Exhibit 5

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Identification of a novel transcriptional repressor element located in the first intron of the human *BRCA1* gene

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Loss or lowered expression of *BRCA1* in non-familial breast cancer has been shown in several recent studies. Understanding how *BRCA1* expression is regulated should provide new insights into the role of *BRCA1* in sporadic breast cancer. We have recently identified a critical 18-base pair (bp) DNA element within the minimal *BRCA1* promoter whereupon the formation of a specific protein-DNA complex and transcription of *BRCA1* is dependent. We now report a non tissue-specific transcriptional repressor activity, located more than 500 bp into the first intron of *BRCA1*. Progressive deletions from the 3′-end of intron 1 and reporter gene assays localized the repressor activity to an 83-bp region. Electrophoretic mobility shift assays with this 83 bp DNA and various sub-fragments of it showed binding of nuclear proteins to a 36 bp *Bst*NI–*Bse*RI fragment. Functional transcriptional repression by this 36 bp DNA could be conferred on a heterologous thymidine kinase promoter. Analysis of multiple reporter gene constructs containing the *BRCA1* genomic region driving transcription in both directions suggests that the putative negative regulatory element functions to block transcription only in the *BRCA1* direction, although the promoter is shared by the divergently transcribed *NBR2* gene. *Oncogene* (2001) **20,** 440–450.

Keywords: *BRCA1*; breast cancer; transcriptional regulation; intron; repressor

Introduction

Mutations of the *BRCA1* gene are responsible for the vast majority of breast and ovarian cancer families, and for one-third of breast cancer only families (Miki *et al*., 1994; Futreal *et al*., 1994; Ford *et al*., 1998). *BRCA1* encodes a gene product of 1863 amino acid residues (for reviews, see Casey, 1997; Bertwistle and Ashworth, 1998), translating into a 220 kd nuclear phosphoprotein (Chen *et al*., 1996; Ruffner and Verma, 1997; Wilson *et al*., 1999). Recent studies using *BRCA1*-deficient mouse embryonic stem cell lines (Moynahan *et al*., 1999), primary skin fibroblasts (Cressman *et al*., 1999), or a *BRCA1*-mutated human breast cancer cell line (Scully *et al*., 1999) have provided strong evidence of a caretaker function for BRCA1 (Deng and Scott, 2000). These studies also demonstrated that loss of *p53* function is a growth-promoting event in the transformation process of *BRCA1* deficient cells (Shen *et al*., 1998; Cressman *et al*., 1999).

BRCA1 can function as a transcriptional activator when fused to the Gal4 protein, both *in vitro* (Haile and Parvin, 1999) and *in vivo* (Chapman and Verma, 1996; Monteiro *et al*., 1996). Its ability to stimulate p21 expression provides direct evidence of its role as a transcription factor (Somasundaram *et al*., 1997). BRCA1 has been shown to physically associate with p53 and co-activate p53-responsive genes (Ouchi *et al*., 1998; Zhang *et al*., 1998). Furthermore, its presence in the RNA polymerase II holoenzyme (Scully *et al*., 1997; Anderson *et al*., 1998), its interaction with the histone deacetylase complex (Yarden and Brody, 1999) and the coactivatior CBP/p300 (Pao *et al*., 2000), firmly establish its role in transcription regulation. Its ability to inhibit the transcriptional activating function of estrogen-receptor-α provides a possible explanation as to why the mammary gland is the major target organ of tumorigenesis when *BRCA1* is mutated (Fan *et al*., 1999).

Several lines of evidence support the hypothesis that BRCA1 functions as a tumor suppressor protein. Most mutations found in *BRCA1* result in truncation of and therefore non-functional protein product (Breast cancer information core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). In addition, mutations are frequently accompanied by loss of the wild type allele in familial breast and ovarian cancer (Smith *et al*., 1992; Neuhausen and Marshall, 1994; Cornelis *et al*., 1995). Increased expression of *BRCA1* blocks the induction of tumors in nude mice bearing xenografts of the human MCF7 breast cancer cell line (Holt *et al*., 1996). Furthermore, reduction of *BRCA1* expression by antisense RNA results in an increase in cellular proliferation and transformation of NIH3T3 fibroblasts (Thompson *et al*., 1995; Rao *et al*., 1996). The generation of mammary gland specific knockout of *BRCA1* in mice provides the ultimate evidence for its tumor suppressor activity (Xu *et al*., 1999).

More than 700 mutations have now been reported throughout the entire *BRCA1* coding sequence (Breast cancer information core). However, the role of *BRCA1* in sporadic cancers is not clear since somatic mutations of the gene are very rare in sporadic breast or ovarian

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cancers (Futreal *et al*., 1994; Hosking *et al*., 1995; Merajver *et al*., 1995; Berchuck *et al*., 1998; Khoo *et al*., 1999; van der Looij *et al*., 2000). Loss of, or lowered, *BRCA1* expression is frequently found in sporadic breast tumors as compared to surrounding normal tissue (Thompson *et al*., 1995; Magdinier *et al*., 1998; Sourvinos and Spandidos, 1998; Wilson *et al*., 1999). An epigenetic mechanism such as methylation of the promoter has been proposed as a mechanism responsible for lowered *BRCA1* expression (Dobrovic and Simpfendorfer, 1997; Mancini *et al*., 1998; Magdinier *et al*., 1998; Rice *et al*., 1998; Catteau *et al*., 1999). However, alterations in methylation pattern between tumor and normal tissues have been found in only a small percentage (Dobrovic and Simpfendorfer, 1997; Mancini *et al*., 1998; Rice *et al*., 1998; Catteau *et al*., 1999) of cases or not at all (Magdinier *et al*., 1998). It therefore remains necessary to determine important regulatory sequences of *BRCA1* transcription. We (Suen and Goss, 1999) and others (Xu *et al*., 1997a; Thakur and Croce, 1999) have performed functional studies of the *BRCA1* promoter. We recently localized an 18 bp DNA element within the minimal *BRCA1* bi-directional promoter whereupon nuclear protein binding is dependent in order for *BRCA1* transcription to occur (Suen and Goss, 1999). We report here a second determinant of *BRCA1* expression, located more than 500 bp into the first intron of *BRCA1*. This *cis*-acting regulatory element functions as a repressor of transcription in multiple cell lines representing various tissues of origin. Protein-DNA complexes formed between this putative repressor element and nuclear extracts isolated from multiple cell lines were detected by electrophoretic mobility shift assays (EMSAs). Additional EMSAs with smaller fragments within the repressor region localized protein binding to a 36 bp DNA, which was able to confer a strong repressor activity on a heterologous thymidine kinase promoter. Analysis of extended promoter constructs going in the opposite direction suggests only transcription in the *BRCA1* direction is controlled by this putative repressor element. Transcription in the direction of the neighboring *NBR2* gene, which shares the same promoter and transcribes in the opposite direction, is unaffected.

