Complex Regulation of the BRCA1 Gene*

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We have analyzed the promoter region of the human BRCA1 gene in detail and demonstrate that the expression of the BRCA1 gene is under complex regulation. First, its transcription is under the control of two promoters generating two distinct transcripts α and β, and second, promoter α is shared with the adjacent NBR2 gene and is bi-directional. Both promoter α and promoter β are responsive to estrogen stimulation. We also discerned that there are striking differences in both the genomic organization and immediate cis-control elements of the BRCA1 gene between humans and mice.

Since its isolation in 1994 (1), much effort has been devoted toward unraveling the biological function of the breast and ovarian cancer susceptibility gene, BRCA1. The fact that many germline mutations have been found has firmly established the involvement of the BRCA1 gene in familial breast and/or ovarian cancer (2). However, unlike the precedent of other tumor suppressor genes where mutant forms of the gene products are responsible for both the inherited and sporadic forms of the same type of cancer, no sporadic breast cancers and only a handful of sporadic ovarian cancers have been found to harbor BRCA1 mutations (3–5). It is thus postulated that either BRCA1 is involved only in the etiology of inherited breast cancer or that BRCA1 disruption in sporadic cancers occurs by mechanisms other than mutations in the coding region. A few lines of evidence are consistent with the latter hypothesis: in sporadic breast cancer the BRCA1 mRNA levels are decreased (6), while in familial breast cancer inferred regulatory mutations are present (2). Therefore, studies aimed at determining the regulation of the BRCA1 gene will be critical in helping to resolve this enigma as well as to understand the normal function of the gene product.

We have previously established a comprehensive map of approximately 50-kilobase pair genomic DNA encompassing not only the 5′ end of the BRCA1 gene but also the nearby NBR1 gene (previously named 1A1-3B) (7), which was isolated as a candidate for the ovarian cancer marker CA125 (8). The 5′ ends of both genes are duplicated with a partial copy of the BRCA1 gene lying head to head with the NBR1 gene and a partial copy of the NBR1 gene lying head to head with the BRCA1 gene (Fig. 1) (7). Further analysis of the expression of the partial copies of both genes showed that the partial copy of the NBR1 5′ exons is in fact a part of a new gene, NBR2 (9). The fact that the transcription start sites of both BRCA1 and NBR2 genes are only 218 bp apart suggests that the intergenic region may function as a bi-directional promoter. In addition, we have previously identified two distinct BRCA1 transcripts differing by the alternative use of the first exons, predicting that the BRCA1 gene is regulated by two promoters (10). This study is therefore aimed at unravelling the regulation of the BRCA1 gene by functional analysis of the BRCA1 promoters.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Plasmid p3ba containing the 5′ region of both BRCA1 and NBR2 genes was described previously (10). Constructs pGL1-4, -6, -8, -9, and -10 were generated by inserting various restriction enzyme-digested fragment sequences derived from p3ba into pGL3-basic vector (Promega), upstream of the firefly luciferase gene in either the BRCA1 or NBR2 orientation. Constructs pGL5, -7, and -11–13 were generated by polymerase chain reaction amplification, using primers polymerase chain reaction (PCR) amplification products, of the relevant promoter fragments (Fig. 2) and subsequently subcloned into the pGL3-basic vector. Plasmid pGL12M and pGL12RM, in which the CCAAT site (nucleotide 1433) was mutated to CACCT, were generated by a standard polymerase chain reaction-based site-directed mutagenesis method (11). Plasmid DNA was prepared with Qiagen columns and the sequences of all the constructs were verified by automated sequencing analysis using a 373A sequencer (ABI).

Cell Culture and Transfections—All cell lines were from stocks maintained by the Imperial Cancer Research Fund. MCF7 between T47D and JAR were grown in RPMI 1640 (Sigma) with 10% fetal calf serum (FCS, Life Technologies, Inc.). SKOV3 was grown in Eagle’s medium (Sigma) with 10% FCS and BT20 in minimal essential medium (Sigma) supplemented with bicarbonate and 15% FCS. Cells were cultured until approximately 70% confluent. Qiagen prepared plasmid DNA (15 μg of pGL3 basic vector, the activity of test plasmid and 10 μg of pGL3 control plasmid) was transfected by electroporation (250–270 volts, 960 μF) into approximately 5 × 10⁶ cells.

Reporter Gene Assays—All assays were carried out using assay kits from Promega. Cells were harvested 40–48 h after transfection by the addition of reporter lysis buffer (Promega), followed by cell scraping. The cell lysates were analyzed for both the luciferase and β-galactosidase activities using assay kits from Promega. After normalization to the β-galactosidase control, the transactivation activity of each test construct was calculated relative to the pGL3 basic vector, the activity of which was arbitrarily defined as 1. Each experiment was done at least twice, and the relative promoter activities shown represent the mean value.

