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Screening for Germ-Line Rearrangements and Regulatory Mutations in *BRCA1* Led to the Identification of Four New Deletions[1]

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ABSTRACT

Most previous *BRCA1* mutation screening studies conducted on breast cancer families were aimed at identifying mutations in the coding sequence and splice sites. Mutations in the promoter and untranslated regions, and large rearrangements are missed by standard mutation detection strategies. To look specifically for such germ-line mutations in the *BRCA1* gene, we have analyzed a series of 27 American and 51 French breast cancer families in which no *BRCA1* mutation was identified by classical techniques. No mutations were detected in either the promoter or untranslated regions, and we did not find any deletion of the whole gene. Four families were found to carry distinct deletions. Two of them, probably generated by Alu-mediated homologous recombination, were internal deletions of 3 and 23.8 kb, encompassing exon 15 and exons 8–13, respectively. These alterations both lead to a frameshift in the mutant mRNA and to premature stop codon-mediated mRNA decay. The other two deletions encompass exons 1 and 2. On the basis of previous and present analyses, rearrangements represent 8% (3/37) of all mutations in our set of *BRCA1* American families. Consequently, the search for rearrangements appears mandatory in *BRCA1* mutation screening studies.

INTRODUCTION

The vast majority of mutations found to date in the *BRCA1* gene in breast and/or ovarian cancer families are point mutations or small insertions and deletions scattered over the whole coding sequence (5592 bp) and the splice junctions.[3] These mutations are detected by PCR-based methods such as direct sequencing, single-strand conformation analysis, HDA,[4] denaturing gradient gel electrophoresis, and the protein truncation test, using mainly genomic DNA as a starting material. In a recent analysis performed on 237 families with at least four cases of breast cancer collected by the Breast Cancer Linkage Consortium, it was estimated that, among the *BRCA1*-linked families tested by one of the screening methods described above, 63% (95% confidence interval, 51–77%) of mutations were detected (1). This relatively low sensitivity estimate is suggested to be due to the fact that a substantial fraction of *BRCA1* mutations may occur outside the coding sequence and splice sites and are, thus, not screened in most studies. Alternatively, some alterations may not be detectable by standard screening methods performed on genomic DNA. Supporting this latter hypothesis, five different large germ-line rearrangements have recently been reported in the *BRCA1* gene in American (2, 3) and Dutch families (4). Three of these were found only once, but the remaining two account for 36% of all *BRCA1* mutations in Dutch breast cancer families (4). Furthermore, in a few families, the presence of regulatory mutations that prevent transcription from the mutated allele has been inferred, based on the observation that at least one carrier was heterozygous for a *BRCA1* polymorphism at the genomic level but apparently homozygous at the cDNA level (5–7). A rearrangement has been identified in a family with an "inferred regulatory" mutation *i.e.*, a 14-kb deletion removing the putative promoter and exons 1a, 1b and 2, which explains, at the genomic level, the absence of transcription of the mutated allele (3). Deletions of this type may be responsible for other reported inferred regulatory mutations in *BRCA1*, as well as mutations in the promoter, in potential regulatory elements in the 5′ and 3′ UTRs or in the polyadenylation signal.

To assess the importance of *BRCA1* alterations that are missed by standard screening techniques, we looked specifically for germ-line mutations occurring in regulatory regions and for germ-line rearrangements in 27 American and 51 French breast or breast/ovarian cancer families negative in a mutation screening analysis of the *BRCA1* coding sequence and splice sites.

MATERIALS AND METHODS

Families. Twenty-seven families, from an original set of 71 families ascertained by Dr. H. T. Lynch at the Creighton University School of Medicine (Omaha, NE), that were screened for mutations in the coding region and splice sites of the *BRCA1* and *BRCA2* genes by HDA and protein truncation test (8, 9)[5] and found to be negative for mutations were used in this analysis. Inclusion criteria for the families were as follows: (a) two first-degree relatives affected with ovarian cancer; (b) three affected first-degree relatives, at least one of which had breast cancer diagnosed at <40 years of age; or (c) four relatives affected with breast cancer, at least either three of which were first-degree relatives or two of which had breast cancer diagnosed at <40 years of age. Among these families, 12 are breast cancer-only families (1 family including one case of male breast cancer), whereas the remaining 15 are breast and ovarian cancer families.

Fifty-one women from a set of 160 individuals referred to a breast/ovarian family cancer clinic at the Institut Curie (Paris, France) that were screened for mutations by denaturing gradient gel electrophoresis in the coding region and splice sites of the *BRCA1* gene (10) and found to be negative were selected for this analysis on the basis of being heterozygous for at most one coding polymorphism (28 of them were homozygous). Inclusion criteria for families were as follows: (a) two affected first-degree relatives, at least one of which had either invasive breast cancer diagnosed at <41 years of age or ovarian cancer diagnosed at any age; (b) at least three first- or second-degree relatives from the same lineage, affected with either invasive breast cancer or ovarian cancer at any age; or (c) at least one woman affected with both breast and ovarian cancers. Forty-four individuals belonged to breast cancer-only families (four include one case of male breast cancer), whereas seven belong to breast/ovarian cancer families.

HDA. The primer pairs used for HDA were as follows: to amplify the promoter and 5′-UTR, forward 5′-GGG ATT GGG ACC TCT TCT TAC/

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[3] The Breast Cancer Information Core on the Internet (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/).

