sample was then digested overnight by *CfoI* or *HpaII* or *MspI*. Restriction endonuclease products were separated by electrophoresis on a 1.2% agarose gel and transferred to Hybond N⁺ Nylon membrane (Amersham, France). After drying, the membranes were hybridized overnight at 65°C to a randomly primed ³²P-labeled probe (Random Primed DNA labeling kit, Roche Diagnostics) in the hybridization solution (0.25 M sodium phosphate, 7% SDS, 1% BSA, 50 µg/ml yeast tRNA). Membranes were washed with increasing stringency (from 2 to 0.1 × SSC; 0.1% SDS) at 65°C and exposed to Hyperfilm (Amersham, France) for 1 to 7 days at -70°C. Membranes could be used for rehybridization after two successive treatments with SDS 1% at 80°C for 30 min.

Gene probes

The probe used for Southern blot analysis of the CpG island of the *BRCA1* gene was synthesized by PCR amplification of a 239 bp region (-1244 to -1005; ref 33). The PCR fragments were cloned into a pGEM-T vector (Promega). After digestion with the appropriate enzyme, the insert was purified by agarose gel electrophoresis, followed by electroelution. Each filter was rehybridized with the exon 1 probe encompassing exon 1a of the *BRCA1* gene and the bidirectional promoter (5) in order to verify that DNA cleavage with restriction endonucleases was complete.

Bisulfite modification

The sodium bisulfite modification method, followed by the sequencing of PCR products, is used to determine the CpG methylation pattern. Sodium bisulfite converts unmethylated cytosines to uracils whereas the methylated cytosines remain unmodified. In the resultant modified DNA, uracils are replicated as thymines during PCR amplification. The sodium bisulfite reaction was carried out on 4 µg of DNA (3 µg of carrier DNA and 1 µg of human genomic DNA). Alkali-denatured DNA was incubated in 3 M NaHSO₃ and 5 mM hydroquinone for 16 h at 50°C. Modified DNA was purified using the Wizard DNA Clean-up System (Promega) and eluted into 50 µl of sterile water. Modification was completed by 0.3 M NaOH; DNA was precipitated by 0.5 M ammonium acetate (pH 4.6) and resuspended in water.

DNA was amplified using strand-specific primers designed to amplify a 258 bp region in the CpG island of the *BRCA1* gene in two separate reaction mixtures. The first round of PCR amplification was accomplished in 100 µl in a buffer containing 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 100 µM of each of the four deoxyribonucleoside triphosphates, 0.25 µM of the primers (forward: 5' TTT TGT TTT GTG TAG GGC GGT T 3'; Reverse: 5' CCT TAA CGT CCA TTC TAA CCG T 3'), and 0.6 units of *Taq* DNA polymerase (Roche Diagnostics) after 35 cycles in an Eppendorf thermocycler (1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C). An aliquot of the first amplification was reamplified with internal primers (forward: 5' TGA GAA TTT AAG TGG GGT GT 3'; reverse: 5' AAC CCT TCA ACC CAC CAC TAC 3') in the same conditions.

PCR products were first analyzed by digestion with restriction enzymes *DdeI*, *EcoRI* (Roche Diagnostics), and *HphI* (Biolabs) in the buffers recommended by the manufacturers.

Then PCR products were cloned in a pGEM-T vector (Promega) and 10 random clones were analyzed by automatic sequencing (Eurogentec, Belgium) to determine the proportion of methylated (CpG) or unmethylated (TpG) sites.

RESULTS

The 5' CpG island of *BRCA1* is regionally methylated in somatic tissues

Significant variations, up to fivefold in cell lines (32) and 30-fold in sporadic breast cancers (5), in the amount of *BRCA1* mRNA are not related to the methylation status of the putative promoter sequence located at the 5' end of the exon 1a (5). However, this unmethylated region (nt -167 to nt +484) is included in a large CpG island, spanning nucleotides -2200 to +500 (33 and Fig. 1E), which suggests that hypermethylation of this CpG island could be associated with gene silencing. Analysis of the methylation status of the -1714 to -1023 region using a PCR-based methylation assay indicated that this CpG island was methylated at *CfoI* and *HpaII* sites in human somatic tissues (including normal and tumoral somatic tissues and fetal tissues, 65 samples analyzed) and cell lines (data not shown). Since this assay is qualitative rather than quantitative, methylation patterns were further determined by Southern blot experiments (representative samples are shown in Fig. 1A).

