Inherited mutations in the \textit{BRCA1} gene, presumably leading to loss of function, confer susceptibility to breast and ovarian neoplasms and are thought to be responsible for approximately 2.5–5% of all breast cancers. It has been suggested that alternative mechanisms, such as disruption of transcription, may also be involved in the suppression of \textit{BRCA1} gene expression/function in breast cancers. Therefore, we initiated studies on the \textit{BRCA1} transcriptional promoter. Utilizing systematic promoter deletions and transient transfection assays, a 36-base pair region was determined to be important for the positive regulation of \textit{BRCA1} transcription. Deletion of this positive regulatory region resulted in a significant loss of promoter activity. Utilizing DNA binding assays, proteins with specific affinities for the positive regulatory region were detected. Disruption of the DNA-protein complexes could affect normal \textit{BRCA1} transcription and may contribute to breast cancer susceptibility.

Breast cancer is the second leading cause of death in American women, accounting for more than 50,000 deaths each year. Current estimates place the average American woman’s lifetime risk of developing breast cancer at approximately 11%. However, women with two or more first degree relatives with breast cancer have an estimated 13-fold increased risk over the general population (1). Breast cancer in such families has an inheritance pattern consistent with a highly penetrant autosomal dominant allele (2, 3). \textit{BRCA1}, the first breast cancer susceptibility gene to be identified (4), was isolated in 1994 (5).

An interesting fact regarding \textit{BRCA1} is that although the mutations in the gene in familial breast cancers are of high penetrance, very few mutations in the \textit{BRCA1} gene have been found in sporadic forms of the cancer. These findings are prompting researchers to study the possibility of disruption of \textit{BRCA1} function through epigenetic mechanisms. Consistent with this notion, it has been suggested that transcriptional dysregulation of \textit{BRCA1} may play a role in suppressing \textit{BRCA1} expression in breast cells, perhaps contributing to the development of a neoplastic phenotype. Two studies have demonstrated a decrease of \textit{BRCA1} expression in sporadic breast cancer (6, 7). Another set of studies describe CpG methylation of \textit{BRCA1} transcriptional promoter in a number of sporadic breast cancers, in contrast to the lack of methylation in normal breast tissues samples (8, 9).

In light of these observations, we initiated a study to characterize the \textit{BRCA1} transcriptional promoter. Previously, the structural features of the \textit{BRCA1} promoter were described (10, 11), and preliminary descriptions of modest and indirect effects of estrogen on the \textit{BRCA1} promoter activity were reported (12, 13). However, no information was available regarding regulatory sites and specific regulatory factors. In this report we provide evidence for a positive regulatory region (PRR) in the \textit{BRCA1} promoter and show data that suggest that multiple proteins bind specifically to the site.

**EXPERIMENTAL PROCEDURES**

**Isolation of the \textit{BRCA1} Promoter**—The \textit{BRCA1} promoter was subcloned from a bacterial artificial chromosome clone, BAC 694 (kindly provided by Dr. Sean Tavtigian, Myriad Genetics) (14). Briefly, PsI linker (5′-GCTGCAGC-3′) was ligated into the blunted \textit{HindIII} site in the pGL2 vector (Promega vector with the firefly luciferase reporter gene). BAC 694 was digested with PsI, and the resulting fragments were shotgun cloned into the pGL2 vector and transformed into competent \textit{DH}5α \textit{Escherichia coli} cells. The transformed bacterial colonies were screened by colony hybridization with a radiolabeled \textit{BRCA1} cDNA probe (\textit{BRCA1} cDNA was kindly provided by Frank Calzone) (15) labeled by the random hexamer method (16), and clones with a 3.8-kilobase insert containing the \textit{BRCA1} 5′ genomic fragment were selected. The cloned fragment was sequenced completely and is identical to the previously described genomic fragment encompassing the \textit{BRCA1} promoter (GenBank accession number U37574) (11). The nucleotide position of mutants are numbers from the P1 promoter initiation site at nucleotide 1582.