[4] The abbreviations used are: HDA, heteroduplex analysis; UTR, untranslated region.

[5] Unpublished results.

reverse 5′-CCA CTC TCC CAC GCC AGT A; and to amplify the 3′-UTR, forward 5′-AGC ACT CTA CCA GTG CCA GG/reverse 5′-AGG TTT CAA GTT TCC TTT TCA and forward 5′-GGA AAA TGA AAC TAG AAG AGA TTT/reverse 5′-AGG CTC TGA GAA AGT CGG CT. HDA of ^{33}P-radiolabeled PCR fragments generated using the Red Hot Taq (Advanced Biotechnologies, United Kingdom) was performed using mutation detection enhancement gels (MDE; Bioprobe Systems) according to the instructions provided by the manufacturer.

Southern Blot Analysis. The *BRCA1* gene is distributed over 81 kb of genomic DNA and is split into 23 exons ranging in size from 41 to 311 bp (mean size, 104 bp), for 21 of them, plus 2 large exons (3425 bp and 1501 bp). To be able to detect deletions or insertions as small as 500 bp, we wanted each exon of *BRCA1* to be comprised in a restriction fragment no larger than 10 kb. Because the gene's structure was not known when we started this study, each intron of *BRCA1* was amplified, and we constructed a restriction map of the whole gene with four different enzymes (data not shown). Single digestion by three different enzymes, *Hin*dIII, *Eco*RI, and *Xba*I, met our criteria for rearrangements screening and, furthermore, gave us the possibility of checking the authenticity of each potential abnormal pattern seen with one enzyme with the other two. We checked the accuracy of our map when the sequence of the whole gene became available (Ref. 11; GenBank accession no. L78833). The presence of extra bands in the Southern blot makes the identification of rearrangements relatively easy, but one needs to use genomic probes covering the whole gene to be able to visualize them in all cases. We, thus, tried to use a combination of eight long-range PCR products of ~10 kb covering the whole gene as a probe: hybridization gave rise to smears, although the probes were preincubated with repetitive sequences, revealing a high concentration of such sequences. Indeed, the percentage of Alu sequences was subsequently shown to be 41.5% in the *BRCA1* gene (11). This led us to change our strategy and to use only the coding sequence of *BRCA1* as a probe, which doesn't systematically allow identification of an extra band in the case of a rearrangement. Therefore, a quantitative Southern blot hybridization approach using densitometry analysis was necessary. Most *BRCA1* exons are very small (14 exons are <100 bp and 4 are <60 bp); consequently, hybridization with the whole *BRCA1* cDNA did not allow the detection of all restriction fragments expected. Furthermore, it led to the comigration of restriction fragments containing different exons; for example, in the case of the *Xba*I digest, four fragments comigrate: a 6.9-kb fragment containing exons 22–24; two 6.5-kb fragments containing, respectively, exon 17 and exon 1; and a 6.2-kb fragment containing exon 20. We wanted to avoid this situation to achieve a reduction of intensity of 50% for each deleted fragment in the densitometry analysis. We, thus, amplified each exon as a ~300-bp genomic fragment and performed a three-step hybridization using, consecutively, the following probes: mix 1, containing exons 3–10 and 15–19; mix 2, containing exons 11–14 and 20–24; and mix 3, containing exons 1 and 2 (Table 1). A 1.75-kb *Eco*RI and a 5.8 kb *Hin*dIII fragment, which we were not able to explain, were systematically recognized with mix 2 (exons 11–14 and 20–24). The 5.8-kb fragment was also visualized by another group when genomic DNA digested with *Hin*dIII was hybridized with a *BRCA1* cDNA probe containing exons 14–24 (4).

Genomic DNA was prepared from either whole blood or EBV-immortalized lymphoblastoid cells. Ten μg were digested with ~30 units of *Eco*RI, *Hin*dIII, or *Xba*I (Promega) for 5 h. Twenty-four-cm-long agarose gels (0.8%) were run 16 h at 75 V in 1× Tris-borate EDTA buffer (12). Denaturation and alkali transfer of the DNA to nylon membranes (Hybond N$^+$; Amersham) was performed according to the instructions provided by the manufacturer. PCR products used as probes were obtained with a GenAmp PCR System 9600 (Perkin-Elmer) using the Red Hot Taq and primers listed in Ref. 13 for exons 1–10 and 12–24; primer pair 11G was used for exon 11 probe. PCR fragments were purified with the Wizard PCR Preps DNA Purification System (Promega) and labeled using the Random Primed DNA Labeling Kit according to the instructions provided by the manufacturer (Boehringer Mannheim), using 100 μCi of ^{32}P-dCTP and ~50 ng of an equimolecular mixture of exons 3–10 and 15–19, 11–14 and 20–24, or 1 and 2. Probes were preincubated 1 h at 65°C with 1 mg of human placental DNA. Hybridizations were performed overnight at 65°C in the Church and Gilbert buffer (14), and washes were conducted at room temperature followed by 65°C as described (12). Filters were exposed 8–15 days to a phosphor screen and scanned with the Phosphorimager 445 SI (Molecular Dynamics), and densitometric analysis was performed using the ImageQuant software.