DNAs were first digested with the methylation-insensitive restriction endonuclease *PstI*. Using probe 1 (position -1244 to -1005, Fig. 1C), *PstI* generates two bands of 3.8 kb for the *BRCA1* gene and 5 kb for the *BRCA1* pseudogene (Fig. 1A, lanes 1 and 9). When DNA extracted from the HBL 100 cell line is digested with the methylation-insensitive enzyme *MspI*, this probe reveals a 1008 bp band corresponding to the *BRCA1* gene (Fig. 1A, lane 2). A faint 202 bp band corresponding to the *BRCA1* pseudogene was detected at the bottom of the gel. DNAs extracted from normal tissues (Fig. 1A) were digested with the methyl-sensitive enzyme *HpaII* that cleaves CCGG sites when the internal cytosine at CpG dinucleotide is unmethylated. Probe 1 maps a 1449 bp band (Fig. 1A, lanes 4-7) corresponding to the methylation of 9 CCGG sites located within the 5' region of the *BRCA1* gene (Fig. 1B and 1C). In spermatozoa, the CCGG sites analyzed using probe 1 are unmethylated (Fig. 1A, lane 8). DNA from the HBL 100 cell line and fetal breast tissue (Fig. 1A, lanes 10 and 11, respectively) were cleaved with the methylation-sensitive enzyme *CfoI* in order to map several other CpG sites. Probe 1 reveals a 924 bp band in the HBL 100 cell line and reveals a 736 bp

