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Breast cancer susceptibility gene; BRCA1 has been implicated in maintenance of genomic integrity. Deregulation of genomic stability is closely associated with tumorigenesis. However, the molecular mechanism by which BRCA1 participates in maintaining genomic fidelity remains unclear. Our current study has demonstrated that BRCA1 transcriptionally activates GADD45, a p53-regulated and DNA damage-inducible gene that may play an important role in cell cycle checkpoints, apoptosis and DNA repair. Activation of the GADD45 gene by BRCA1 is independent of tumor suppressor p53. Most recent findings demonstrate that upregulation of GADD45 mRNA by BRCA1 is mediated through activation of the GADD45 promoter. Using 5'-deletion analysis, the BRCA1-regulatory elements are mapped at the region of the GADD45 promoter from -107 to -57. Deletion of this region abrogates the BRCA1 activation of the GADD45 promoter. Inspection of DNA sequence shows that there are two OCT-1 and CAAT motifs located at this region. Mutations of OCT-1 and CAAT motifs also disrupt BRCA1 activation of the GADD45 promoter, indicating these two motifs are involved in the BRCA1 activation of the GADD45 promoter. The current study demonstrates a novel pathway (BRCA1-GADD45) involved in cellular response to DNA damage.

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1. INTRUDUCTION

More than half of the hereditary human breast cancer can be attributed to mutations in the breast susceptibility gene (1-3). BRCA1. BRCA1 has been implicated in cellular response to DNA damage, including cell cycle checkpoints, apoptosis and DNA repair (4-10). These biological events are thought o maintain genomic stability. Deregulation of genomic fidelity is closely associated with malignant transformation and tumorigenesis. However, the molecular mechanism by which BRCA1 plays a role in maintaining genomic integrity remains to be elucidated. The current project is to define the molecular pathway, which mediates BRCA1's role in the control of cell cycle G2-M checkpoint. The proposed studies will provide the understanding of how BRCA1 participates in maintenance of genomic stability and prevents the onset of breast cancer, as well as provide the insight to development of novel new anticancer drugs. Our previous and current work indicates that BRCA1 transcriptionally activates Gadd45, a p53-regulated and DNA damage-inducible gene that may play an important role in cell cycle G2-M checkpoints, apoptosis and DNA repair in response to DNA damage (11-17). We speculated that the role of BRCA1 in cell cycle G2-M checkpoint is mediated through Gadd45. Therefore, two major tasks have been proposed in this study. (1). To define the role Gadd45 in BRCA1-induced cell cycle G2-M arrest. (2). To determine the biochemical and molecular mechanism by which BRCA1 regulates Gadd45.

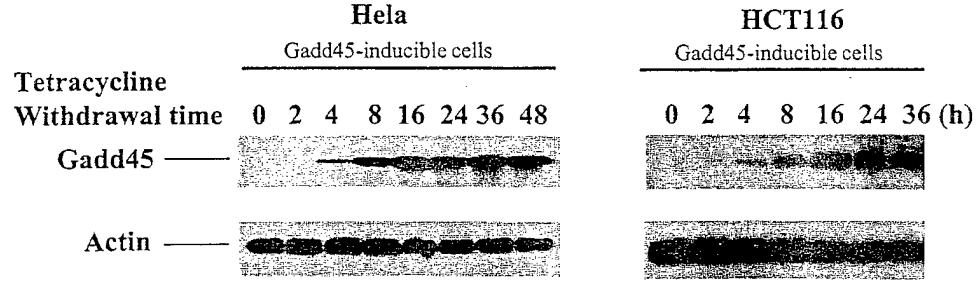
2. BODY

During the first year of effort on this grant, significant progress has been achieved. These will be summarized as the following individually.

In Task 1:

1. To study the role of Gadd45 in the BRCA1-induced cell cycle G2-M checkpoint, we have successfully developed two GADD45-inducible cell lines via tet-off system in Hela (p53 negative status) and HCT116 (with wt p53) cells. In these cell lines, expression of Gadd45 protein is precisely controlled through the presence of tetracycline. As shown in Figure 1A, Gadd45 protein is highly induced following withdraw of tetracycline and induction of Gadd45 protein significantly suppresses cell growth (Figure 1B). Recently, we have found that induction of Gadd45 protein in HCT116 cell line (wt p53) results in cells arrest in G2-M phase, but induction of Gadd45 protein in Hela cells (deleted p53) failed to generate G2-M accumulation, suggesting that the Gadd45-activated cell cycle G2-M arrest is dependent of normal cellular p53 function. Therefore, these results demonstrate that Gadd45, as a BRCA1-targeted effector, is capable of mediating BRCA1-induced cell cycle arrest.