Results

Detection of a transcriptional blocking activity in the first intron of BRCA1

A 56 bp DNA located within the intergenic region between *BRCA1* and its neighboring gene *NBR2* could function as a bi-directional minimal promoter (Suen and Goss, 1999). Reporter gene constructs containing the 56 bp minimal region (its relative position is shown by the closed box on the 2.7 kb *Pst*I – *Xba*I fragment in Figure 1; nucleotides are numbered the same way as a genomic *BRCA1* sequence that was deposited in the GenBank with accession no. U37574) displayed a tissue-specific transcriptional activity in the *BRCA1* direction. Consistent with our previous study (Suen and Goss, 1999), the intergenic sequences between the *Eco*RI and *Sst*I sites were required for the high level of expression detected with constructs 2, 3 and 7. Only a low background level of expression (construct 8) was observed when this intergenic region was excluded (constructs 4 and 6 in Figure 1). Transcriptional activity was maintained when the promoter was extended 3′ into the first exon and also included 172 bp of the first intron of *BRCA1* (construct 2, and several other constructs in Suen and Goss, 1999). Complete abolition of promoter activity was however observed, when a further 3′ – 820 bp sequence from the first intron of *BRCA1* was included (constructs 1 and 5). These data show that transcriptional activity from the *BRCA1* promoter was blocked by DNA sequences between the *Nru*I and *Xba*I sites.

Transcriptional repressor activity could be detected in various cell lines that express BRCA1

To confirm the intronic transcriptional repressor activity, the four reporter gene constructs shown in Figure 2 were transfected into three different cell lines which were shown to express *BRCA1* in our previous study (Suen and Goss, 1999). The pBR(*Spe*I – *Xba*I)-CAT (construct 1) was found to express 36 – 48-fold lower activity than pBR(*Spe*I – *Nru*I)CAT (construct 2) in all three cell lines. When the activity of pBR (*EcoR*I – *BamH*I)CAT (construct 3) was compared to that of pBR(*EcoR*I – *Sst*I) CAT (construct 4), a 12-fold suppression was observed in both Caco2 and HeLa cells, while a less pronounced but significant fourfold suppression was seen in the breast cancer cell line MDA-MB453.

Localization of the transcriptional repressor activity to a smaller fragment

In order to localize the repressor activity to a smaller region, a series of progressive 3′ end deletions were introduced from the *Xba*I site near the end of intron 1, and their activities were determined in Caco2 cells (Figure 3a). Consistent with results from the last two experiments, pBR(*Spe*I – *Xba*I)CAT (construct 1) expressed only at background level, similar to the empty vector (construct 9). A strong increase in CAT activity was observed when 3′ deletion was extended to the *Ava*II site at 2255 (compare constructs 3 and 4). A similar profile of CAT activities was observed with these constructs when they were analysed in HeLa cells (data not shown). These data suggest that the sequence between the *Ava*II and *Afl*II sites was responsible for transcriptional repression of the *BRCA1* promoter.

Sequence of the putative repressor element

A 90 bp *Nla*IV – *Nla*IV (2253 – 2342) DNA fragment that encompasses the putative repressor activity (as detected in Figure 3a) was cloned. It is located at 553 bp downstream from the first nucleotide of intron

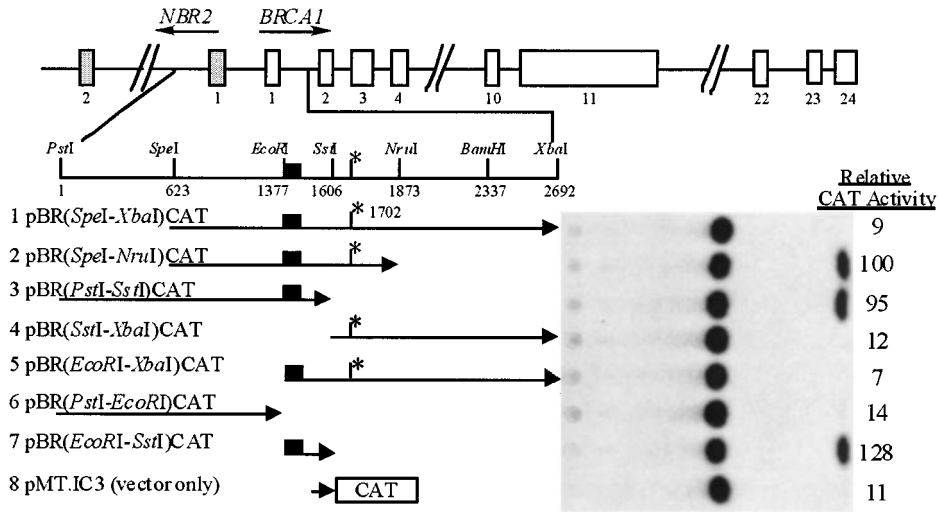

Figure 1 The intron 1 of *BRCA1* contains a transcriptional blocking activity. The top line shows the genomic organization of *BRCA1* and its neighboring gene, *NBR2*. Exons are marked as open and shaded boxes, and transcription proceed toward the right and left sides for *BRCA1* and *NBR2*, respectively. The translation start site for *BRCA1* is located in exon 2, while that of *NBR2* is located in exon 3 (not shown). The relative position and restriction map of a 2.7 kb *Pst*I – *Xba*I fragment is shown below the genomic scheme. Nucleotides are numbered the same way as a Genbank sequence (accession no. U37574). The closed box represnts a 56 bp minimal region which can function as a bi-directional promoter for the two divergently transcribed genes (Suen and Goss, 1999). The asterisk at position 1702 marks the beginning of intron 1 of *BRCA1*. Numbered solid rightward pointing arrows (constructs 1 – 7) correspond to the indicated restriction fragments that were cloned into the empty CAT vector (construct 8) and driving transcription in the *BRCA1* direction. These constructs were transfected into HeLa cells and a typical result of CAT assay is shown. Activities of the constructs are shown as a relative number to that of construct 2, which was assigned as 100. Experiments were repeated three times and a s.d. < 10% was observed

1 of *BRCA1* (nucleotide 1702). Interestingly, this putative repressor region contains three *GA*-rich sequences (each longer than 10 bp and marked by striped boxes above the nucleotides in Figure 3b), which are potential binding sites for both the *ets* (Sharrocks *et al*., 1997) and the *Sp1* (Philipsen and Suske, 1999) families of transcription factors.