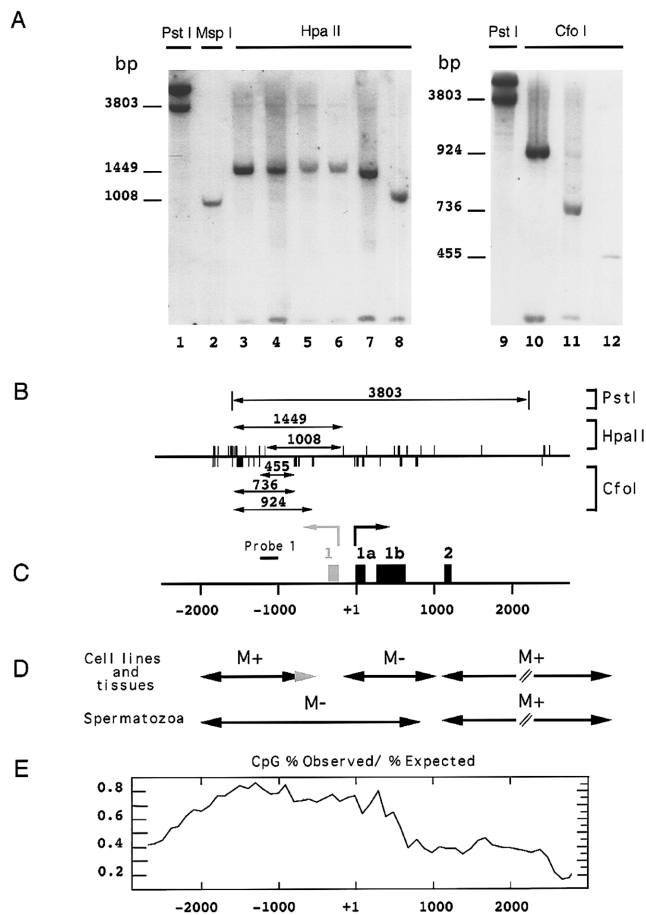


Figure 1. DNA methylation pattern of the 5' region of the *BRCA1* gene in human cells and tissues. **A)** DNA was extracted from the HBL 100 cell line (lanes 1–3; 9–10); adult breast tissue (lane 4), fetal breast tissue (lane 5; 11), normal ovary (lane 6), normal kidney (lane 7), and spermatozoa purified from semen sample on Percoll gradient (lane 8; 12). Southern blot experiments were performed as described in Materials and Methods. After *Pst*I digestion (lanes 1 and 9), probe 1 reveals two bands, the 3.8 kb band corresponding to the *BRCA1* gene and the 5 kb band to the *BRCA1* pseudogene. When cleaved with *Msp*I (lane 2), these two bands produce a 1008 bp band for the *BRCA1* gene and a 202 bp band for the pseudogene. *Hpa*II digestion (lanes 4–7) generates a 1449 bp band, indicating that the 9 CCGG sites at the 5' end of the region analyzed are methylated. In spermatozoa (lane 8), *Hpa*II patterns are identical to *Msp*I patterns, indicating that these sites are unmethylated in these samples. The methylation status of GCGC sites was investigated using *Cfo*I digestion (lanes 10–12) and probe 1. The HBL 100 cell line (lane 10) exhibits a 924 bp band and in somatic tissues (one representative sample is shown on lane 11) a 735 bp band, indicating that the proximal *Cfo*I sites are unmethylated in normal tissues (see Fig. 1B). In spermatozoa, the CpG dinucleotides located within the GCGC sites are unmethylated, giving rise to a 455 bp band. **B)** Position of enzymatic sites in the 5' region of the *BRCA1* gene. *Hpa*II sites are indicated by upward strokes, *Cfo*I sites by downward strokes. Arrows indicate the portion of DNA recognized by the probe and the size of the bands as revealed by autoradiography is indicated for the different couples of enzymes. **C)** Schematic representation of the promoter region of the *BRCA1* gene. The *BRCA1* gene spans 81 kb of genomic DNA and is located head to head with the *NBR2* gene. The two genes are separated by a bidirectional promoter. The arrows point to

band in tissues, indicating that the proximal *Cfo*I sites are unmethylated in normal tissues. In spermatozoa, CpG dinucleotides located within GCGC sites are unmethylated, giving rise to a 455 bp band after hybridization with probe 1.

Taken together, these data indicate that DNAs extracted from somatic tissues and cell lines were methylated at CCGG and GCGC sites within the region spanning nucleotides -2000 to -1000 or -720 for the HBL 100 cell line, with the exception of human spermatozoa DNA, which was unmethylated at these sites (Fig. 1A).

Analysis of the methylation pattern of the body of *BRCA1* gene was carried out, as described previously (5), on several fetal and adult human tissues, cell lines, and male germ cells by Southern blot after digestion by the methylation-sensitive enzymes *Hpa*II and *Cfo*I (Fig. 1D). The filters were successively hybridized with several probes corresponding to the different exons present in the 3' region of the *BRCA1* gene. The body of the gene (from the end of exon 1b to the end of exon 24) is methylated in somatic tissues (5) and mature male germ cells, as expected for non-CpG island regions. In somatic cells, the promoter region of the gene is unmethylated, as described previously (5). In cell lines and tissues, the 5' region is methylated at CCGG and GCGC sites in the various samples. On the other hand, this region is unmethylated in spermatozoa as expected for a CpG island.

The 5' CpG island of *BRCA1* is unmethylated in human gametes

The gamete-specific pattern was further confirmed by scaling-down the bisulfite sequencing method in order to investigate CpG methylation from very small amounts of DNA, and experimental conditions were

the transcription start of both genes. The position of the first exons of the *BRCA1* gene is indicated by black boxes (exons 1a, 1b, 2). The first exon of the *NBR2* gene is indicated by a gray box. The scale indicates the position of the exons starting from the initiation of transcription of the *BRCA1* gene (exon 1a). The black line represents the position of the 239 bp probe (probe 1). **D)** Methylation pattern of the *BRCA1* gene in human somatic and germ cells. Data obtained from Southern blot experiments and ref 5 are summarized. The methylation status of *BRCA1* DNA segments (symbolized by the arrows) is indicated: M⁺: methylated region; M⁻: unmethylated region. The gray arrow corresponds to the HBL 100 pattern. **E)** Distribution of the CpG sites in the 5' region of the *BRCA1* gene. A graph of the percentage of CpG observed divided by the expected frequency. Dinucleotide frequencies were calculated in 1000 nucleotide windows moved at 100 nucleotide intervals. The ratio (CpG-% observed/CpG-% expected) indicates that the 5' region of the *BRCA1* gene is embedded within a CpG island beginning upstream from the transcription start site and encompassing the first exons of the gene.

chosen for obtaining a quantitative assay of the ratio methylated CpG vs. unmethylated CpG.