A.



B. Hela GADD45-inducible cell HCT116 GADD45-inducible cell

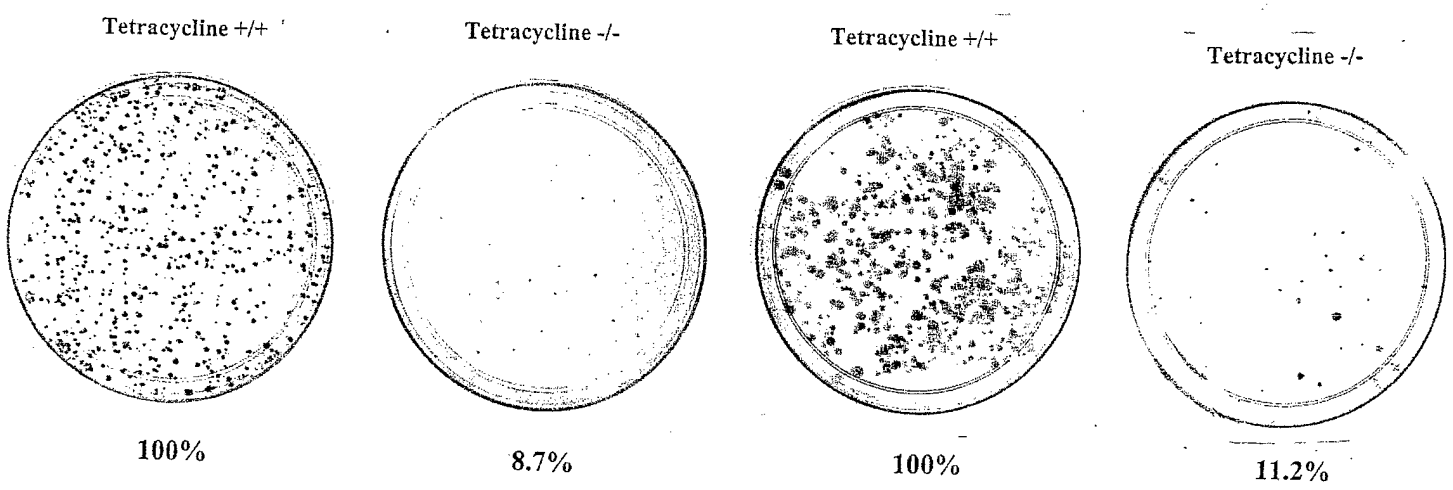


Fig. 1 (A). Inducible expression of Gadd45 protein in Hela and HCT116 controlled by the Tet-off system. Hela and HCT116 *GADD45*-inducible cells were placed in 100 mm dishes at a density of 4 X 10^5 per ml and grown containing tetracycline at a concentration of 4 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated times for preparation of cellular protein. 100 μg of whole cell protein was used for immunoblotting analysis with anti-*GADD45* antibody. As a loading control, anti-actin antibody was included. **(B). Induction of *GADD45* suppresses cell growth.** Hela and HCT116 *GADD45*-inducible cells were seeded at a density of 1000 cells per 100 mm dish and grown in the medium containing 4 μg/ml of tetracycline. 16 hours later, medium was removed and plates were washed three times with PBS, then fresh medium containing no tetracycline was added into plates. The cells were fixed and stained at 14 days and scored for colonies containing at least 50 cells.

2. The work of establishing the BRCA1-inducible cell lines via tet-off inducible system is actively being carried out at the current time. Following completion of the BRCA1-inducble cell line, analysis of cell cycle G2-M arrest will be conducted, including flow cytometric analysis, evaluation of mitotic index and cyclinB1/Cdc2 kinase activity.

3. In a typical cell growth suppression assay (colony formation assay), we have found co-expression of antisense Gadd45 mRNA is able to reduce the BRCA1-induced growth suppression. In addition, BRCA1-induced growth suppression is also seen reduced in the cells stably expressing antisense Gadd45 mRNA (Gadd45-deficient cell lines), indicating that Gadd45 plays a role in mediating growth inhibition by BRCA1.