Fine Characterization of the 23.8- and the 3-kb Deletions. PCR were performed using the Expand Long Template PCR System (Boehringer Mannheim) using the manufacturer's conditions. In the case of F568, forward primer 7F (5′-AAA GTA TGG GCT ACA GAA ACC G) and reverse primer 14R (5′-TGC AGA CAC CTC AAA CTT GTC AGC) only amplified a ~3.8 kb mutant genomic fragment, the normal fragment being too large (27.6 kb) to be amplified. After digestion with several restriction enzymes (Promega), we managed to work out that the breakpoint junction was comprised in a ~400-bp *Pst*I fragment. After recovery from a 1% Low Melting Point (Life Technologies, Inc.) agarose gel, this fragment was purified using the Wizard PCR Preps DNA purification kit (Promega) and cloned in the pBluescript vector (Stratagene). Positive clones were sequenced with primers M13F and M13R using an Applied Biosystems Prism 377 semiautomated sequencing system. In the case of F788, amplification of 5.5- and ~2.5-kb genomic fragments from wild-type and mutant alleles, respectively, was performed using forward primer 14F (5′-CAG AAT CCA GAA GGC CTT TC) and reverse primer 16R (5′-GCA TTA TAC CCA GCA GTA TC). The reaction was digested with *Hae*III (Promega); an extra band of ~600 bp containing the breakpoint junction was isolated, cloned, and sequenced as described above.

mRNA was extracted from EBV-immortalized lymphoblastoid cell lines using the QuickPrep mRNA Purification Kit, according to the instructions provided by the manufacturer (Pharmacia). cDNA synthesis was carried out with the Expand Reverse Transcriptase, using the protocol given by the manufacturer (Boehringer Mannheim). Primers 7F/14R and 14F/16R were used with cDNAs from F568 and F788, respectively, and the Expand Long Template PCR System. The PCR fragments obtained from the mutant transcripts were checked for the absence of exons 7–13 or exon 15 by sequencing with the primers used for the PCR, after recovery from the agarose gel and purification, as described previously.

Haplotype Analysis. Haplotype analysis was performed by typing individuals with three *BRCA1* intragenic microsatellite markers: D17S1323 in intron 12, D17S1322 in intron 19 (15), and D17S855 in intron 20 (16). All markers were assayed by PCR with the Red Hot Taq using standard procedures. ^{33}P-radiolabeled PCR products were electrophoresed on 6% acrylamide denaturing gels.

RESULTS

Search for Mutations in the Promoter and the UTRs. Twenty-seven American and 51 French individuals (see "Materials and Methods") were screened by HDA in a region of 532 bp, containing a 267-bp fragment with strong promoter activity (17) and *BRCA1* exon 1a representing most of the 5′-UTR (the rest of the 5′-UTR is located in exon 2, which contains the start codon and has been screened

Table 1 *BRCA1 probes used in the Southern blot analysis and sizes (in kb) of the EcoRI, HindIII, and XbaI restriction fragments detected*

Exons :	ψ1	ψ2	1	2	3	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
EcoRI	~9.5		7.1		18.1					3.4		8.2		5.8	1.5	5.7		9.8			16.1				
HindIII	~14		8.8		1.5	10.4			4.0	1.7		7.2		6.4		19.0					3.2	3.6	9.7		
XbaI	~6	?	6.5	13.1	18.5									3.5	2.2	5.3		6.5	1.7		6.2	1.1	6.9		

, mix 1: exons 3–10 and 15–19; , mix 2: exons 11–14 and 20–24; , mix 3: exons 1 and 2 (exons 1 and 2 of *ψBRCA1* are also detected; see text).

previously). No variation was found either in the promoter or in the 5′-UTR of the *BRCA1* gene.

Because the sequence of the 3′UTR was not known when we started our analysis, we cloned it by performing the 3′ rapid amplification of cDNA ends technique and sequenced it (GenBank accession no. Y08864): it was found to be 1376 bp long and to contain an Alu repeat. The entire 3′-UTR, with the exception of the Alu repeat, was screened for mutations in our set of 79 samples by HDA; 3 variants were found. Two of these variants, G→T at position 424 (referring to the GenBank accession no. Y08864 sequence) or position 83481 (referring to the sequence of the whole *BRCA1* gene described in Ref. 11; GenBank accession no. L78833) and C→T at position 1290 (Y08864) or 84346 (L78833), were found to be frequent and were, thus, classified as polymorphisms. The third one, a 16-bp deletion at nucleotide 375 (Y08864) or 83432 (L78833), was found only once, but it did not segregate with the disease and was, thus, classified as a rare variant. No mutation was found in the polyadenylation signal.

Search for Internal Rearrangements by Southern Blot. The 78 DNA samples were analyzed by quantitative Southern blot hybridization with three probes used successively (Table 1): mix 1, containing exons 3–10 and 15–19; mix 2, containing exons 11–14 and 20–24; and mix 3, containing exons 1 and 2 (for a detailed description of the strategy used, see "Materials and Methods").