Specific nuclear proteins bind to the putative repressor element

To determine if there are protein transcription factors that bind to the putative repressor element, the 84 bp *Nla*IV – *Nla*IV DNA (Figure 3b) was labeled and subjected to EMSAs (Figure 4a). Several slow migrating bands representing protein-DNA complexes were observed when the *Nla*IV – *Nla*IV fragment (lane 1) was incubated with nuclear extract isolated from HeLa cells (lane 2). The strong competitive effect of a 100-fold excess of the same unlabeled fragment (lane 3), but not a non-specific DNA (*ns*, lane 4) suggests that a major (marked with a solid arrow in all EMSAs, likely to be equivalent to C3 in Figure 4d) and a minor (marked with open arrowhead, likely to be equivalent to C1 in Figure 4d) bands were protein-DNA complexes that form specifically with this fragment. Additional bands representing other nuclear proteins (such as C2 and C4 in Figure 4d) that bind to this DNA were detectable after longer exposure (not shown and Figure 4d).

To localize the protein-binding activity to a smaller region, the labeled *Nla*IV – *Nla*IV fragment was cut with the restriction enzymes *Bst*NI or *Afl*II; and the four resulting fragments (Figure 4b, fragments 2 – 5) were gel-purified and subjected to EMSAs. Specific binding similar to those bands detected with the *Nla*IV – *Nla*IV fragment (Figure 4a) was observed with the *Bst*NI – *Nla*IV fragment (Figure 4c, lanes 4 – 6). Weak but specific binding was also detected with the *Nla*IV – *Afl*II fragment (lanes 1 – 3, position marked by a solid arrow. A long exposure of the dotted region (lanes 2 and 3) revealed another band migrating slightly faster than the one marked with the solid arrow (boxed region shown on the left), thus matching the two lower bands (one major and one minor) as detected with the *Bst*NI – *Nla*IV fragment (lane 5). The faint band detected with the *Afl*II – *Nla*IV fragment (its position is marked by an asterisk) was determined to be non-specific as the addition of the unlabeled competitor had no effect on the intensity of this band (lanes 11 and 12).

Specific binding of nuclear proteins to the *Bst*NI – *Nla*IV fragment was further confirmed by its strong competitive effect against formation of protein-DNA complexes with the *Nla*IV – *Nla*IV fragment (Figure 4d, lane 3), as compared to that of the *Nla*IV – *Nla*IV fragment itself (lane 2) and the adjacent *Nla*IV – *Bst*NI fragment (lane 4).

Delineation of the protein-binding activity to a 36 bp fragment

We next attempted to delineate a minimal region that could account for the protein-binding activity. The

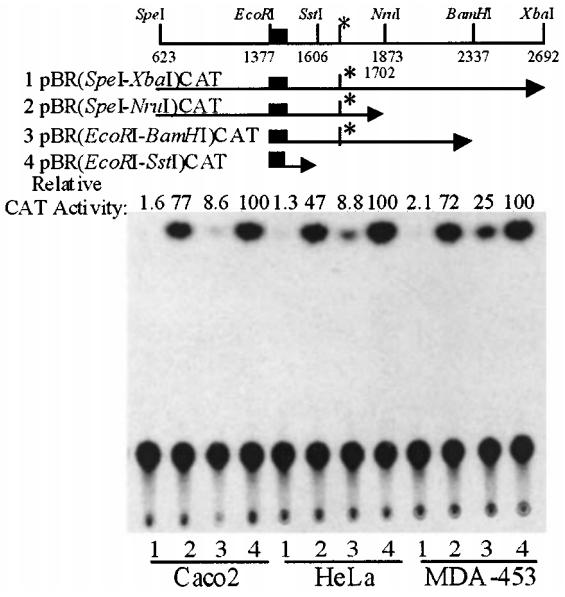

Figure 2 Putative repressor activity is found in various cell lines. The four CAT constructs (constructs 1–4) were transfected into the three indicated cell lines. The activities of the correspondingly numbered constructs are shown on top of a typical CAT assay. As in Figure 1, the closed box represents the minimal promoter region and the asterisk marks the beginning of intron 1 of *BRCA1*. Activity of the pBR(*Eco*RI–*Sst*I)CAT (construct 4) was assigned as 100 for reference. No cross comparison of activities should be made among the cell lines, as transfection was not normalized as such

62 bp *Bst*NI–*Nla*IV fragment (Figure 4b, fragment 3) was cut by *Bse*RI, the resulting 36 bp *Bst*NI–*Bse*RI and 26 bp *Bse*RI–*Nla*IV fragments (fragments 6 and 7, respectively in Figure 4b) were cloned. These fragments were first tested for their ability to compete against formation of protein-DNA complexes with the *Bst*NI–*Nla*IV fragment (Figure 5a). In agreement with its strong-competitive effect against formation of protein-DNA complexes between HeLa nuclear extract and the *Nla*IV–*Nla*IV fragment (Figure 4d), the *Bst*NI–*Nla*IV fragment yielded the same pattern of binding (protein-DNA complexes labeled as C1–C4) as detected with the *Nla*IV–*Nla*IV fragment (compare lane 2 of Figure 5a with lane 1 of Figure 4d). A progressive loss of effectiveness in competition was observed from the *Bst*NI–*Nla*IV (lane 3) to *Bst*NI–*Bse*RI (lane 4), and *Bse*RI–*Nla*IV (lane 5) fragments. These data were confirmed by testing the individual fragments in a separate EMSA (Figure 5b). The labeled *Bst*NI–*Bse*RI DNA (lanes 1–4) was able to form the two faster migrating complexes C3 and C4 (Figure 5b, lane 2). These complexes were strongly competed away by the inclusion of a 100-fold excess of its unlabeled self (lane 3), and less effectively by a similar quantity of the adjacent *Bse*RI–*Nla*IV fragment (lane 4). The cross-competitive effects of the *Bst*NI–*Bse*RI and *Bse*RI–*Nla*IV fragments and the apparent difference in their effectiveness in competition against the formation of protein-DNA complexes (Figure 5a and lanes 3 and 4 in Figure 5b) was also reflected by the much weaker ability of the *Bse*RI–*Nla*IV fragment to form specific