In Task 2:

1. We have found expression of BRCA1 is able to upregulate Gadd45 mRNA when BRCA1 expression vector is introduced into human breast carcinoma MCF-7 cells (Figure 1D in Appendix 1).

2. We have demonstrated that BRCA1 can activate the Gadd45 promoter and BRCA1 activation of the Gadd45 promoter requires normal transcriptional activity of BRCA1

since the tumor-derived mutants of BRCA1, which lack normal transcriptional property, are unable to induce the Gadd45 promoter (Figure 1 and 1B in Appendix 1).

3. We have further demonstrated that BRCA1 activation of the Gadd45 promoter is a BRCA1-mediated specific effect and in a p53-independent manner (Figure 2 in Appendix).

4. Importantly, we have mapped the BRCA1-regulatory elements in the Gadd45 promoter. These BRCA1-responsive elements are localized at the region of the Gadd45 promoter between –107 to –57 (see Figure 3 in appendix 1 and Figure 1 in Appendix 2). Deletion of this region has been shown to abrogate BRCA1 activation of the Gadd45 promoter (Figure 3 in Appendix 3).

5. Based on the sequence analysis, we have found that there are two OCT-1 motifs and one CAAT box located at the region of the Gadd45 promoter from –107 to –57. Disruption of these motifs abolishes activation of the Gadd45 promoter by BRCA1.

3. KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments of this project over the past year include: (1). We have made first demonstration that BRCA1 activates the promoter of the Gadd45 gene that play an important role in cell cycle checkpoint, apoptosis and DNA repair. These results indicate that implication of BRCA1 in transcriptional regulation may contribute to its role as a tumor suppressor in maintaining genomic stability. (2). We have made first demonstration that BRCA1 play a role in gene regulation through OCT-1 and CAAT box. This finding will significantly broaden the role of BRCA1 in regulation of its targeted genes. (3). Through the established Gadd45 inducible cell lines, we demonstrated that Gadd45 is capable of mediating BRCA1' s role in the control of cell cycle checkpoint and growth suppression.

4. REPORTABLE OUTCOMES

The reportable outcomes related to this project include:

(1). One paper has been published in ONCOGENE, which is a prestigious journal in the cancer research field (Appendix 1).

(2). One manuscript has recently been submitted to Cancer Research (Appendix 2).

(3). One poster presentation was taken place in the Annual meeting of the American Association for Cancer Research, at New Orleans, 2001 (Appendix 3).

(4). We have established two Gadd45 tet-off inducible cell lines, which are useful resources and available to the scientists in this field.

(5). Under the support from DOD, one postdoctoral fellow is hired to obtain training in my laboratory.

5. CONCLUSION

Overall, this DOD-funded project has been going very well during the first year. The finding from this project has demonstrated a novel pathway (BRCA1-Gadd45) in cellular response to DNA damage, indicating that role of BRCA1 in maintenance of genomic stability may be mediated through Gadd45. Future work will be focused on defining the detailed biochemical and molecular mechanism by which the pathway plays important role in preventing breast cancer. Completion of this project will provide insight into development of new anti-breast cancer drugs.

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Appendices

1. Reprint published in Oncogene
2. Manuscript submitted to Cancer Research

Oncogene (2000) 19, 4050–4057

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BRCA1 activation of the *GADD45* promoter

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Breast cancer susceptibility gene BRCA1 has been implicated in the control of gene regulation and such regulated genes are thought to mediate the biological role of BRCA1. Overexpression of BRCA1 induces *GADD45*, a p53-regulated and stress-inducible gene. However, the molecular mechanism by which BRCA1 induces the expression *GADD45* remains unclear. In this report, we have shown that the *GADD45* promoter is strongly activated following expression of wild-type BRCA1. In contrast, both the tumor-derived BRCA1 mutants (p1749R and Y1853insA) and truncated BRCA1 mutant protein (Δ500–1863 BRCA1), which lack transactivation activity, were unable to activate the *GADD45* promoter, indicating that the BRCA1-mediated activation of the *GADD45* promoter requires normal transcriptional properties of BRCA1. BRCA1 did not induce the c-Jun and c-fos promoters, which rules out a general effect of BRCA1 on other immediate-responsive genes. Expression of the human papillomavirus E6 and the dominant-negative mutant p53 proteins had no effect on the induction of the *GADD45* promoter by BRCA1, suggesting that activation of the *GADD45* promoter by BRCA1 is independent of cellular p53 function. With the 5′-deletion analysis, the BRCA1-responsive element of the *GADD45* promoter was mapped at the region from −121 to −75. Disruption of this region resulted in the abrogation of BRCA1 activation of the *GADD45* promoter. Taken together, these results demonstrate that the mechanism by which BRCA1 induces *GADD45* is mainly through the transactivation of the *GADD45* promoter, further demonstrating the evidence that *GADD45* acts as one of the BRCA1-regulated genes. *Oncogene* (2000) **19,** 4050–4057.