**Mutants of \textit{BRCA1} Promoter**—Systematic promoter deletions were constructed by unidirectional exonuclease III digestion. 10 µg of \textit{BRCA1} promoter-luciferase construct was digested with \textit{MluI} restriction enzyme and blunted with α-phosphoribothioate nucleotides using Klenow enzyme. This treatment rendered the ends of the linearized plasmid resistant to exonuclease III digestion. The linearized DNA was purified by phenol/chloroform/isomyl alcohol extraction and digested with \textit{XhoI} restriction enzyme, generating a 5′ end susceptible to the exonuclease digestion. The fragment was purified and then subjected to exonuclease III digestion. Aliquots of the reaction were removed at regular intervals, and the reactions were terminated. Finally, the fragments were blunted using S1 nuclease, religated, and transformed into \textit{DH}5α competent cells. DNA preparations (using Qiagen columns) made from selected colonies were screened by analytical restriction enzyme digestions. Mutants –202 and +20 were constructed by exploiting the restriction enzyme sites present on the \textit{BRCA1} promoter (EcoRI, –202; \textit{SacI}, +20) and also the sites present in the polylinker of the luciferase vector. The \textit{BRCA1}-luciferase construct was digested with EcoRI/\textit{XhoI} (\textit{XhoI} site is present in the polylinker) and \textit{SacI} \textit{Sacl} site is also present in the polylinker), respectively, and blunted with Klenow enzyme, and the larger fragment gel was purified and religated.

Additional mutants to generate progressive deletions were constructed by a polymerase chain reaction based strategy. 5′ primers were designed at regular intervals along the sequence of the \textit{BRCA1} promoter: –245, 5′-CTC AGG CGT TAG AGG CTA GAG GGC AGG-3′; –198, 5′-CTC AGG CGT TCC TCT TCC GTC TCT TTC-3′; –177, 5′-CTC AGG CGT TCC TCT TCC GTC TCT TTC-3′.
5'-CTC ACG CGT TTA CAT CGG CCG GGA-3'; –162, 5'-CTC AGT CGG GAC TGG TGC GTT AAT-3'; –152, 5'-CTC AGT CGG TGG CCA ATC AGG CCC-3'; –118, 5'-CTC AGT CGG CTT TCT GTC CCT CCC ATC-3'; –86, 5'-CTC AGT CGG GAT TTA GTC TTT TGA-3'. This primer included TAG TGC GTT AAT-3' followed by a XhoI site, which is underlined, at the 5' end. The amplification conditions used were as follows (1 cycle for 2 min at 94°C and 35 cycles at 94°C for 15 s, 60°C for 15 s, 72°C for 30 s, and 1 ng of the cloned BRCA1 promoter plasmid was used as template DNA. The amplified products were digested with MluI and XhoI restriction enzymes and ligated into the restricted MluI-XhoI site of the pGL3 basic vectors (Promega). In addition, two synthetic primers (+5, 5'-GCC CGT T-3') and a complementary palindromic C/EBP canonical site (5'-TGG G-3') were annealed. The resulting double-stranded annealed double-stranded oligonucleotides dATP, dGTP, dTTP, and dCTP containing a-32P label.

RESULTS

BRCA1 Minimal Promoter—In order to identify the minimal promoter containing the essential regulatory regions, systematically deleted mutants of the BRCA1 promoter region were constructed (Fig. 1). The transcriptional activities of these mutants were tested in TK-TS13 and MCF-7 cells by luciferase reporter gene assays (Fig. 2). The TK-TS13 cells were used in initial studies (Fig. 2A), due to the ease with which they are transfected and because of their ability to support high levels of BRCA1 promoter activities. Subsequent detailed studies were performed in MCF-7 breast epithelial cell line (Figs. 2B and 3A).

Results of transfections of the mutants in TK-TS13 cell indicated that on the deletion of 1380 bases from mutant –1582 (and generating –202), there is a 25-fold drop of luciferase activity, which is not significant considering the high sensitivity of the luciferase assays and the number of bases deleted (Fig. 2A). Within the same tract of promoter DNA (–1582 to –202), the two most wide ranging luciferase values were 6127 (for –1244) and 308 (for –329) normalized light units, a difference of 20-fold. However, this difference was accompanied by a loss of 915 bases from –1244.

Overall, the data suggest that short deletions within the segment –1582 to –202 do not cause a significant change in promoter activity. However, gross deletions (–1582 to –202) do alter the configuration of the promoter sufficiently to affect the promoter activities significantly, because several weak regulatory sites (both enhancers and repressors) with additive effects may be deleted.

Transfections of selected BRCA1 promoter mutants in MCF-7 cells also indicated that essential transcriptional regulatory sites (which could have a strong effect on the transcription of BRCA1) were not present in the tract from –1582 to –202 (Fig. 2B). Interestingly, in MCF-7 cells mutant –202 was observed to possess a transcriptional activity that was comparable (and slightly higher) to that of construct –1582.