DNA extracted from purified human spermatozoa was modified by sodium bisulfite (34). This chemical treatment converts unmethylated cytosines to uracils while methylated cytosines remain unmodified. After modification, DNA was amplified by a two step PCR method, as described in Materials and Methods. The PCR product was digested by specific restriction endonucleases to determine the global methylation status of the sample. Completeness of the modification was monitored by digestion with *DdeI*, which cleaves only unconverted DNA. PCR products obtained from methylated molecules exhibit a new *EcoRI* site at position 138, whereas unmethylated molecules exhibit a new *HphI* site at position 165.

The sensitivity of PCR amplification after bisulfite modification was monitored by mixing different proportions of unmethylated DNA from spermatozoa (from 25 to 100%) and methylated DNA from HBL 100 (from 0 to 75%). For each assay, an aliquot of the PCR product was cleaved with *DdeI* (unmodified DNA), *EcoRI* (methylated DNA), or *HphI* (unmethylated DNA), loaded on a 2% agarose gel, and visualized by ethidium bromide staining. The results indicate that the amount of PCR product cleaved by enzymatic digestion is directly related to the percentage of methylated or unmethylated DNA used in the coamplification assay (Fig. 2A). DNAs from somatic tissues and gametes were therefore modified using this method and PCR products were cloned and sequenced.

Within the region analyzed, -1643 to -1358, all the 24 CpG sites were unmethylated in DNA from human oocytes and spermatozoa (Fig. 2B). As expected from the Southern blot experiments, all these CpG sites were methylated in all somatic tissues and cell lines, including the somatic cells of the corona radiata surrounding the oocytes (Fig. 2B). The absence of DNA methylation within the CpG island in human gametes did not extend to the body of the *BRCA1* gene, since control experiments indicated that two regions of the exon 11 are methylated both in somatic tissues and gametes (data not shown), suggesting that the methylation of the CpG island might play regulatory role in *BRCA1* expression.

Chemically induced hypomethylation elevates *BRCA1* expression

To test the potential role of DNA methylation in the control of *BRCA1* expression, demethylation was induced by 5-aza-2'-deoxycytidine (5aza-dC) treatments. Human cell lines (three breast cell lines: BT20, MCF7, and HBL 100; one cervix cell line, HeLa; and one kidney cell line, Bosc 23) were grown for 72 h in the presence of 1 μ M of 5aza-dC. The