Keywords: *GADD45*; BRCA1; p53; gene regulation

Introduction

Breast cancer is the most frequent malignancy in women. More than half of the hereditary breast cancer can be attributed to mutations in the breast cancer susceptibility gene BRCA1, (Easton *et al.*, 1995; Ford and Easton, 1995; Miki *et al.*, 1994). Human BRCA1 encodes an 1863 amino acid nuclear phosphoprotein (Miki *et al.*, 1994) that is expressed in a variety of human tissues (Marquis *et al.*, 1995). BRCA1 levels are cell cycle-dependent, with peak levels at the G1/S border and low levels in late S or G2 (Rajan *et al.*, 1996; Vaughn *et al.*, 1996). Normally, BRCA1 associates and colocalizes with Rad51, the human homologue of the *Escherichia coli* RecA protein, and may be involved in the process of DNA recombination. Following exposure to DNA damage, BRCA1 becomes hyperphosphorylated and then dynamically redistributes to the complexes containing proliferating cell nuclear antigen (PCNA), suggesting that BRCA1 may participate in a replication checkpoint response (Scully *et al.*, 1997). It has also been found that BRCA1 plays an important role in the transcription-coupled repair of DNA damage (Gowen *et al.*, 1998). Several lines of evidence demonstrate that BRCA1 plays an important role in the control of cell cycle progression. Introduction of the COOH terminus of BRCA1 (residues 1293–1863), which behaves in a dominant-negative manner, into nontumorigenic human breast epithelial cells can cause a reduction in the doubling time and abrogation of the G2-M block induced by the spindle inhibitor colchicine (Larson *et al.*, 1997). Additionally, Murine embryos carrying a BRCA1 null mutation exhibit hypersensitivity to DNA damage and chromosomal abnormalities (Shen *et al.*, 1998). Disruption of endogenous BRCA1 protein through introducing antisense oligonucleotides to BRCA1 mRNA accelerated the growth of normal and malignant mammary cells (Thompson *et al.*, 1995). Similarly, NIH3T3 cells with a stable expression of antisense BRCA1 mRNA displayed an accelerated growth rate and tumorigenesis in nude mice (Rao *et al.*, 1996). In addition, introduction of wild-type BRCA1 into cells resulted in the growth suppression in both breast and ovarian tumor lines (Holt *et al.*, 1996).

A number of observations have implicated BRCA1 in the control of transcriptional regulation. BRCA1 has an N-terminal ring finger domain and a C-terminal transcription activation domain that activates transcription when linked to a DNA-binding domain (Chapman and Verma, 1996). It has been shown that BRCA1 may interact with RNA helicase (Anderson *et al.*, 1998), and the transcriptional regulators p53 (Ouchi *et al.*, 1998; Zhang *et al.*, 1998) and c-Myc (Wang *et al.*, 1998). Transient transfection of BRCA1 expression vector results in transcriptional activation of the p21 promoter via both p53-dependent and -independent pathways (Somasundaram *et al.*, 1997). Recent finding by Harkin *et al.* has demonstrated that overexpression of BRCA1 induces endogenous *GADD45* mRNA, leads to the JNK/SAP-dependent apoptosis (Harkin *et al.*, 1999). This evidence suggests that *GADD45* should be one of BRCA1-targeted genes

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and might play a role in mediating BRCA1's biological functions. However, the mechanism by which BRCA1 induces *GADD45* mRNA remains unclear.