Genomic Sequence Analysis—The mouse sequence used in this analysis was from cosmid clone, MCHCA1, as described previously (12) with additional mouse genomic sequence generated from further sequencing of this clone (GenBank™ accession number U73040). Human and mouse sequences were compared using the “BestFit” program (GGC). Potential transcription factor binding sites within the human and mouse sequences were identified by searching against the “TFsites.dat” data base using the “FindPatterns” program (GGC).

Estrogen Stimulation of Promoter Activity—To examine the promoter activities in response to estrogen stimulation, MCF7 cells were cultured in phenol red-free RPMI 1640 supplemented with 10% charcoal/dextran-stripped fetal calf serum for at least 3 days. Ten micrograms of the luciferase constructs and 10 μg of pGL3 plasmid were co-transfected by electroporation into MCF7 cells. After 16–18 h, fresh medium containing 10 ng 17β-estradiol was added to the transfected cells. Twenty-four hours later, the cells were harvested and analyzed for both the luciferase and β-galactosidase activities as above. Relative luciferase activities upon estrogen stimulation were calculated with respect to that

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The abbreviations used are: bp, base pair(s); FCS, fetal calf serum; ER, estrogen response element; ER, estrogen receptor.
The transcription directions of these genes.

Activity of pGL10R compared with that of pGL11R (259 and 1191. This is reflected by the sharp increase in promoter activity of pGL9R compared with that of pGL10R (37 and 16, a 16-fold increase) and significant decreases in the activity of pGL8 (2.5-fold).

The promoter activities of pGL11 and pGL11R are less active than that of pGL12 and pGL12R, respectively, indicating that the sequence from positions 1191 to 1357 (GenBank™ number U37574, Fig. 2) contains a silencer negatively regulating the expression of both genes. The inhibitory effect of this silencer is 3.5-fold more potent than that orientated in the opposite direction (Fig. 2). These results confirm our hypothesis that the promoter a of the BRCA1 gene is bi-directional, and the cis-control elements harbored in the intergenic region are of crucial importance in co-ordinated expression of the BRCA1 and NBR2 genes.

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Fig. 1. Comparison of the genomic structure of the BRCA1/NBR1 region between humans and mice. Solid boxes represent the BRCA1 and NBR1 genes, which are conserved between humans and mice. Hatched boxes represent the NBR2 gene and the BRCA1 pseudogene, which are present in humans but not in mice. Arrow lines indicate the transcription directions of these genes.

obtained in the absence of the hormone, which was defined as 1. Each experiment was performed in duplicate and repeated at least twice.

RESULTS

Functional Analysis of the BRCA1/NBR2 Promoters—We have previously isolated a genomic clone p3ba containing the 5′ region of the BRCA1 and NBR2 genes (Fig. 2) (9, 10). The expression of the two distinct BRCA1 transcripts α and β (where exon 1A and exon 1B are the first exons respectively) is predicted to be controlled by their respective promoters a and β (Fig. 2). In addition, due to the close proximity of the transcription start sites for BRCA1 transcript α and the NBR2 transcript, BRCA1 promoter a is therefore predicted to act as a bi-directional promoter. To address these issues, a series of luciferase reporter constructs were generated by inserting various fragments of p3ba into the pGL3-basic vector (Fig. 2). The promoter activities were assessed by transient transfection of these reporter constructs initially into the breast carcinoma cell line, MCF7. All of the 5′-deletion constructs of the BRCA1 and NBR2 promoters tested were expressed in MCF7 cells, but with different efficiencies (Fig. 2). For the BRCA1 promoter a, the strongest activity was detected from the 267-bp fragment (pGL12) encompassing 43 bp of the BRCA1 exon 1A, the 218-bp intergenic region, and 6 bp of the NBR2 exon 1 (Fig. 2). When tested in the NBR2 orientation, the same 267-bp fragment also displayed the strongest promoter activity (pGL12R), which, interestingly, is 2.5-fold more potent than that orientated in the BRCA1 direction (Fig. 2). These results confirm our hypothesis that the promoter a of the BRCA1 gene is bi-directional, and the cis-control elements harbored in the intergenic region are of crucial importance in co-ordinated expression of the BRCA1 and NBR2 genes.

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For the BRCA1 promoter β (pGL7 and pGL6), the activity was much weaker (approximately 1/150) than that of promoter a (pGL12) (Fig. 2). The activity of promoter β can, however, be modulated by upstream cis-elements located in promoter α, as reflected by a 12-fold increase in promoter activity of pGL5 compared with that of pGL7 (24 versus 2, Fig. 2). This suggests that the expression of transcripts a and β of the BRCA1 gene may be co-regulated.