The Southern analysis led, for two samples, to the observation of extra bands (Fig. 1). In the case of F568, a French patient with breast cancer diagnosed at 49 years of age and ovarian cancer at 61 years of age and no family history of breast or ovarian cancer, an extra band was present in digestions with each of the three enzymes with the mix 1 probe (covering exons 3–10 and 15–19) and with *Hin*dIII and the mix 2 probe (covering exons 11–14 and 20–24); the *Eco*RI blots are shown in Fig. 1*a*. Quantitative studies showed loss of several restric-

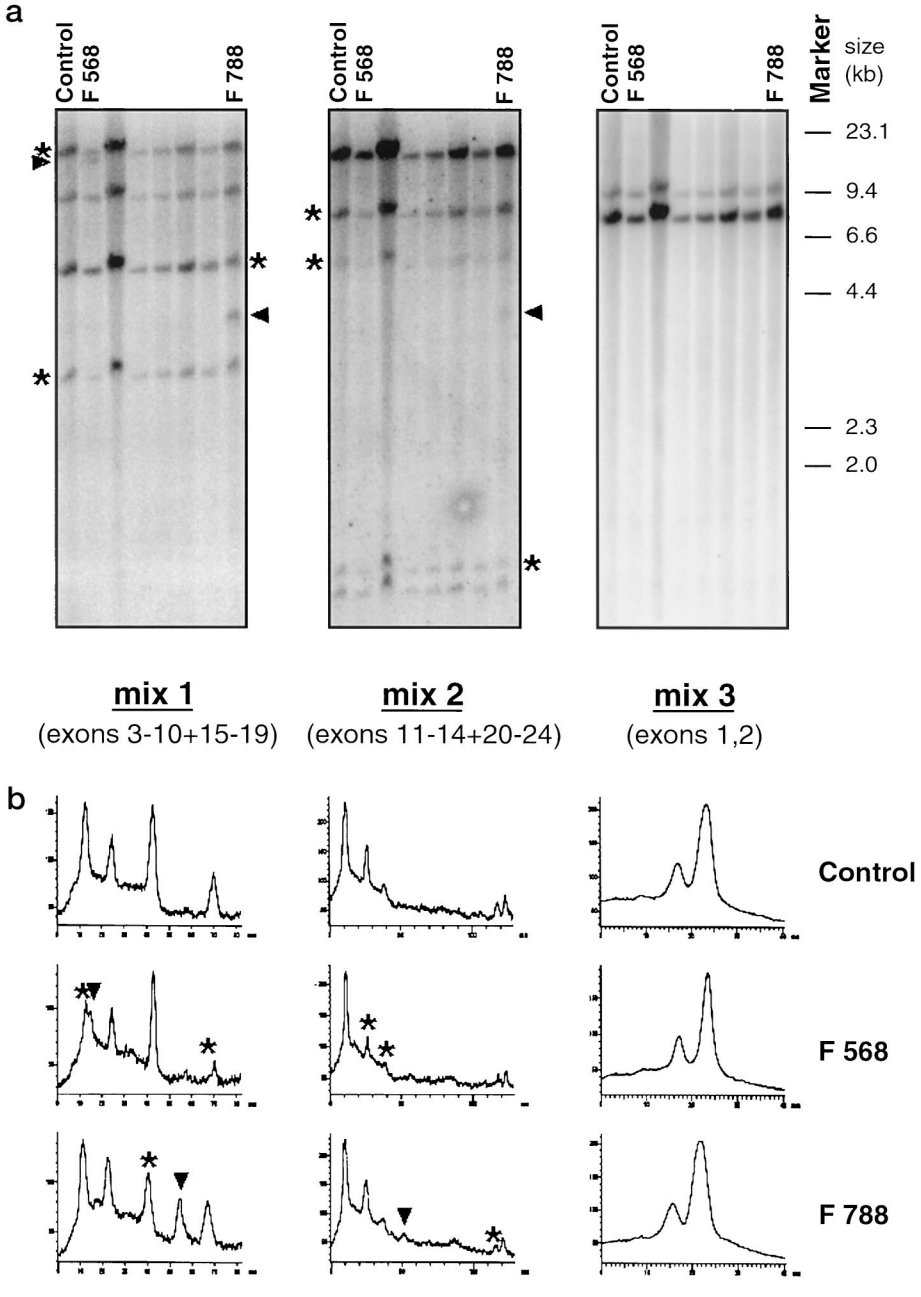

Fig. 1. Southern blot analysis showing the 23.8- and the 3-kb germ-line deletions in the *BRCA1* gene in F568 and F788, respectively. *a*, genomic DNA was digested with *Eco*RI and hybridized with the indicated probes (*mix 1*, *mix 2*, and *mix 3*). One control, five samples without rearrangement, and samples from F568 and F788 are presented. *Arrowheads*, extra bands containing the breakpoint junction (sizes, ~15 kb for mix 1 with F568; ~4 kb for mixes 1 and 2 with F788); ∗, bands with a reduced signal intensity; they are presented on the *left* of the blot for F568 and the *right* for F788. *b*, densitometric analysis of the Southern blots shown above with the ImageQuant software is presented for the control and for samples from F568 and F788. *Arrowheads* and ∗, extra bands and bands of reduced intensity, respectively.