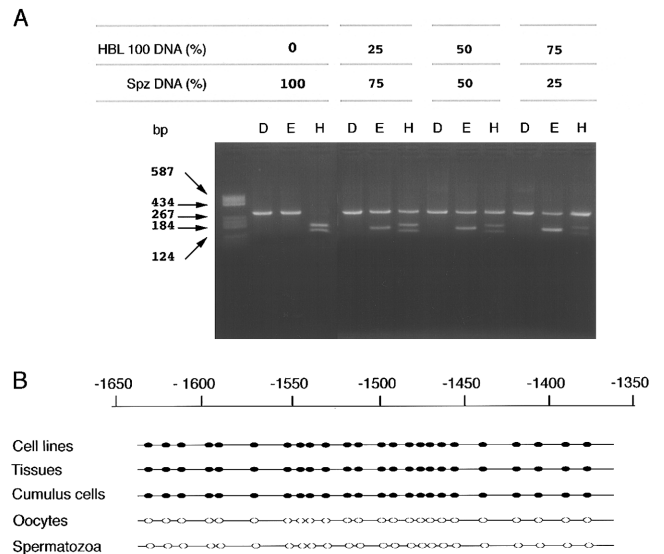


Figure 2. Methylation status of the 5' region of the *BRCA1* gene in human somatic tissues and germ cells. *A*) Sensitivity of PCR amplification after bisulfite modification. After bisulfite modification, a DNA segment of the *BRCA1* CpG island (position -1643 to -1358) was amplified (see Materials and Methods) using a two step PCR method. Ten μ l of PCR products obtained from DNA of spermatozoa (unmethylated) mixed with the appropriate amount (from 0 to 75%) of methylated DNA (HBL 100 cells) was digested with *DdeI* (lanes D), which cuts unmodified DNA; *EcoRI* (lanes E), which cuts at position 138 the PCR products obtained from methylated DNA; and *HphI* (lanes H), which cuts at position 165 the PCR products obtained from unmethylated DNA. The intensity of the corresponding bands was measured by densitometry. *B*) Enzymatic digestion of PCR products after bisulfite modification gives an estimation of the methylation status for only a few CpG sites (at enzymatic sites). Therefore, the methylation profile of a 258 bp region (position -1643 to -1358 from the initiation of transcription) of the CpG island of *BRCA1* containing 24 CpG sites was analyzed at the nucleotide level after cloning and sequencing the PCR products obtained from various human tissues, cell lines, and male and female germ cells. Somatic cells of the corona radiata separated from the oocyte by enzymatic digestions were also analyzed as a control of the purity of the oocyte samples. Bisulfite sequences obtained from individual cloned fragments are represented as horizontal lines, with individual CpGs represented by white circles (when at least 80% of CpGs are unmethylated) or black circles (when at least 80% of the CpGs are methylated). Ten clones were analyzed for each sample. All CpG sites are methylated in somatic tissues (cumulus cells included) and human cell lines whereas the 5' region of the *BRCA1* gene is unmethylated in both male and female human germ cells.

PCR-based methylation assay indicated that the 5' end of *BRCA1* was efficiently demethylated at the end of the treatment. Then the amounts of *BRCA1* mRNA were determined using a competitive RT-PCR method (32).

5aza-dC treatment invariably elevated *BRCA1* expression in the cell lines analyzed by up to fivefold in BT20 cells and from 1.5 to 2.5 in the other cell lines (Table 1), indicating that CpG methylation is associated with a low level of *BRCA1* expression.

TABLE 1. Effect of 5aza-dC on BRCA1 expression

Cell line	BRCA1 mRNA molecules/ μ g of total RNA		Ratio
	- 5aza-dC	+ 5aza-dC	
HBL 100	150×10^4	233×10^4	1.6
MCF7	83×10^4	183×10^4	2.2
BT20	23×10^4	110×10^4	4.8
Bosc23	50×10^4	116×10^4	2.3
HeLa	40×10^4	100×10^4	2.5

In vitro methylation induces a MeCP2-dependent repression of the BRCA1 promoter activity

The methylated domain of the *BRCA1* CpG island is relatively far (~ 0.7 to 1 kb, Fig. 1C, E) from the start site of the transcription. MeCP2 is able to drive a long-range repression and might down-regulated *BRCA1* expression. This hypothesis was investigated using an expression vector (p5'-*BRCA1*-Luc) containing the -1714 to +43 region of *BRCA1* fused to the luciferase enzyme as a reporter gene.

Analysis of the 5' end *BRCA1* sequence indicates that the CCGG sequences (*HpaII* sites) are located outside the minimal promoter region required for full promoter activity and only one GCGC sequence (*HhaI* sites) is located at the 3' end of this region, deletion of this latter sequence inducing only a weak inhibition of the promoter activity (15). Therefore, we take this opportunity to investigate whether the methylation of surrounding sequences might affect *BRCA1* promoter activity. p5'-*BRCA1*-Luc vector was *in vitro* methylated using *HpaII* methylase or *HhaI* methylase and transfected in Bosc 23 cells in the presence or absence of a vector (MeCP2-Tag-HA) encoding for MeCP2. In the absence of the MeCP2-Tag-HA vector, *in vitro* methylation induced only a two- to threefold drop in luciferase activity; meanwhile, in the presence of MeCP2, the *in vitro* methylated vectors exhibited an almost total loss of transcriptional activity (Fig. 3). Control experiments using expression vectors containing the β -galactosidase gene driven by a CMV-promoter (pCMV- β -gal plasmid) and a pGL3-control plasmid containing a SV40 promoter fused to the luciferase gene indicated that the expression of these reporters genes was not or was only minimally affected by the over-expression of the pCMV-MeCP2-HA-vector.