To confirm these results, the promoter activities of a subset of the constructs presented in Fig. 2 were determined in a number of other human cell lines: breast carcinoma cell lines T47D and BT20, an ovarian cancer cell line SKOV3, and a placenta choriocarcinoma cell line JAR. Similar data to that observed in the MCF7 cell lines were obtained (Table I).

Delineation of Transcription Factor Binding Sites in the BRCA1/NBR2 Promoters—In light of the results from the above deletion analysis of the BRCA1 and NBR2 promoters, we directed our attention to the 862-bp region encompassing both the BRCA1/NBR2 bi-directional promoter and the BRCA1 promoter β, i.e., from positions 1191–2052 (GenBank™ number
TABLE I
Relative activities of the BRCA1/NBR2 promoter constructs in human cancer cell lines

| Construct       | MCF7 | T47D | BT20 | SKOV3 | JAR |
|-----------------|------|------|------|-------|-----|
| pGL3-basic      | 1    | 1    | 1    | 1     | 1   |
| pGL7            | 2    | 1.2  | 2.7  | 1.3   | 5   |
| pGL11           | 97   | 47   | 147  | 66    | 120 |
| pGL12           | 335  | 361  | 310  | 296   | 225 |
| pGL13           | 109  | 67   | 97   | 54    | 32  |
| pGL10R          | 259  | 187  | 215  | 301   | 104 |
| pGL11R          | 16   | 11   | 17   | 17    | 19  |

U37574). DNA sequence homology search of this region with a transcription factor data base indicates the presence of several CCAAT boxes, GC boxes and PEA3 binding sites, and one putative binding site for each of the transcription factors CREB, GH, and AP1 (Fig. 3). These sites were obviously candidates for co-ordinated regulation of the BRCA1 and NBR2 genes. Since the BRCA1 and NBR2 promoters are TATA-less promoters, and the significance of the CCAAT element in modulating the function of such promoters has been documented (13), we chose to examine the regulatory effect of the CCAAT box located in the intergenic region (nucleotide 1433–1437, Fig. 13), we chose to examine the regulatory effect of the CCAAT box located in the intergenic region (nucleotide 1433–1437, Fig. 3). This CCAAT box present in reporter constructs pGL12 and pGL12R was targeted by site-directed mutagenesis changing CCAAT to CACCT. Compared with the respective wild type constructs, the activities of the mutated pGL12M and pGL12RM decreased by 65 and 56%, respectively, demonstrating a role for the CCAAT element in the co-ordinated activation of both the BRCA1 and the NBR2 genes.

Hormonal Regulation of BRCA1 Expression—Estrogen is known to modulate the growth and differentiation of human breast epithelium (14) and several studies have shown that BRCA1 levels are elevated upon estrogen stimulation (15, 16). We therefore examined the responses of the BRCA1 promoters to estrogen induction. Sequence homology searches reveal that neither promoter α nor promoter β contains a classic estrogen response element (ERE) (5’-GGTCA(N)3TGGTC(N)9TG-3’) (17). However, an alternative ERE (5’-GGTCA(N)3TGTCG(N)2N-3’) (18), was identified in the BRCA1 promoter β (Figs. 2 and 3). Upon estrogen stimulation, the promoter activity of the ERE-containing construct pGL7 was induced by 1.5-fold, while no change in activity was detected in the non-ERE-containing construct pGL6 (Fig. 4). This result implies that BRCA1 promoter β is regulated by estrogen. For BRCA1 promoter α, sequence homology search recognized a putative AP1 site (Fig. 3), which is known to bind the cellular proto-oncogene products c-Fos and c-Jun and has recently been shown to mediate estrogen effects (19). As presented in Fig. 4, a 2-fold increase in promoter activity of the pGL12 construct was observed upon estrogen induction, while no significant effect was seen for the pGL12R construct. The magnitudes of increase in activities upon estrogen stimulation in both BRCA1 promoters are in line with that observed in other promoters mediated by the alternative ERE and the AP1 site, which ranged from 1.4- to 8-fold (18, 19).