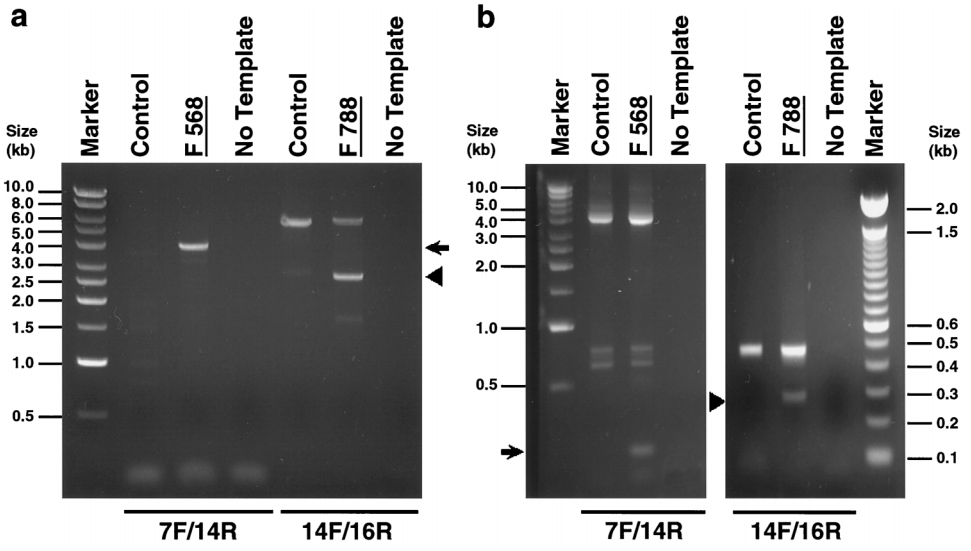

Fig. 2. PCR detection of germ-line deletions identified in the *BRCA1* gene in F568 and F788. *a*, genomic DNA was amplified with the primers indicated (see "Materials and Methods"). In the case of 7F/14R, only the mutant allele in F568 gives rise to a ~3.8-kb fragment (*arrow*) because the wild-type allele is too large (27.6 kb) to be amplified. In the case of 14F/16R, a 5.5-kb fragment is generated in the control, whereas in the patient from F788, an extra band of 2.5 kb (*arrowhead*) is also produced. *b*, cDNA was amplified with the same primers used previously. In the case of 7F/14R, a 4.1-kb fragment is generated in the control, whereas in the patient from F568, an extra band of 151 bp (*arrow*) missing exons 8–13 as determined by sequencing (see "Materials and Methods") is also produced. Nonspecificity around 0.7 kb was observed both in control and in F568 sample. In the case of F788, a 481-bp fragment is generated in the control, whereas in the patient from F788, an extra band of 290 bp (*arrowhead*) missing exon 15, as determined by sequencing, is also produced.

tion fragments, which indicated that a ~24-kb deletion comprising exons 8–13 was present in one allele of the screened individual (Fig. 1*b*). We then performed a long-range PCR to amplify the *BRCA1* gene from exon 7 to exon 14 (Fig. 2*a*). In control individuals, no fragment was generated (the expected fragment of 27.6 kb is too large to be amplified in our conditions), whereas in the patient from F568, a band of ~3.8 kb was obtained. The PCR product was then digested by different enzymes, which showed that the deletion breakpoint was comprised in a ~400 bp *Pst*I fragment (data not shown). This fragment was cloned and sequenced: it revealed that a 23,763-bp fragment comprising exons 8–13 is deleted in F568 (from nucleotide 26967 to nucleotide 50729 inclusive, referring to the whole gene sequence; GenBank accession no. L78833). The absence of exons 8–13 in the *BRCA1* mutant transcript produces a frameshift and leads to the presence of a premature stop codon (ter 150). The mutant mRNA is expressed at a much lower level than the wild-type transcript (Fig. 2*b*). Both breakpoints occurred in a region of perfect identity of 12 bp within two Alu sequences in the same orientation, which suggests that the deletion is probably the result of a homologous recombination. The Alu sequence located in intron 7 belongs to the Sx subfamily, whereas the one located in intron 13 belongs to the Sp subfamily; they share a 82% homology.

In the case of F788, a French family with three sisters affected with breast cancer diagnosed at 40, 41, and 46 years of age, an extra band was present with the three enzymes with the mix 1 probe (covering exons 3–10 and 15–19) and with *Eco*RI and the mix 2 probe (covering exons 11–14 and 20–24); the *Eco*RI blots are shown in Fig. 1*a*. Quantitative studies showed loss of intensity of one fragment on each blot hybridized with mix 1 and on the *Eco*RI blot hybridized with mix 2 (Fig. 1*b*). Together with the size of the extra bands, it indicated that a ~3 kb deletion comprising exon 15 was present in one allele of the screened individual. We then performed a long-range PCR to amplify the *BRCA1* gene from exon 14 to exon 16 (Fig. 2*a*). In control individuals, a 5.5-kb fragment was generated, whereas in the patient from F788, an extra band of ~2.5 kb was also present. The PCR product was then digested by *Hae*III: it produced an extra band, containing the deletion breakpoint, of ~600 bp in the patient only (data not shown). This fragment was cloned and sequenced: it revealed that a 2998-bp region comprising exon 15 is deleted in F788 (from nucleotide 53102 to nucleotide 56099 included, referring to the L78833 sequence). The *BRCA1* mutant transcript, which contains a premature stop codon (ter 1509) due to the absence of exon 15, is expressed at a much lower level than the wild-type transcript (Fig. 2*b*). Here again, the deletion is probably the result of a homologous recombination because both breakpoints occurred in a region of perfect identity of 10 bp within two Alu sequences with the same orientation sharing a 88% homology, a Sg one in intron 14 and a Sp one in intron 15.