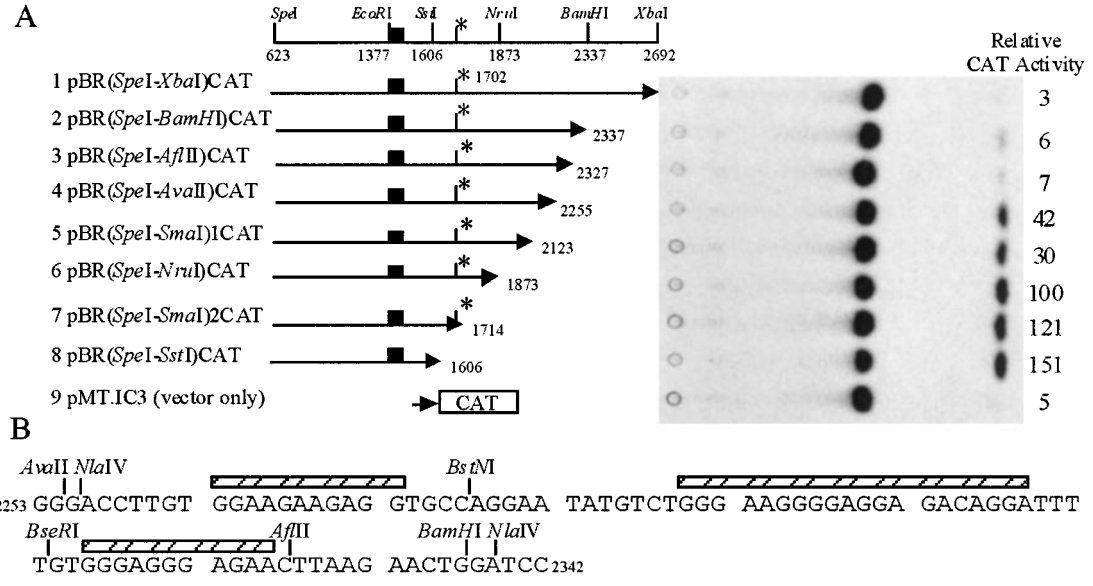

Figure 3 Localization of a putative repressor region and its sequence. (**a**) A series of deletion was obtained by cloning the indicated restriction fragments (shown as solid rightward pointing arrows, constructs 1–8) into the CAT vector pMT.IC3 (construct 9). A typical result of a transfection into Caco2 cells and subsequent CAT assay is shown. The activities of all constructs are expressed as a relative number to that of pBR(*Spe*I–*Nru*I)CAT (construct 6) which was chosen as a reference and assigned an activity of 100. Note that this construct was used in all CAT assays in this study so that comparison of relative activities of all constructs is possible after simple calculations. (**b**) The sequence between the *Ava*II (nucleotide 2255) and *BamH*I (nucleotide 2337) sites, which was mapped to contain the repressor activity in (**a**). Nucleotides are numbered the same way as a Genbank sequence (accession no. U37574). Several *GA*-rich sequences are marked by striped boxes above nucleotides and represent potential binding sites for *ets* or *Sp1* families of transcription factors