GADD45 is a genotoxic stress-responsive gene and is induced by a variety of DNA damaging agents, such as UV radiation, methyl methanesulfonate (MMS) and ionizing radiation (IR) (Fornace *et al.*, 1988; Papathanasiou *et al.*, 1991). In response to IR, *GADD45* induction is regulated by the tumor suppressor p53 probably through the p53-binding motif located at the third intron of the *GADD45* gene (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). Expression of Gadd45 protein suppresses cell growth in multiple tumor lines (Zhan *et al.*, 1994b). Gadd45 protein has been found to interact with PCNA (Smith *et al.*, 1994), p21 (Kearsey *et al.*, 1995), Cdc2 (Zhan *et al.*, 1999), core histone (Carrier *et al.*, 1999) and MTK/MEKK4 (Takekawa and Saito, 1998), indicating that *GADD45* may be involved in multiple important cellular events. Most recently, *GADD45* has been shown to play a role in the control of cell cycle G2-M checkpoint following certain DNA damaging treatments (Wang *et al.*, 1999).

In the present study, we investigated the mechanism of how BRCA1 induces expression of *GADD45* mRNA. When the *GADD45* promoter was linked to a chloramphenical acetyltransferase (CAT) reporter gene and cotransfected with the BRCA1 expression vector into several human cell lines, we have found that *GADD45* promoter, as measured by CAT activity, was strongly activated following BRCA1 expression. In contrast, BRCA1 does not activate c-*Jun* and c-*fos* promoters, which rule out a general effect of BRCA1 on all the immediate-responsive genes. Importantly, the induction of the *GADD45* promoter by BRCA1 was shown to require the transcriptional properties of BRCA1 since either mutations or deletion of the transcriptional domain in BRCA1 protein abrogated the BRCA1 activation of the *GADD45* promoter. The induction of *GADD45* promoter by BRCA1 was not affected by the HPVE6 and the dominant-negative mutant p53, suggesting the BRCA1-mediated activation of the *GADD45* promoter is p53-independent. Furthermore, using the deletion analysis, the BRCA1 responsive-elements of the *GADD45* promoter were mapped at the promoter region from −121 to −75.

Results

BRCA1 strongly activates GADD45 *promoter*

To explore the mechanism(s) of how BRCA1 transcriptionally up-regulates *GADD45* expression, we tested the effect of BRCA1 on activation of the *GADD45* promoter. In the initial experiments, two *GADD45* promoter reporter constructs (pHG45-CAT1 and pHG45-CAT2) were co-transfected with pCR3-BRCA1 (BRCA1) expression vector into human breast carcinoma MCF-7 or human colorectal carcinoma HCT116 cells and followed by the employment of CAT assay. As a control, pCMV-neo vector was included. As shown in Figure 1a, two *GADD45* promoter CAT reporter constructs (pHG45-CAT1 that contains the *GADD45* promoter region from −2256 to +144 and pHG45-CAT2 that contains the *GADD45* promoter from −909 to +144) were strongly activated following expression of BRCA1 in both cell lines. In contrast, pCMV-neo did not exhibit activating effect on the *GADD45* promoter. To determine transfection efficiency, 4 μg of GFP (green fluorescence protein) expression vector was co-transfected with each tested plasmid. The expression of GFP protein indicated that transfection efficiency is similar among different samples and the variations were seen less than 20%. To confirm the activation of the *GADD45* promoter by BRCA1 is specifically due to the BRCA1 transactivation activity, several constructs expressing the tumor-derived BRCA1 mutants (P1749R and Y1853insA) and truncated mutant (Δ500−1863 BRCA1) were employed. Those mutants are transactivation-deficient. As a positive control, the pWW-CAT, a p21 promoter CAT reporter construct, was included in the experiments. In Figure 1b, mutants of BRCA1 lacking normal transactivation domain were deficient in activating both the *GADD45* and p21 promoters, indicating that the intact transcriptional capability of BRCA1 is required for the induction of the *GADD45* promoter.

It should be noted here that there is a discrepancy between these results and the report by Harkin *et al.* (1999). In Harkin's report, there was no activation of the *GADD45* proximal promoter (from −994 to +1) by expression of BRCA1. Therefore, we constructed two GADD45 promoter CAT reporters spanning −994 to +1 and −909 to +1, and tested them in four different cell lines including MCF-7, H1299, HCT116 and Hela. Similarly, the proximal promoter CAT reporters were strongly activated by BRCA1 and results were shown in Figure 1c. In agreement with Harkin's observation that intron 3 of *GADD45* was BRCA1-responsive, we found REX5, which contains five copies of a p53 consensus that is identical to *GADD45* intron 3, was activated following BRCA1 expression. In our experiments, the *GADD45* promoter seems to be more potent in mediating BRCA1-dependent transcriptional activation compared to the intron 3 element of GADD45 (Figure 1b).