Densitometric evaluation of the Southern blots hybridized with the mix 3 probe (covering exons 1 and 2) showed deletions of these two exons in two American breast and ovarian cancer families, 32 and 3514 (the *Eco*RI and *Hin*dIII blots are shown in Fig. 3). F32 contains 10 cases of breast cancer (mean age at diagnosis: 45 years old) and 2 cases of ovarian cancer; F3514 contains two cases of breast cancer, one case of ovarian cancer, and one case of bilateral breast cancer and ovarian cancer. Analyses of individuals belonging to F32 and F3514 failed to reveal any heterozygosity in the *BRCA1* coding sequence; therefore, we were not able to show that the absence of exons 1 and 2 in the mutant allele prevents transcription. Haplotype analysis with three *BRCA1* intragenic markers, D17S1323 in intron 12, D17S1322 in intron 19, and D17S855 in intron 20, revealed that these two families do not share a haplotype. Consequently, these deletions are expected not to be identical. A 14-kb deletion containing *BRCA1* exons 1 and 2 was recently reported in a breast/ovarian American family (3). To check if one of our families bore this 14-kb deletion, we used the PCR primers described in the publication; they normally anneal to regions separated by ~15 kb but produce a 1.3-kb fragment in carriers. No PCR product was produced when using DNA from individuals belonging to families 32 and 3514 (data not shown). Furthermore, the hybridization results we obtained with DNA of these families and probes covering the 5′ region of *BRCA1* (data not shown) did not match those obtained with DNA bearing this 14-kb deletion. Taken together, these results rule out the possibility that the same mutation was present in our two families. A *BRCA1* pseudogene has been shown to lie just telomeric to *BRCA1*, due to the duplication of a region containing *BRCA1* exons 1 and 2 (18). This leads to the detection of extra bands corresponding to exons 1 and 2 of *ψBRCA1* in Southern blotting when using probes for exons 1 and 2 (Fig. 3). Densitometric analysis allowed us to ascertain that the deletion does not include *BRCA1* exon 3 or exons 1 and 2 of *ψBRCA1*. Although the telomeric region of the human *BRCA1* gene has been examined in detail, and a gene, *NBR2*, has been reported to lie between *BRCA1* and *ψBRCA1* (18), data are missing concerning the size and the sequence of this region. Consequently, we were not able to define the location

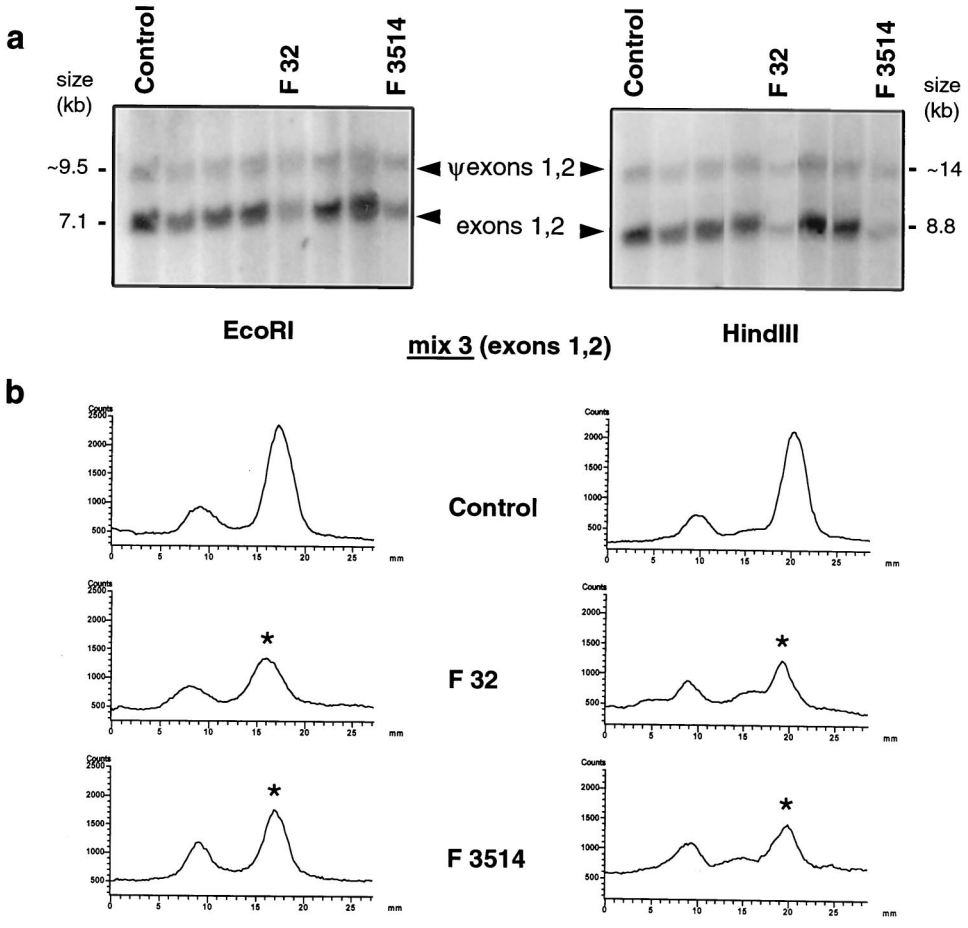

Fig. 3. Southern blot analysis showing exons 1 and 2 germ-line deletions in the *BRCA1* gene in F32 and F3514. *a*, genomic DNA was digested with *Eco*RI and *Hin*dIII and hybridized with the mix 3 probe. One control, five samples without rearrangements, and samples from F32 and F3514 are presented. *b*, densitometric analysis of the Southern blots shown above with the ImageQuant software is presented for the control and for samples from F32 and F3514. ∗, bands of reduced intensity.

of the breakpoints and the extent of these deletions. Further work is in progress to characterize them. A summary of identified deletions is presented in Table 2.