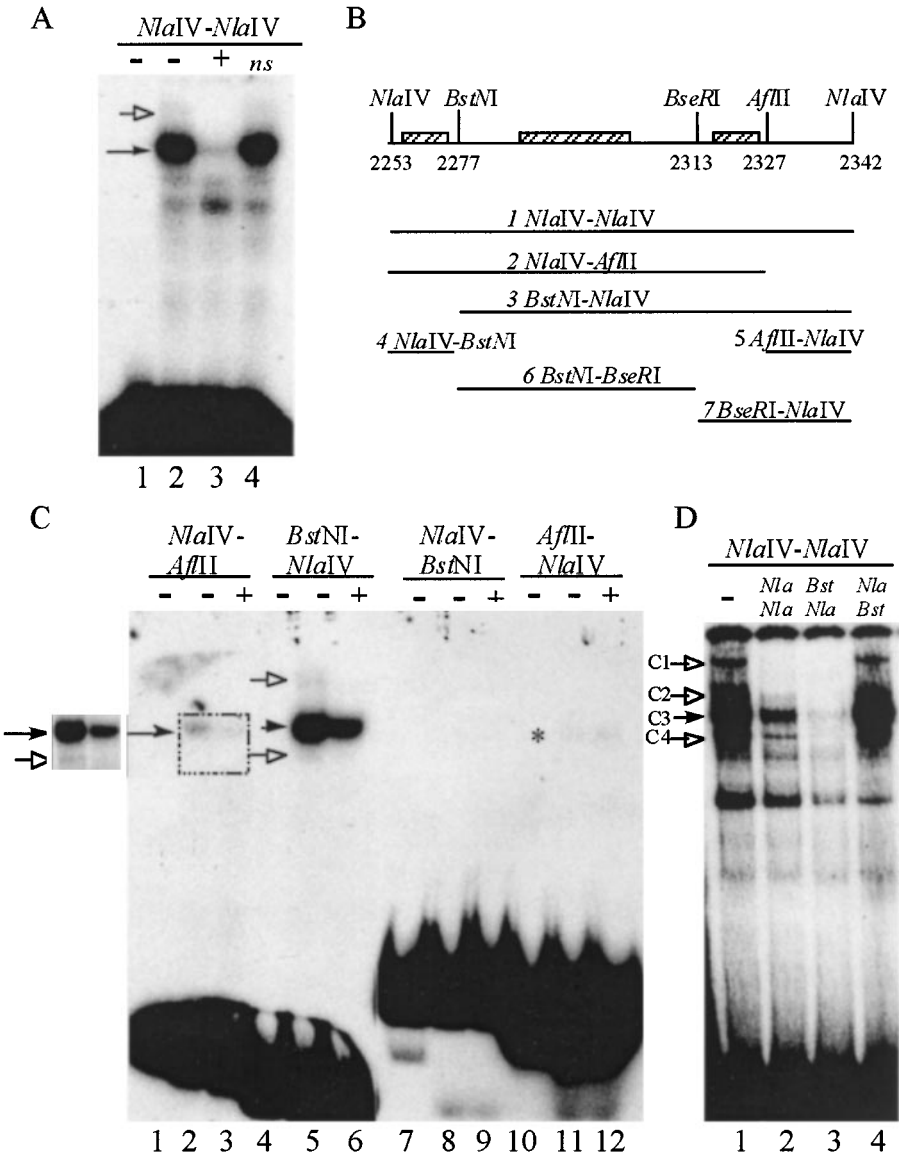

Figure 4 Nuclear proteins bind to the putative repressor element. (**a**) An 84 bp *Nla*IV–*Nla*IV fragment which encompasses the putative repressor DNA element (sequence and restriction recognition sites are shown in Figure 3b) was labeled and analysed by an electrophoretic mobility shift assay (EMSA). Slower migrating complexes were detected when the probe (lane 1) was incubated with nuclear extract isolated from HeLa cells (lanes 2–4). No additional DNA ('minus' sign, lanes 1 and 2), a 100-fold excess of the unlabeled self-fragment ('plus' sign, lane 3), or an irrelevant DNA (*ns* for nonspecific , lane 4) was added as competitor to determine the specificity of the protein-DNA complexes (a major band marked by the solid arrow; a minor band marked by the open arrowhead can be seen more readily with longer exposure). (**b**) Schematic representation of the different fragments used in additional EMSAs. The *Nla*IV–*Nla*IV fragment (fragment 1) is shown on top with positions of the relevant restriction enzyme recognition sites marked. The striped boxes correspond to the positions of the three *GA*-rich sequences, marked the same way as in Figure 3b. Smaller restriction fragments that were used in additional EMSAs are shown below the *Nla*IV–*Nla*IV fragment (fragments 2–7). (**c**) The *Nla*IV–*Nla*IV fragment was labeled on both ends, cut with either *BstN*I or *Afl*II, and the resulting restriction fragments (fragments 2–5) were separated on a 6% polyacrylamide gel, visualized by autoradiography and purified. An EMSA of the four indicated fragments (fragments 2–5), *Nla*IV–*Afl*II (lanes 1–3), *BstN*I–*Nla*IV (lanes 4–6), *Nla*IV–*BstN*I (lanes 7–9), and *Afl*II–*Nla*IV (lanes 10–12) is shown. No nuclear extract was added for lanes 1, 4, 7, and 10. Either no competitor (lanes with 'minus' sign) or a 100-fold excess of the unlabeled *Nla*IV–*Nla*IV fragment (lanes with 'plus' sign) was added to the incubation. A long exposure of the dotted region (lanes 2–3) si shown to the left. (**d**) The *Nla*IV–*Nla*IV fragment was labeled and incubated with nuclear extract isolated from HeLa cells in the absence (lane 1) or presence of an 100-fold excess of the indicated unlabeled DNAs as competitor (lanes 2–4). The solid arrow and open arrowheads indicate the positions of the major and minor specific protein-DNA complexes, respectively, in all EMSAs

complexes in the same EMSA (Figure 5b, lanes 5–8, marked by a solid arrow). This complex is likely to be C3 based on the banding pattern and its mobility which is similar to C3 (minus the difference in size of the two probes). *GA*-rich sequences could be found in both the *BstN*I–*BseR*I and the *BseR*I–*Nla*IV frag-

ments (Figure 3b). The vast number of transcription factors in the *ets* family which can recognize *GA*-rich sequences (Sharrocks *et al*., 1997) with different affinities may underlie the cross-competitive effects of the two fragments against formation of protein-DNA complexes with the repressor DNA (Figure 5). It is also possible that a weaker binding protein can only be detected when a stronger binding protein is competed away from binding to the same *GA*-rich region. This may explain the strong increase in intensity of a faster migrating band (Figure 5b, lane 3, band marked with an asterisk) when the *Bst*NI – *Bse*RI fragment was used to compete for formation of protein-DNA complexes against itself.

The 36 bp BstNI – BseR*I fragment conferred a transcriptional repressor activity onto a heterologous promoter*

The combined results from EMSAs (Figures 4 and 5) suggest that the 36 bp *Bst*NI – *Bse*RI fragment is responsible for two (a major complex C3 and a minor complex C4) of the four complexes which form on the putative repressor DNA (*Nla*IV – *Nla*IV fragment). It was important to determine if it has functional effects on transcription. A reporter gene construct driven by a thymidine kinase (*TK*) promoter was used to test if repressor activity could be transferred onto a heterologous promoter. As shown in Figure 6, the *Bst*NI – *Bse*RI fragment was able to suppress the *TK* promoter activity (construct 1) by 4 – 12-fold when it was cloned immediately downstream in either orientation (constructs 2 and 3). This transcriptional repressive effect was however, not observed when the same DNA was cloned upstream to the *TK* promoter (data not shown).

The neighboring gene transcribed in the opposite direction might also be negatively regulated by a repressor element in its intron

The above result suggests that the repressor element may function in a position- or oreintation-dependent manner. To characterize the function of the repressor element within its native genomic alignment, we made use of the fact that *BRCA1* is in close proximity with its neighboring gene (*NBR2*) (Xu *et al*., 1997b) and that they share a common promoter (Xu *et al*., 1997a; Suen and Goss, 1999). Multiple reporter constructs either including or excluding the putative repressor region were analysed for their activities. As shown in Figure 7a, all constructs excluding the putative repressor element (its position is indicated by a square and labeled with an *X*) were functional. Consistent with our previous work (Suen and Goss, 1999), promoter constructs transcribing in the *NBR2* direction (constructs 4 – 6) were always more active than their equivalent constructs transcribing in the *BRCA1* direction (constructs 1 – 3). However, when the repressor region was included, only transcription in the *BRCA1* direction was stopped (Figure 7b, compare constructs 4 and 5), while transcription in the *NBR2* direction was not affected (compare contructs 8 and 9 with construct 10). This suggests that the repressor region may function to stop transcription in the *BRCA1* direction specifically.