Finally, results from both cell lines indicated the presence of a sensitive region of 222 bases (–202 to +20), which on deletion resulted in a 100% loss of BRCA1 promoter activities (Fig. 2). These experiments strongly suggested that the essential regulatory elements of the BRCA1 promoter reside within the deleted segment. Furthermore, the mapped segment encompassed the P1 promoter region, suggesting that the activity of the P1 promoter was predominant in both the transfected cell lines. Curiously, P2 did not demonstrate any functional activity in either of the cell lines tested (Fig. 2). It is possible that sequences within the P1 promoter region may regulate the transcriptional initiation from P2.

Identification of the BRCA1 PRR—Following the identification of the –202 to +20 segment as essential for BRCA1 transcription, detailed characterization of the segment was undertaken. Additional unidirectional, polymerase chain reaction-based deletion mutants were constructed, and their activities were tested (Fig. 3A). With the aid of these promoter mutants, it was determined that deletion of a short 22-base pair region between –198 and –177 (Fig. 3B) resulted in a significant loss (14.5-fold) of luciferase activity. Further removal of 15 more nucleotides (–162) led to an additional 4-fold loss in activity.
Overall, the removal of 36 bases (from $2198$ to $2162$) results in a 56-fold loss in luciferase activity, indicating a PRR.

Interestingly, the PRR contains a short polypyrimidine-polypurine (Py-Pu) tract (the majority of nucleotides on the sense strand are pyrimidines and by extension the complementary strand is mostly purine) (Fig. 3B). The first 22 bases in the site are almost exclusively Py-Pu (21 of 22 or 95%). This Py-Pu-rich tract is followed by a putative CREB site. Overall the pyrimidines in the sense strand make up 75% (27 of 36) of PRR.

DNA Binding Assays—In order to characterize the proteins binding to the PRR site, electrophoretic mobility shift assays were performed using MCF-7 nuclear extracts. Retarded protein-DNA complexes were detected (Fig. 4A, lanes 2–8). Lane 2 lacked poly(dI-dC), and therefore the factors binding the probe largely represented nonspecific proteins. Addition of poly(dI-dC) (lanes 3–9), cleared nonspecific bands, and three protein-DNA complexes were detected (except in lane 9); a single intense, higher mobility band and two weaker bands with lower mobility, were observed. Addition of 50-fold excess of double-stranded nonlabeled oligonucleotides or sheared salmon sperm DNA (lanes 5–8) did not compete away the protein-DNA complexes. In sharp contrast, the proteins binding the labeled probe were efficiently competed out by a 50-fold excess of nonlabeled PRR probe (lane 9). This experiment indicates that the PRR-binding proteins bind in a sequence-specific manner.

In order to further characterize the components of the protein complex that assembled on the PRR, UV cross-linking experiments (involving DNA-protein linkage) were performed (Fig. 4B). This involved incubation of radiolabeled, BrdUrd-containing PRR probe, with MCF-7 nuclear extracts in the presence of UV radiation. The radiation induced covalent linking of proteins to the BrdUrd residues present in the PRR probe. All the reactions contained 3 µg of poly(dI-dC), and one of the reactions was supplemented with 0.5 µg of double-stranded oligonucleotides to increase the stringency of binding to the probe (lane 3). Lane 2 exhibits three diffuse and indistinct bands. The intensity of these bands increased on the addition of 0.5 µg of double-stranded oligonucleotides (lane 3), reinforcing the possibility that they represent specific protein-DNA interactions. The approximate molecular masses of the protein-DNA complexes observed were 55, 150, and 230 kDa.

Additional experiments were performed to exclude the possibility that the covalent protein-DNA complexes were formed due to nonspecific interactions. A random oligonucleotide labeled with α-32P and BrdUrd residues was used in UV cross-linking experiments (Fig. 4C). Lane 2 of the figure demonstrates the characteristic three-band pattern representing proteins bound to the PRR. In contrast, no significant protein-DNA complexes were observed when the random probe was incubated with MCF-7 nuclear extract (lane 4). This experiment suggests that the proteins detected by cross-linking experiments recognized and bound the PRR specifically.

Finally, it is probable that each of the DNA-protein complexes observed do not represent multiple proteins linked to a single DNA probe but a single protein molecule bound to one molecule of the PRR probe. This conclusion is based on the fact that the UV-induced protein-DNA cross-linking is inefficient; therefore it is unlikely that more than one protein molecule will link to a single molecule of DNA probe.

DISCUSSION

Evidence for important roles of BRCA1 in normal functioning of cells is accumulating. Specifically, a strong role for BRCA1 in DNA repair mechanisms (18–20) as well as in transcriptional regulation (21–23) has been suggested. This theme is reinforced in a recent report that cites the involvement of BRCA1 in transcription-coupled repair of DNA (24). Therefore,
it has been suggested that suppression of BRCA1 expression may cause defects in the DNA repair machinery, leading to chromosomal defects and tumorigenesis.