We next examined whether introduction of BRCA1 expression vector into human cells induces the expression of endogenous *GADD45* mRNA. To do that, BRCA1 and pCI Tac expression vectors were co-introduced into MCF-7. Thirty-six hours later, Tac-positive cells were isolated for RNA analysis. As shown in Figure 1d, using RNase protection assay, the levels of endogenous *GADD45* mRNA were found to evidently elevate in MCF-7 cells. Taken together, these results indicate that induction of *GADD45* mRNA by BRCA1 is mediated through the transcriptional activation of *GADD45* promoter following BRCA1 expression and activation of the *GADD45* promoter depends on normal transcriptional function of BRCA1.

BRCA1 activates GADD45 *promoter in a p53-independent manner*

In cellular response to DNA damaging agents, *GADD45* induction by ionizing radiation (IR) strictly depends on normal cellular p53 function (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). However, following exposure to certain non-IR agents such as UV irradiation and MMS, p53 is not required for the *GADD45* induction

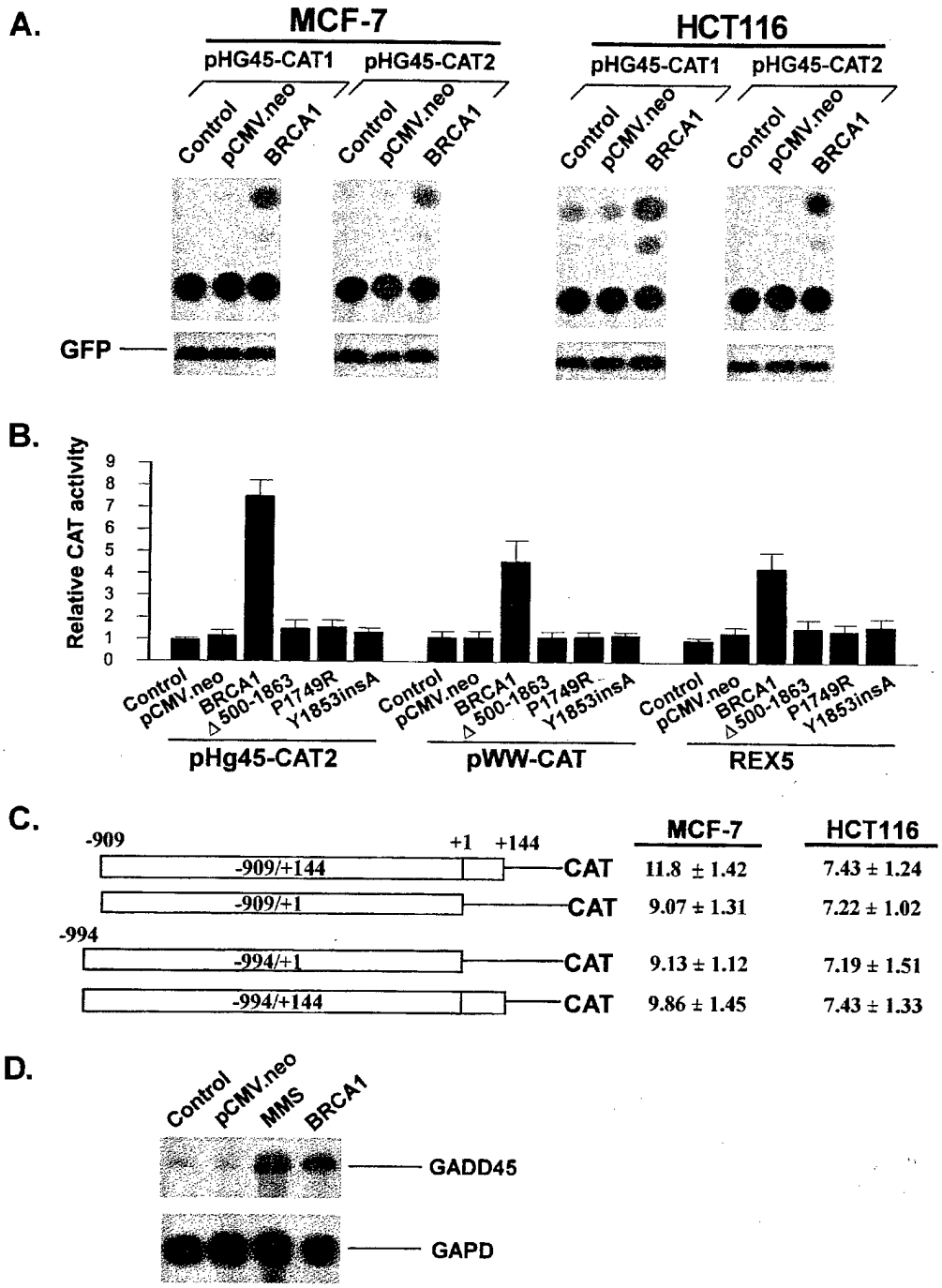

Figure 1 Activation of the *GADD45* promoter following expression of BRCA1. (**a**) 4 μg of the *GADD45* promoter CAT reporter constructs (pHG45-CAT1 and pHG45-CAT2) were cotransfected with 4 μg of either pCR3.BRCA1 (BRCA1) and pCMV·neo expression vectors into MCF-7 and HCT116 cells via the calcium phosphate method. Forty-eight hours later, cells were collected for the CAT assay (see Materials and methods). To determine transfection efficiency, 4 μg of GFP (green fluorescence protein) expression vector was co-transfected