Therefore, cotransfection experiments in the Bosc 23 cell line indicated that partial methylation of the vector containing a part of the 5' end of *BRCA1*, which mimics the methylation pattern observed in somatic cells, induced a MeCP2-dependent repression of the transcriptional activity of this vector (Fig. 3).

DISCUSSION

In mammalian genomes, the CpG dinucleotides are underrepresented (~ 5 to 10% of their predicted frequency) and a high proportion (60 to 80%) is methylated. However, short regions exhibit a ratio of CpG observed vs. CpG expected of at least 0.6 and a G+C content greater than 50%. These regions, called CpG islands, are associated with all the housekeeping genes and $\sim 40\%$ of tissue-restricted genes. Most frequently, the CpG islands are located at the 5' end of these genes and contain the promoter and the first exon of the associated gene (for a review, see ref 22). Although these sequences are usually unmethylated, hypermethylation of CpG islands associated with gene silencing has been described for crucial regulator of growth during cancer progression (20). In addition, abnormal methylation at multiple CpG-rich sequences and gene silencing are also characteristic of human and murine cell lines (35).

In normal somatic tissues, methylated CpG islands have been found for X chromosome-inactivated genes and for imprinted genes, suggesting that the methylation of a subset of CpG islands also occurs during normal physiological process. In line with this observation, it has been shown in colon tissue that DNA methylation at CpG islands takes place during the aging process (36).

In this study we have found that the CpG island of *BRCA1* is regionally methylated in all somatic human cells and tissues analyzed, suggesting that this epigenetic modification might have some consequences on *BRCA1* gene expression. Since some promoters are not or are only minimally inhibited by *in vitro* methylation, we have investigated the methylation sensitivity of the p-5'-*BRCA1*-Luc expression vector

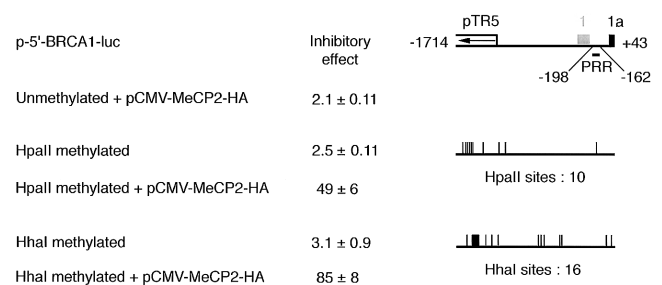


Figure 3. Effect of *in vitro* methylation on the promoter activity of *BRCA1* in the presence or absence of MECP2. The 5' end (nt -1714 to nt +43) containing the promoter region of *BRCA1* was cloned upstream from the luciferase reporter gene. The constructs were methylated *in vitro* by *M.HpaII* or *M.HhaI* and transfected in Bosc 23 cells in the presence (ratio, 1/1) or absence of the pCMV-MeCP2-HA vector. The inhibitory effect (mean value of 4 independent experiments \pm standard deviation) was determined from the ratio between the value obtained with the unmodified vector and the value obtained for the sample analyzed. PRR, positive regulatory region (15); pTR5, long terminal repeated element.

(containing the 5' end of *BRCA1*). *In vitro* methylation using *M-HpaII* generates a methylated site at position -167 that is not methylated *in vivo*, whereas the other nine sites are methylated in the cell lines and tissues analyzed.

Several papers have described a bidirectional promoter in this region. For example, Thakur and Croce (15) have localized the promoter region at position -195 to -162 and a minimal positive regulatory region at position -195 to -177. This short region seems to be involved in the binding of the nuclear proteins. More recently, Suen and Gross (37) also mapped the bidirectional *BRCA1* promoter in this region at position -204 to -149, and band-shift assays indicated that within this promoter the -167 to -149 region was the target of nuclear proteins. These data might suggest that the nucleotide 'C' at position -167 is not involved in the binding of potential transcription factors.