The construct which contains extended sequences from the *NBR2* intron (construct 1) drove transcription

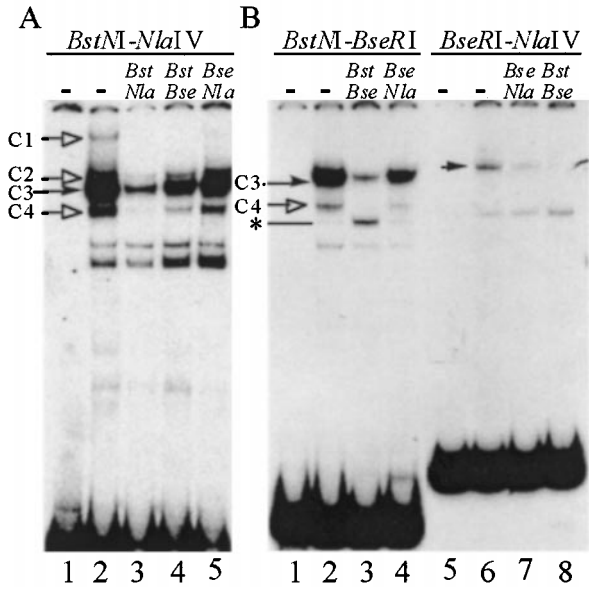

Figure 5 Binding of nuclear proteins is localized to a 36 bp *Bst*NI – *Bse*RI fragment. (**a**) The *Bst*NI – *Nla*IV fragment (Figure 4b, fragment 3) identified in the previous experiment (Figure 4c, lanes 4 – 6) was labeled and incubated with HeLa nuclear extract. Similar pattern of binding (lane 2, one major complex C3 and three minor complexes C1, C2 and C4) was observed as with the *Nla*IV – *Nla*IV fragment (Figure 4d). 100-fold excess of unlabeled self (lane 3), the *Bst*NI – *Bse*RI (lane 4, fragment 6 in Figure 4b), or the adjacent *Bse*RI – *Nla*IV fragment (lane 5, fragment 7 in Figure 4b) was included to examine the specificity of the protein-DNA complexes. (**b**) The *Bst*NI – *Bse*RI (lanes 1 – 4) and the *Bse*RI – *Nla*IV (lanes 5 – 8) fragments were labeled and tested as in (**a**). The incubation was carried out in the absence (lanes with 'minus' sign) or presence of the indicated unlabeled competitor (lanes 3, 4, 7 and 8). The solid arrow marks the same major complex (C3) that was detected in Figures 4 and 5a. An asterisk marks the position of a fast migrating complex that yielded stronger intensity only after self-competition (lane 3)

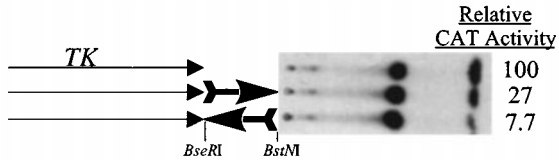

Figure 6 The 36 bp *Bst*NI – *Bse*RI fragment is sufficient for a repressor activity which could be transferred onto a heterologous promoter. The thymidine kinase (*TK*) promoter is shown as a solid arrow driving the CAT reporter gene (construct 1). The *Bst*NI – *Bse*RI (shown as a block arrow) was cloned as in its native (construct 2) or opposite orientation (construct 3) downstream to the *TK* promoter. The three constructs were transfected into HeLa cells and the result of a typical CAT assay is shown. The activity of the *TK* promoter was assigned as 100 for comparison

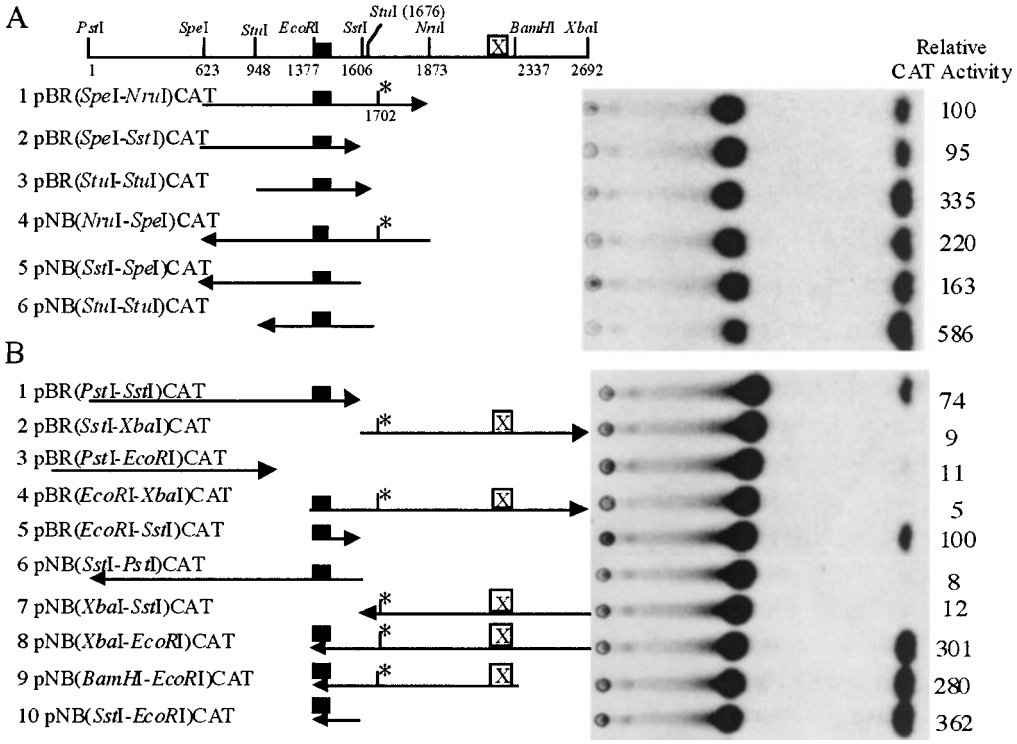

Figure 7 Transcriptional repressor element is also found in the first intron of the neighboring *NBR2* gene and the two transcriptional repressor elements only affect expression of their respective genes. (**a**) Comparison of transcriptional activities of multiple constructs in their native genomic configuration, driving expression in either *BRCA1* (rightward pointing arrows, constructs 1–3) or *NBR2* direction (leftward pointing arrows, constructs 4–6) when the *BRCA1* intronic repressor region (approximate position is indicated by a square labeled with an *X*) was excluded. (**b**) Examination of further reporter constructs which include the *BRCA1* intronic repressor region and driving in either the *BRCA1* (constructs 2 and 4) or the *NBR2* direction (constructs 7–9). Constructs extending far into the intron 1 of *NBR2* gene were also tested in their ability to drive transcription in either the *BRCA1* (constructs 1 and 3) or *NBR2* (constructs 6) direction. The intergenic promoter region (*EcoR*I–*Sst*I) driving transcription in the *BRCA1* direction (construct 5) was used as a reference (activity assigned as 100)

in the *BRCA1* direction as efficiently as the intergenic sequence (construct 5). As shown in earlier experiments (Figure 1), construct 2 and 7 were non-functional because the intergenic region was not included (Suen and Goss, 1999). Most interestingly, when the *Pst*I–*Sst*I (as in construct 1) fragment was tested in the opposite orientation (construct 6), the strong promoter activity in the *NBR2* direction was extinguished (compare constructs 6 and 10). These results imply that transcription in the *NBR2* direction may be controlled in a similar manner as is transcription in the *BRCA1* direction, namely that the first intron of the respective genes plays an important negative regulatory role in their transcription.