Search for Deletion of the Whole Gene. Deletion of the whole gene would not be picked up by our Southern assay and thus needed a separate, specific detection method. Because all of the patients used in this analysis were previously screened for mutations in the coding sequence of *BRCA1*, we were able to exclude deletion of the whole gene in 18 American families and 23 French families on the basis of heterozygosity for at least one polymorphism. The remaining 37 individuals were typed with three microsatellite markers located in introns of the *BRCA1* gene (D17S1323, D17S1322, and D17S855): only five individuals were found to be homozygous for all three markers and were thus suspected to be hemizygous. However, when more affected individuals belonging to the same families and carrying the linked haplotype were tested with these markers, hemizygosity remained possible in three families only. This possibility was then tested by quantitative Southern blot hybridization: a *BRCA2* probe (exon 11), which maps to 13q12.3, was used as an internal hybridization standard for densitometric analysis with *Eco*RI, *Hin*dIII, and *Xba*I digests probed with *BRCA1* exon 11. No anomaly in the ratio of the corresponding signals was detected with all three enzymes (data not shown). Therefore, deletion of the whole *BRCA1* gene was excluded for all families.

DISCUSSION

Two years ago, we reported the first deleterious genomic rearrangement in the *BRCA1* gene, a 1-kb Alu-mediated germ-line deletion removing exon 17 (2). Four more different large germ-line rearrangements have since been reported (3, 4). Three of them comprise 36% of all *BRCA1* mutations found in Dutch breast-cancer families (4). Recently, it was estimated, in an analysis performed by the Breast Cancer Linkage Consortium on 237 families, that, among those families linked to *BRCA1*, mutations were detected in the coding sequence or splice sites in 63% only (1). Altogether, these data led us to examine a series of 27 American and 51 French breast and breast/ovarian cancer families in which no mutation had been found by PCR-based techniques in the coding sequence and the splice sites of the *BRCA1* gene. We searched mutations in the promoter and the 5′- and 3′-UTR and rearrangements in the entire *BRCA1* gene.

The important role of 5′- and 3′-UTR of eukaryotic mRNAs in gene regulation and expression is described extensively (19). Nevertheless, no mutation was found in the 5′- and 3′-UTR or in the promoter of the *BRCA1* gene in any of our families. We did not find any deletion of the entire *BRCA1* gene, although such mutations were found in the case of other tumor suppressor genes as the retinoblastoma gene (20), the neurofibromatosis 1 gene (21, 22), and the von Hippel-Lindau gene (23). This could derive from the fact that this region is very gene rich (11, 24, 25); consequently, germ-line deletion

Table 2 *BRCA1 rearrangements identified*

Family no.	No. of breast cancers (mean age dg[a])	No. of ovarian cancers (mean age dg[a])	Mutation
F568[b]	1 (49)	1 (61)	del24kbEx8–13
F788	3 (42)	0 (—)	del3kbEx15
F32[b]	10 (45)	2 (61)	del?kbEx1–2
F3514[b]	3 (50)	2 (48)	del?kbEx1–2

[a] dg, diagnostic; ages are given in yrs.
[b] Families with one breast/ovarian cancer patient.

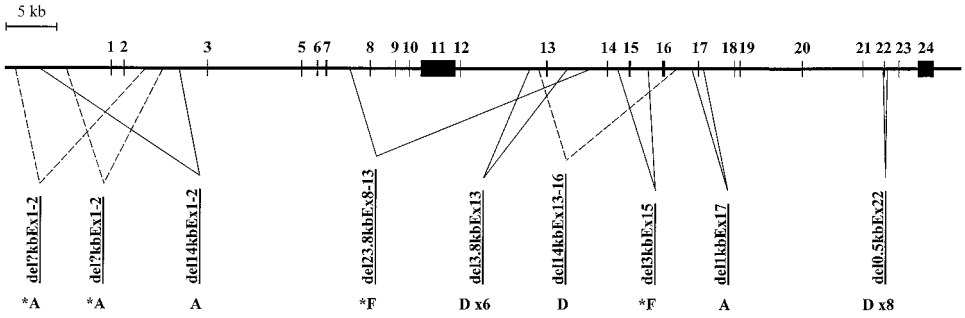

Fig. 4. Summary of all germ-line rearrangements described in the *BRCA1* gene. The location and extent of each characterized deletion are represented below the *BRCA1* gene. - - - - -, deletions which remain to be characterized. Families' origin is indicated (*A*, American; *D*, Dutch; *F*, French), along with the number of families in which each recurrent rearrangement has been found. *, rearrangements described in this paper.

of one or more important gene(s) localized close to *BRCA1* could be lethal.