In addition, the methylation of this site (-167) does not seem to be crucial for the expression of p-5'-*BRCA1*-Luc vector since the methylation of the *HhaI* sites (the more proximal sites are at positions -564 and -20) has a more pronounced inhibitory effect. Furthermore, the methylation of all CpGs induces (using *SssI* methylase) a very strong inhibition of expression of the p-5'-*BRCA1*-luc vector, even in the absence of pCMV-MeCP2-HA vector, suggesting that the density of methylated sites is the main point of the inhibitory effect.

Although MeCP2 can bind DNA segments containing only one methylated CpG, it had been shown that the inhibitory effect of MeCP2 is strongly dependent on the density of methyl-CpGs (38). Using p-5'-*BRCA1*-Luc vector cotransfected with a *MeCP2* expression vector, in our experiments the inhibition seems dependent on the number of methylated CpGs, since the inhibition observed with *HhaI* methylase (16 sites) is stronger than the inhibition obtained after methylation with *HpaII* methylase (10 sites). These data also suggest that the inhibition of the expression of the p-5'-*BRCA1*-Luc vector by DNA methylation is not the result of the methylation of a specific site, but seems to be dependent on the methylation density.

The regional methylation of the 5' end *BRCA1* CpG island is clearly not the consequence of a pathological process, since this pattern was observed in all normal somatic tissue samples analyzed. In contrast to the imprinted genes, this region is unmethylated in human spermatozoa and in oocytes. Furthermore, in fetal and adult tissues differential methylation between alleles was not detected. The presence, at the 5' end of *BRCA1*, of the complete long terminal repeat element pTR5, spanning nucleotides -3123 to -1273 (33), which is partially included in the *BRCA1*-CpG island (nt -2200 to

+500), might account for the partial methylation of this region. In addition, the 5' end of *BRCA1* exhibits a complex organization since the transcription start site of *BRCA1* is separated from that of the *NBR2* gene by 218 bp and, therefore, its 5' end region is included in the intron 1 of *NBR2* gene (13). In line with this hypothesis, the promoter region of the mouse *Brca1* gene, which lacks this repetitive element and complex organization (39), appears to be unmethylated in mouse somatic tissues and in the NIH 3T3 cell line (unpublished data), suggesting that insertion of the LTR element in somatic cells might have some consequences on the methylation patterns of the human *BRCA1* gene.

This methylation pattern of the *BRCA1* CpG island confers potential regulatory features. Our results show that *BRCA1* expression is regulated at least in part by methyl-CpG binding proteins. Among this family, MeCP2 might be a good candidate since transient expression of the corresponding gene led to a methylation-dependent inhibition of *BRCA1* expression. Despite the low level of *MeCP2* expression (unpublished data) in the cell lines analyzed, inhibition of DNA methylation resulted in an elevated level of *BRCA1* mRNA. This chemically induced hypomethylation by 5aza-dC might suggest that other members of the methyl-DNA binding proteins family are also involved in this regulation.

Transient transfection of fusion proteins have identified a MeCP2 domain that is capable of long-range repression of vectors (19, 24). This protein, which binds to methylated CpG, forms a complex with histone deacetylase and the transcription repressor Sin3A, leading to formation of transcriptionally repressive chromatin architecture (27, 28). MBD2 and MBD3, two other methyl-DNA binding proteins, have recently been characterized and shown to participate in other histone deacetylase complexes and in gene silencing mechanisms (40, 41). The expression patterns of these methyl-DNA binding protein genes are not fully determined, but it has been shown in the rat that the level of *MeCP2* expression is dependent on the tissues (42), MBD3 is associated with metastatic-associated protein 1, a protein that is overexpressed in several human cancers (43), and MBD2 belongs to the MeCP1 deacetylase complex, which is also present at various levels depending on the cell type and the differentiation state (40).

Although, genes possessing a 5' end methylated CpG island should represent only a very small part of the mammalian genomes, differences in the amount of methyl-DNA binding proteins between tissues or cells could represent an additional mechanism for tissue-specific gene expression. [F]

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