with each tested plasmid and the expression of GFP protein was detected as the internal control of transfection. (**b**) pHG45-CAT2, pWW-CAT (p21 promoter reporter construct) and REX5 (a reporter construct that contains five copies of a p53 consensus that is identical to *GADD45* intron 3) were transfected with the BRCA1 mutants of lacking transactivation activity into HCT116 cells. The CAT assays were performed as in (**a**). (**c**) The activating effect of BRCA1 on the *GADD45* proximal promoter. pHG45-CAT23 (+1 to −909 bp upstream of transcriptional start site) and pHG45-CAT30 (containing the region from +1 to 994) were co-transfected with the BRCA1 expression vector into MCF-7 and HCT116 cells. The CAT assays were carried out as in (**a**). (**d**) MCF-7 cells were transfected with 1 μg of pcI Tac and either 10 μg of pCMV·neo (lane 2) or pCR3·BRCA1 (lane 4). The Tac-positive cells were isolated. 10 μg of whole-cell RNA were hybridized with the human *GADD45* and *GAPD* RBI-probes by RNase protection assay (see Materials and methods). As a positive control, an RNA sample from MCF-7 cells treated with MMS at 100 μg/ml for 4 h was included (lane 3)

but p53 can greatly contribute to those cellular responses (Zhan *et al*., 1996). To test whether activation of the *GADD45* promoter by BRCA1 involves normal p53 function, several human lines with known p53 status were utilized. pHG45-CAT2 plasmid was transfected into MCF-7 that contains wt p53 and its isogenic line, MCF7-E6 where HPV16-E6 is stably integrated and much of the p53's biological function has been abrogated. As shown in Figure 2a, BRCA1 was shown to strongly activate pHG45-CAT2