BRCA1 expression may be suppressed through transcriptional silencing in a subset of sporadic breast cancers, underscoring the importance of studies to elucidate the transcriptional mechanisms involved in the regulation of BRCA1 expression. The present studies strongly suggest that intact and functional PRR may be crucial for normal transcription of BRCA1. Hindrance of the PRR function may occur either by methylation of proximate sequences or by alterations in the properties of the regulatory factors, leading to suppression of BRCA1 expression. Furthermore, elucidation of the factors that regulate BRCA1 transcription could provide additional clues regarding its function.

It is interesting that the PRR encompasses a CpG dinucleotides reported by Mancini et al. (9) to be methylated in one case of sporadic breast cancer. The methylated cytosine was present in the putative CREB site present in the PRR (Fig. 3B). CREB proteins are known to mediate hormone stimulation of a variety of genes (25, 26), and BRCA1 is known to be indirectly responsive to estrogen stimulation (27), prompting speculation of a regulatory role of CREB in BRCA1 transcription. However, treatment of cells with forskolin (a reagent that stimulates post-translational activation of CREB by phosphorylation; Ref. 28) did not show any effect on the activities of transiently transfected BRCA1 promoter (data not shown). In addition, forskolin treatment and cotransfection of a CREB-binding protein (a CREB coactivator; Refs. 29 and 30) expression plasmid elicited no response from the BRCA1 promoter (data not shown).

Fig. 2. Minimal BRCA1 promoter. Luciferase activities of the deletion mutants in TK-TS13 (A) and MCF-7 cells (B). Deletions that result in loss of promoter activities are indicated and are found to be consistent for the two cell lines. The data are representative of three independent transfections for each line.

Fig. 3. PRR in the BRCA1 promoter. A, transfections of polymerase chain reaction-generated BRCA1 promoter mutants with short deletions in the minimal promoter region were performed. All the mutants had the 3’ end at nucleotide +36 of the transcription start site. The 5’ ends of each mutant are indicated. The data are representative of four independent transfections performed in triplicate for each sample. B, the 5’ ends of the deletion mutants in close proximity to the PRR (which is boxed). The pyrimidine-rich region and the putative CREB site are indicated.

---

**A**

![Minimal BRCA1 promoter](image1)

---

**B**

![PRR in the BRCA1 promoter](image2)
FIG. 4. Proteins specifically bind the PRR sequence. A, electrophoretic mobility shift assay was performed with a radiolabeled PRR probe. The amounts of poly(dI-dC) added to each reaction are indicated. Nonspecific competitions were performed with 50-fold excess of double-stranded, unlabeled oligonucleotides with indicated binding affinities (lanes 5–7). In addition, 50-fold excess of sheared salmon sperm DNA was also used as a nonspecific competitor (lane 8). Specific competition was performed by the addition of 50-fold excess of unlabeled PRR-binding oligonucleotide (lane 9). B, DNA-protein UV cross-linking experiments with PRR probe and MCF-7 nuclear extracts. The protein-DNA complexes are indicated (arrows). The free PRR probe is at the bottom of the lanes. In lane 3, 0.5 μg of double-stranded oligonucleotide (DS Oligo) containing the binding site for the p53 protein was added. C, comparison of protein affinities of PRR and random probes in UV cross-linking assays. All the reactions contain 3 μg of poly(dI-dC) and 0.5 μg of annealed double-stranded oligonucleotide containing the binding site for the C/EBP proteins.
shown). Attempts to identify CREB proteins in the DNA-protein complex, either by supershift assays or immunoprecipitation (of cross-linked DNA-protein complexes) with CREB antibodies, were not successful (data not shown).

The composition of the PRR (21 of the first 22 bases are pyrimidines on the sense strand) provides possible hints regarding mechanisms involved in transcriptional regulation of BRCA1. Previous studies have described the tendency for such Py-Pu domains to form triplex DNA, which influence transcription (31, 32). These tracts have been reported to be sensitive to S-1 nuclease digestion and are believed to influence the conformation of the chromatin assembly in the promoter region. In addition, a nuclear factor has been reported to bind a Py-Pu tract in the c-Ki-ras promoter (31). Therefore, it is possible that 1) the Py-Pu domain may alter the chromatin structure of the BRCA1 promoter region and 2) it may also be involved in specific recognition and binding by transcription factors.