Comparison of the Regulatory Region of the BRCA1 Gene in Humans and Mice—To gain insight into the biological function of the BRCA1 gene in the human, several studies have been performed using the mouse as an animal model (20–23). However, the relevance of expression and function of the BRCA1 gene in mice to that in humans is, at present, unknown. To address this issue, we analyzed the 5’-end of the mouse Brca1 gene. Dramatic differences in genomic organization at the 5’-flanking region of the BRCA1 gene between the two species are observed (12) (Fig. 1). A fragment of approximately 30 kilobase pairs of genomic DNA housing both the NBR2 gene and a pseudocopy of the 5’ end of the BRCA1 gene found in humans is absent in mice. Since eukaryotic transcriptional machinery functions in a chromatin environment, the difference in the chromosomal organization of the 5’ end of the BRCA1 gene between humans and mice raises the possibility that the expression of the BRCA1 gene is regulated differently. Furthermore, alignment of the 5’ sequence of the BRCA1 gene from the human and the mouse revealed that the human exon 1B is not conserved in the mouse (GenBank™ number U73040), which is further supported by the fact that exon 1B of the human BRCA1 transcript β contains the primate-specific Alu sequence. This result indicates there is no mouse homologue of the human BRCA1 transcript β, implying that the mouse Brca1 gene is unlikely to be regulated by two promoters. Last, sequence homology comparison between the cis-control elements in the promoter regions of the human and mouse BRCA1 genes revealed that only a SP1 binding site and a CCAAT box are conserved (Fig. 3). In particular, neither the ERE nor the AP1 site is found in the 5’-flanking region of the mouse Brca1 gene, indicating that the regulation of the BRCA1 levels by estrogen is unlikely to be the same across species. Therefore for both genomic organization and immediate cis-control elements.
in the BRCA1 promoters, significant differences have been found between humans and mice.

**DISCUSSION**

This study analyzed the BRCA1 promoters in detail and demonstrates that the expression of the human BRCA1 gene is under the control of two promoters, one of which is bi-directional. The different transcripts may have distinct biological functions, and the maintenance of a correct ratio between them may be important for normal function. Further studies aimed at determining the proportion of the distinct BRCA1/NBR2 transcripts to each other in the growth and/or differentiation of human breast epithelial cells and in mammary tumorigenesis may be important for normal function. Further studies aimed at determining the proportion of the distinct BRCA1/NBR2 promoters, significant differences have been reported that estrogen induces BRCA1 expression in ER-positive breast cancer cell lines. Our data show that both BRCA1 promoters α and β are responsive to estrogen stimulation, albeit being a less pronounced effect than that observed for a classic perfect ERE (17). We detected a newly described alternative ERE (18) in BRCA1 promoter β (Figs. 2 and 3). It is therefore possible that the estrogen stimulation effect seen in promoter β is mediated by the classical ER pathway, where estrogen-bound ER interacts with DNA and subsequently interacts with the basic transcription machinery to stimulate transcription. In contrast, no conventional ERE was detected in BRCA1 promoter α, suggesting that more complicated mechanisms may underlie the estrogen regulation of this promoter. It has been proposed that estrogen-ER complex can activate transcription directly through the AP-1 motif (19, 25, 26). In BRCA1 promoter α, a putative AP-1 site is detected. Estrogen may therefore regulate BRCA1 expression via protein-protein interaction between ER and the c-Fos-Jun complex at the AP1 site. Alternatively, the estrogen activation of the BRCA1 promoter α may be indirect, secondary to hormone-induced synthesis of other transcription factors, which in turn transactivate the BRCA1 promoter α. This hypothesis is supported by the observation that estrogen regulation of BRCA1 expression is blocked by cycloheximide (27), suggesting de novo protein synthesis is required for estrogen-induced BRCA1 expression. However, these investigators failed to detect any response to estrogen induction using genomic fragments near the 5′ end of the BRCA1 gene (27). Neither of the two promoter constructs made by these authors contains the transcription initiation sites of the BRCA1 gene; therefore no direct comparison to our results can be made.

Through targeted germline inactivation, mice have provided a valuable animal model to study the role of tumor suppressor genes in normal growth control pathways and in human cancer. For the BRCA1 gene, however, contrasting data regarding the function of the encoded protein in development and in mammary tumorigenesis have been documented between mice and humans. Brca1-null mice die during early embryogenesis (20–22), suggesting that Brca1 is indispensible for normal cell growth and differentiation during murine embryonic development. This is in contrast with the human phenotype observed, in which a woman homozygous for a BRCA1 mutation is normal in growth and development with the only mutation-associated phenotype being a predisposition to breast/ovarian cancer (28). In addition, mice heterozygous for the BRCA1 deletion were phenotypically normal and did not develop any type of cancer at least by 1 year of age, whereas humans heterozygous for BRCA1 mutations are susceptible to early onset breast and/ovarian cancer. We demonstrate that both the genomic organization and the immediate cis-control elements of the BRCA1 gene are significantly different between humans and mice. This implies that the temporal and spatial expression pattern of the BRCA1 gene may differ between the two species, and caution needs to be exercised when interpreting experiments involving either endogenous murine BRCA1 expression or transgenic models of BRCA1 function.

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