To perform a genomic rearrangement search that was as comprehensive as possible, we optimized the conditions for the Southern analysis: three sets of digestion were useful to screen the whole gene, and consecutive hybridizations using probes made from three different combinations of small genomic fragments encompassing each *BRCA1* exon were necessary to achieve a reliable densitometric analysis. Such an analysis is useful not only to detect deletions and insertions in the *BRCA1* gene but also, once identified, to work out which part of the gene is involved. It should be noted, however, that Southern blot analysis is not sensitive enough to detect partial gene duplications (1.5-fold signal strength difference compared to a 2-fold difference in deletions) unless abnormal restriction fragment patterns are generated. Similar conclusions were drawn when the retinoblastoma locus was screened for the presence of rearrangements in patients with bilateral retinoblastoma (20).

We identified by Southern blot analysis four distinct *BRCA1* large deletions in three breast/ovarian cancer families and in one breast cancer-only family. Two of these alterations result in the absence of exons 1 and 2 in the mutant allele. Further work is needed to identify precisely the breakpoint junctions. The remaining two alterations were characterized: they consist of internal deletions of 3 and 23.8 kb removing exon 15 and exons 8–13, respectively. In both cases, the breakpoint junctions occurred between two repetitive Alu elements sharing >80% homology and oriented in the same direction. Up to now, nine different germ-line *BRCA1* rearrangements, including these latter four, have been described by us or others (Fig. 4), of which six have been entirely characterized: they range in size from 0.5 to 23.8 kb. Five are probably the result of a homologous recombination between two Alu sequences oriented in four cases in the same direction, whereas the sixth, a 0.5-kb deletion removing exon 22, probably occurred by recombination between nonhomologous sequences involving one Alu repeat. A compilation of rearrangements supported the idea that a 26-bp core sequence in Alu elements functions as a recombinational hot spot (26). However, in the case of *BRCA1*, only one site of cross-over fell in this core element, the other four being scattered throughout the Alu sequence (Fig. 5), suggesting that these gene rearrangements may have not been induced by specific sequences stimulating genetic recombination. The 11 Alu repeats involved in the six *BRCA1* germ-line deletions belong to various Alu subfamilies (Sx, 3; Sp, 3; Y, 2; Sc, 2; and Sg, 1). The density of Alus is rather high in the *BRCA1* gene (41.5%, compared to the average repeat element content as calculated in 326 genes, 30.4%; Ref. 11). We, thus, expected such a high number of repetitive elements to lead to genetic instability in this locus, as has been seen in the case of the human C1 inhibitor locus (density of Alu repeats of 31.4%): Alu-mediated rearrangements have been shown to account for ~20% of the hereditary angioedema chromosomes (27, 28). To obtain an estimate of the representation of rearrangements in the *BRCA1* mutation spectrum, we summarized the data obtained in our panel of 71 American families recruited by Dr. H. T. Lynch. Mutations in *BRCA1* were found in 37 of them (Refs. 2, 8, 9, and 29 and this study).[5] The proportion of families with *BRCA1* mutations in this series is in very good agreement with the results of the heterogeneity analyses performed using *BRCA1* linkage data (1): 37.5% in families with male breast cancer (expected, 19%), 68% in breast and ovarian cancer families (expected, 80%), and 20% in female breast cancer families (expected, 28%). Among these 37 germ-line alterations, 3 are rearrangements and 34 are mutations in the coding sequence and in splice sites. Thus far, rearrangements represent 8% of all *BRCA1* mutations in our sample. This percentage is likely to be more representative of the proportion of large rearrangements in the *BRCA1* mutation spectrum in most populations, as compared to the 36% found in Dutch families, due to a strong founder effect.

In summary, our results indicate that mutations in regulatory regions of the *BRCA1* gene are rarely expected to be the basis of genetic predisposition to breast and ovarian cancer. However, mutation screening efforts which focus only on genomic sequencing of all *BRCA1* exons (30) run the risk of missing a significant number of deleterious mutations. Indeed, screening for germ-line rearrangements appears mandatory in studies that aim to detect all mutations in this gene.

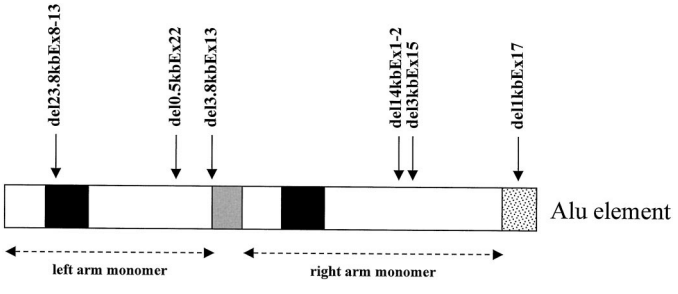

Fig. 5. Localization of breakpoint junctions of all characterized *BRCA1* rearrangements along a schematic Alu element. ▨ and ▩, adenine-rich linker and tail, respectively. ■, 26-bp core element reported to stimulate recombination (see text), one in the left arm monomer and one in the right one.

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Note Added in Proof

Following the submission of this paper, a duplication was identified in three American families of this set (N. Puget, O. M. Sinilnikova, D. Stoppa-Lyonnet, C. Audoynaud, S. Pagès, H. T. Lynch, D. Goldgar, G. M. Lenoir, and S. Mazoyer. An Alu-mediated 6 kb duplication in the *BRCA1* gene: a new founder mutation? Am. J. Hum. Genet., in press, 1999). Consequently, rearrangements represent 15% (6 of 40) of all *BRCA1* mutations in our samples.

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