There is no additional information available at present regarding the factors binding the PRR. Studies are being initiated to definitively identify and characterize the binding proteins in order to investigate their effects on BRCA1 transcription and their potential role in breast cancer.

Acknowledgments—We thank Kay Huebner for critical reading of the manuscript. We also thank Joseph F. Tamburrino for assistance in the maintenance of cell lines.

REFERENCES

1. Sattin, R. W., Rubin, G. L., Webster, L. A., Huezo, C. M., Wingo, P. A., Ory, H. W., and Layde, P. M. (1985) J. Am. Med. Assoc. 253, 1968–1973
2. Newman, B., Austin, M. A., Lee, M., and King, M. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3044–3048
3. Claus, E. B., Risch, N., and Thompson, W. D. (1991) Am. J. Hum. Genet. 48, 232–242
4. Hall, J. M., Lee, M. K., Newman, B., Morrow, J. E., Anderson, L. A., Huey, B., and King, M. C. (1989) Science 250, 1684–1689
5. Miki, Y., Swensen, J., Shattuck, E. D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., et al. (1994) Science 266, 66–71
6. Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. (1995) Nat. Genet. 9, 444–450
7. Sourninos, G., and Spandidos, D. A. (1998) Biochem. Biophys. Res. Commun. 245, 75–80
8. Dobrovic, A., and Simpfendorfer, D. (1997) Cancer Res. 57, 3347–3350
9. Mancini, D. N., Rodenhisier, D. I., Ainsworth, P. J., O'Malley, F. P., Singh, S. M., Xing, W., and Archer, T. T. (1998) Oncogene 16, 1161–1169
10. Brown, M. A., Nitsch, H., Xu, C.-F., Griffiths, B. L., Jones, K., Solomon, E., Hocking, L., Trowsdale, J., Black, D. M., and McFarlane, R. (1994) Nature 372, 733
11. Xu, C.-F., Brown, M. A., Chambers, J. A., Griffiths, B., Nicolai, H., and Solomon, E. (1995) Hum. Mol. Genet. 4, 2259–2264
12. Marks, J. R., Huper, G., Vaughan, J. P., Davis, P. L., Norris, J., McDonnell, D. P., Wiseman, R., Futreal, P. A., and Inglehart, J. D. (1997) Oncogene 14, 115–121
13. Xu, C.-F., Chambers, J. A., and Solomon, E. (1997) J. Biol. Chem. 272, 20994–20997
14. Neuhansen, S. L., Swensen, J., Miki, Y., et al. (1994) Hum. Mol. Genet. 3, 1919–1926
15. Wilson, C. A., Payton, M. N., Elliott, G. S., Buaas, F. W., Cajulis, E. E., Grosshans, D., Ramos, L., Reese, D. M., Slamon, D. J., and Calzone, F. J. (1997) Oncogene 14, 1–16
16. Feinberg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267
17. Thakur, S., Lin, H.-C., Tseng, W.-T., Kumar, S., Bravo, R., Gelinas, C., and Rabson, A. B. (1994) Oncogene 9, 2335–2344
18. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Fuenteun, J., Ashley, T., and Livingston, D. M. (1997) Cell 88, 265–275
19. Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997) Cell 90, 425–435
20. Thomas, J. E., Smith, M., Tonkinson, J. L., Rubinfield, B., and Pelakis, P. (1997) Cell Growth Differ. 8, 801–809
21. Chapman, M. S., and Verma, I. M. (1996) Nature 382, 678–679
22. Monteiro, A. N., August, A., and Hanauska, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1595–9
23. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5605–5610
24. Gower, J. C., Avrutskaya, A. V., Lateur, A. M., Koller, B. H., and Leadon, S. A. (1998) Science 281, 1009–1012
25. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682–6686
26. Comb, M., Birnberg, N. C., Seascholtz, A., Herbert, E., and Goodman, H. M. (1986) Nature 323, 353–356
27. Marks, J. R., Huper, G., Vaughan, J. P., Davis, P. L., Norris, J., McDonnell, D. P., Wiseman, R. W., Futreal, P. A., and Iglehart, J. D. (1997) Oncogene 14, 115–121
28. Gonzalez, G. A., and Montminy, R. (1989) Cell 59, 675–680
29. Kwek, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226
30. Arias, J., Alberts, A. S., Brindle, P., Clar, F. X., Smeal, T., Karin, M., Ferrarisno, J., and Montminy, M. (1994) Nature 370, 226–229
31. Hoffmann, E. K., Trusko, S. P., Murphy, M., and George, D. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13595–9
32. Sanda, M., Danielson, K. G., and Ita, R. V. (1994) J. Biol. Chem. 269, 579–587