Discussion

An early study of the *BRCA1* promoter suggested the existence of an alternative promoter located within the first intron of the *BRCA1* gene (Xu *et al*., 1997a). Recent studies by us (Suen and Goss, 1999) and others (Rice *et al*., 1998) have shown contradictory results and implied an insignificant role of such an alternative promoter. We now present strong evidence that the first intron of *BRCA1* functions in a negative regulatory manner in the control of *BRCA1* transcription.

A transcriptional blocking activity was first located to an 819 bp *Nru*I–*Xba*I fragment within the first intron of the *BRCA1* gene (Figure 1). This repressor activity appears not to be tissue-specific as it could be found in multiple cell lines representing various tissues of origin (Figure 2). Although strong repressor activity could be mapped to a smaller 464 bp *Nru*I–*BamH*I fragment, longer sequences might be necessary to achieve the full potential of transcription repression in the breast cancer cell line MDA-MB453 (Figure 2). Further experiments with multiple mammary epithelial cell lines are required to determine if the observed effect is cell line-, tissue-, or cancer-specific. Nevertheless, analysis of the activities of a series of 3′ deletion constructs confirmed the existence of the putative repressor element and localized it to an 83 bp *Ava*II–*BamH*I DNA fragment (Figure 3a). Since alternative splicing upstream of exon 2 of the *BRCA1* gene has been described (Xu *et al*., 1995), the repressor element may be considered as either 553 bp downstream from the first nucleotide of intron 1a, or 19 bp from intron 1b. To avoid confusion and allow easy

understanding by others, the *BRCA1* genomic sequence deposited in Genbank (accession no. U37574) was chosen as a reference numbering system. In this sense, the *Ava*II – *BamH*I fragment encompasses the sequence between 2254 and 2337 (Figure 3b).

Analysis of the *Nla*IV – *Afl*II and the *BstN*I – *Nla*IV fragments by EMSAs (Figure 4c, lanes 1 – 6) suggests that binding of nuclear proteins to the putative repressor region can be localized to the overlapping region, between the *BstN*I and *Afl*II sites. However, the strong difference in their abilities to form the same protein-DNA complexes (Figure 4c, compare lanes 2 and 3 to lanes 5 and 6) also indicates that possible interactions among the different fragments might play a role in the binding activity. It is possible that the *Nla*IV – *BstN*I fragment may contain an inhibitory activity against proteins binding to the *BstN*I – *Afl*II region, thus the observed weak binding activity of the *Nla*IV – *Afl*II fragment. On the other hand, the *Afl*II – *Nla*IV fragment may contribute positively to proteins binding to the *BstN*I – *Afl*II region, and thus the observed strong binding to the *BstN*I – *Nla*IV fragment. Although the two possibilities are not mutually exclusive, the fact that the *BstN*I – *Nla*IV was a stronger competitor than the complete *Nla*IV – *Nla*IV fragment in EMSAs (Figure 4d) favors the former mechanism. Furthermore, the presence of a *GA*-rich sequence within the *Nla*IV – *BstN*I, but not the *Afl*II – *Nla*IV fragment (Figures 3b and 4b) may also contribute to the apparent difference in the binding activity (also see below, discussion on *GA*-rich sequences).

EMSAs using various smaller fragments within this putative repressor element localized a 36 bp *BstN*I – *BseR*I (2278 – 2313) fragment which was capable of forming specific protein-DNA complexes effectively with nuclear extracts isolated from HeLa cells (Figures 4 and 5) and Caco2 cells (data not shown). The apparent progressive lowering in the ability of the *BstN*I – *Nla*IV and its two sub-fragments to compete against the formation of protein-DNA complexes with the *BstN*I – *Nla*IV fragment (Figure 5a) might be explainable from the analysis of the *Nla*IV – *Nla*IV sequence. *GA*-rich sequences are found throughout the *Nla*IV – *Nla*IV fragment (Figure 3b) and they can be recognized by numerous transcription factors of the *ets* (Sharrocks *et al*., 1997) and *Sp1* (Philipsen and Suske, 1999) families. The possible involvement of these proteins and their large range of affinities for *GA*-rich sequences (Sharrocks *et al*., 1997) may explain the observed cross-competitive effects and protein-binding ability of the individual DNA fragments (Figure 5). It is impossible to speculate which one of these transcription factors may be responsible for the binding activity that we observed. More detailed characterization of the binding sites may provide some clues as to the identity of the binding factors. Cloning of transcription factors interacting with the putative repressor element might be necessary to reveal their identity.

Nevertheless, the ability of the 36 bp *BstN*I – *BseR*I fragment to attenuate the transcriptional activity of the heterologous *TK* promoter confirmed the repressor function (Figure 6). The apparent positional effects of this 36 bp repressor element distinguish it from a classical silencer element. Indeed, analyses of multiple reporter gene constructs in their native genomic alignment suggest that the *BRCA1* intron repressor element does not affect transcription in the opposite direction (Figure 7). Functional analyses within the context of both the heterologous *TK* and the native *BRCA1* promoters suggest the repressor element is only functional when it is located downstream to the promoter. Most interestingly, intron 1 of the *NBR2* gene also appears to contain a repressor activity which blocks only transcription in the *NBR2* direction (compare pBR(*Pst*I – *Sst*I)CAT and pNB(*Sst*I – *Pst*I)-CAT in Figure 7b with constructs 1 – 6 in Figure 7a). Further studies are necessary to confirm and localize this repressor activity within intron 1 of *NBR2*.

BRCA1 expression is known to be induced during the S phase of the cell cycle (Gudas *et al*., 1996; Vaughn *et al*., 1996) and altered by DNA damaging agents (Andres *et al*., 1998; Husain *et al*., 1998). The expression pattern of *BRCA1* throughout development also suggests its importance in tissues that undergo rapid proliferation and terminal differentiation (Lane *et al*., 1995; Marquis *et al*., 1995; Blackshear *et al*., 1998; Magdinier *et al*., 1999). Removal of the transcriptional block at the position of intron 1 which we have identified in this study would be an effective way to increase BRCA1 levels in a timely manner for subsequent molecular events. Mutations in any important transcriptional regulatory element or its interacting transcription factor(s), regardless of whether it is a positive or a negative element, could result in an absence of *BRCA1* expression, which has been reported in non-inherited breast cancer (Wilson *et al*., 1999; Thompson *et al*., 1995; Magdinier *et al*., 1998; Sourvinos and Spandidos, 1998). Recently, elevated levels of Brn-3b have been shown to correlate with reduced *BRCA1* expression in mammary tumors (Budhram-Mahadeo *et al*., 1999). Our numerous *BRCA1* promoter-reporter constructs will be very useful in identifying the possible site of interaction of this or other transcription factors that may regulate *BRCA1* expression.

Materials and methods

Enzymes and reagents

Restriction enzymes and other DNA modifying enzymes such as T4 kinase, T4 polymerase, T4 ligase, Klenow fragment, and calf intestinal phosphatase were purchased from Life Technologies Inc., New England Biolabs (Mississauga, Ontario, Canada), Roche Molecular Biochemicals, or Amersham Pharmacia Biotech. Chemicals used for the chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Thin Layer Chromatography (TLC) plates were products of Eastman Kodak Co. Cell culture medium and reagents were obtained from Life Technologies Inc. All isotopes were products from Amersham Pharmacia Biotech.

Plasmids

The plasid pBluescripts(IIKS) (Stratagene, La Jollla, CA, USA) was used for general subcloning purposes. pMT.1C3 is a plasmid containing multiple cloning sites placed upstream of the CAT gene (Suen and Hung, 1990). Most of the *BRCA1* DNA restriction fragments were cloned into pBluescripts(IIKS), and were then shuffled into the matching unique restriction sites on the polylinker of pMT.IC3. DNA fragments were blunt ended with Klenow fragment or T4 polymerase when no appropriate restriction enzymes could be used for directional cloning. In addition, reversed orientation of a subcloned fragment in the pMT.IC3 plasmid could easily be obtained by cutting with *Hind*III, which flank the polylinker, followed by religation. The 2.7 kb *Pst*I – *Xba*I fragment containing intron 1 and upstream sequences of *BRCA1* gene has been described in our previous study (Suen and Goss, 1999). Various restriction fragments within this region were subcloned into both pBluescript(IIKS) and pMT.IC3. All the CAT constructs were named according to the direction of transcription followed in parenthesis by their flanking restriction sites in a 5′- to 3′-direction. Therefore the pBR- and pNB-series of CAT plasmids indicate a promoter transcribing towards the *BRCA1* and *NBR2* gene (Xu *et al*., 1997b), respectively. Putative repressor fragments from the *BRCA1* intron were also cloned into the pBLCAT2 (Luckow and Schutz, 1987) reporter plasmid where a possible effect on the heterologous thymidine kinase promoter could be analysed. pCMVβ (Clontech, Palo Alto, CA, USA), a plasmid which contains the *lacZ* gene driven by the cytomegalovirus enhancer (MacGregor and Caskey, 1989), was used for monitoring transfection efficiency. Detailed maps of all the plasmids used in this study will be distributed along with the reagents upon request.

Sequencing

Dideoxy-sequencing of double-stranded plasmids was performed with a T7 polymerase sequencing kit using either [α-^{35}S]dATP or [α-^{35}S]dCTP (Amersham Pharmacia Biotech). Most of the *BRCA1* promoter subclones, particularly those with DNA inserts of less than 300 bp in size, were confirmed by sequencing. Sequencing primers for either the pMT.IC3 or pBluescript(IIKS) series of plasmids has been described previously (Suen and Goss, 1999).

Cell culture

All cell lines that were used in this study are available from American Type Culture Collection (ATCC, Manassas, VA, USA). This includes HeLa, a human cervical carcinoma cell line; Caco2, a human colon carcinoma cell line; and MDA-MB453, a human mammary carcinoma cell line. All cell lines were cultured in Dulbecco's modified Eagle's/F12 medium (Life Technologies Inc.), supplemented with 10% fetal calf serum, and kept in a humidified, 37°, 5% CO_2 incubator.

Transfections and CAT assays

A calcium phosphate precipitation method (Chen and Okayama, 1987) was used for transfection as modified and described previously (Suen and Goss, 1999). Briefly, cells were split at a predetermined ratio into 100 mm tissue culture dishes (Falcon) the day before transfection. Unless otherwise indicated, 1 μg of pCMVβ and 10 μg of a CAT reporter DNA were added to 0.5 ml of 0.25 M $CaCl_2$. This was followed by dropwise addition of 0.5 ml of 2 × BBS buffer (50 mM BES, 280 mM NaCl and 1.5 mM Na_2HPO_4) and gentle mixing. After 25 min at room temperature, the mixture of DNA-precipitate was added to the cells. The cells were incubated at 37°C for 16 – 20 h, after which they were washed three times with phosphate-buffered saline, re-fed with fresh medium, and returned to the 37°C incubator. Cells were washed and harvested after 20 – 24 h and several freeze/thaw/vortex cycles were carried out to lyse the cells. One-fifth of the cell lysate was used for the β-galactosidase assay using ONPG (*O*-Nitrophenyl-β-D-Galactopyranoside) as substrate. The results were used to adjust the amount of lysate for CAT assay. The thin layer chromatography (TLC) method of CAT assays was performed as previously described, where the standard [^{14}C]chloramphenicol was replaced with 1-Deoxy[dichloro-acetyl-1-^{14}C]chloramphenicol (Amersham Pharmacia Biotech.) (Suen and Goss, 1999).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described (Suen and Goss, 2000). Nuclear extract was isolated from the different cell lines by means of homogenization under hypotonic conditions (Dignam *et al*., 1983). DNA fragments were isolated by digesting a plasmid subclone with appropriate restriction enzymes, gel purified, and labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP (depending on the restriction site) by Klenow-fragment. A final volume of 30 μl of reaction mixture was added in the order of H_2O, 10 × binding buffer (1 × 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol), 3 μg of poly(dI-dC).poly(dI-dC), 10 μg of nuclear extract, an appropriate amount of unlabeled competitor if desired, and finally, 20 000 c.p.m. of probe. The mixture was incubated at room temperature for 25 min, after which it was loaded onto a 6% native polyacrylamide gel. After electrophoresis, the gel was dried under vacuum in a gel dryer, and exposed to a Kodad XOMAT-AR film at −80°C.

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