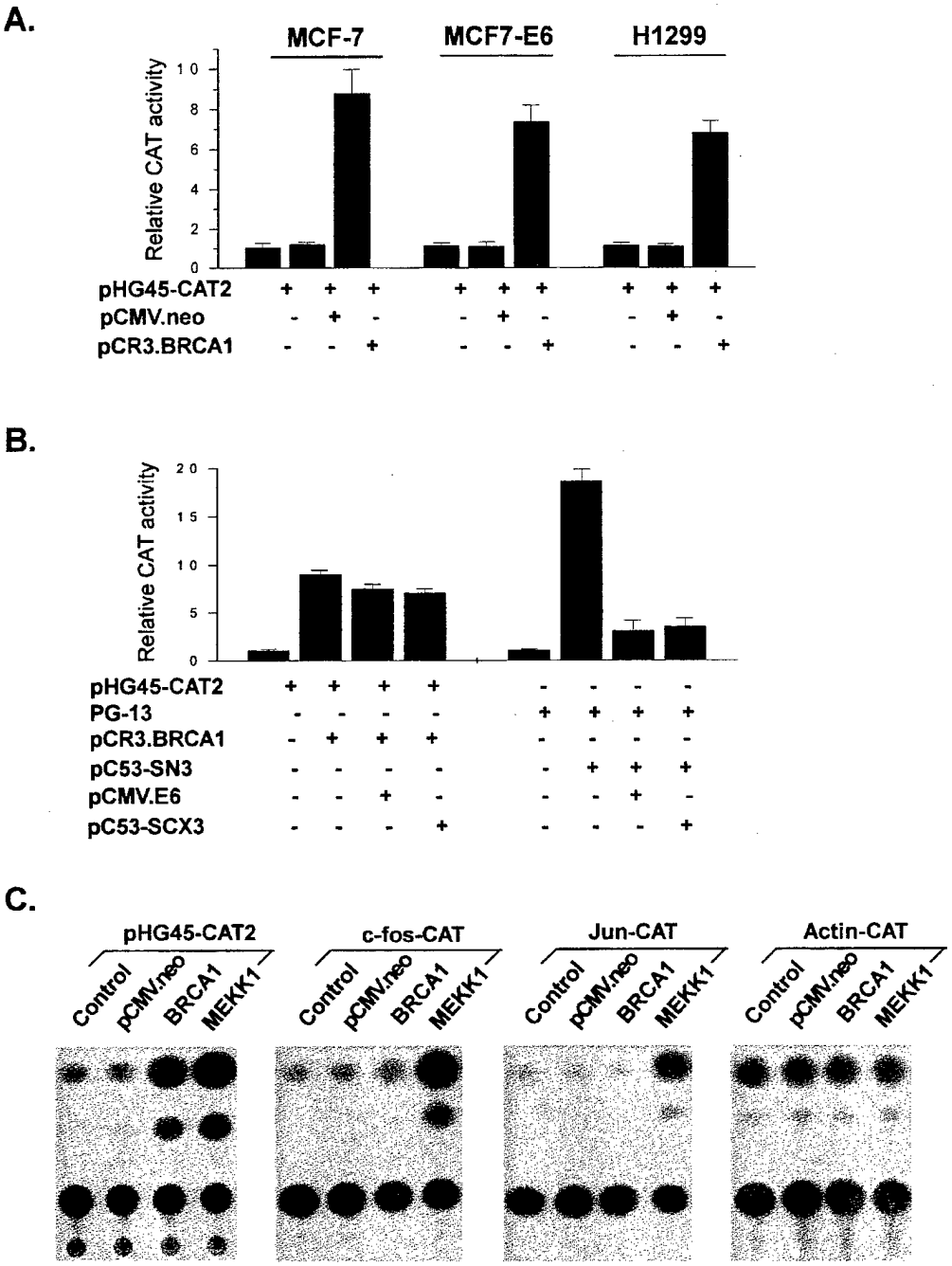

Figure 2 Induction of the *GADD45* promoter by BRCA1 is p53-independent. (**a**) pHG45-CAT2 was cotransfected with pCR3·BRCA1 into MCF-7, MCF7-E6 (with stable integration of HPVE6 gene) and H1299 cells, which has deletions of the p53 gene. The CAT assay was performed as in Figure 1. (**b**) pHG45-CTA2 or PG-13 (p53 reporter construct) were cotransfected with the indicated plasmids into MCF-7 cells and the CAT assays were followed 40 h post-transfection. (**c**) The c-*Jun*, c-*fos*, actin and MEKK1 (an activator of JNK and p38 kinase) promoter CAT reporter constructs were cotransfected with the pCR3.BRCA1 expression vector into MCF-7 cells as in (**a**). The CAT activities were measured as described in Materials and methods

in both MCF-7 and MCF7-E6 lines. Expression of E6 protein did not attenuate the induced-CAT activity of pHG45-CAT2 following BRCA1 expression. In support of these findings, pHG45-CAT2 was also strongly responsive to BRCA1 expression in human H1299 cells, which have a deletion of endogenous p53. Similarly, when the E6 or the dominant-negative mutant p53 expression vectors were transiently co-transfected into MCF-7 cells with pHG45-CAT2, induction by BRCA1 remained unchanged (see Figure 2b). In contrast, activation of PG-13, a p53 reporter construct, by p53 was abrogated following expression of E6 and mutant p53 proteins. Therefore, the results indicate that activation of *GADD45* promoter by BRCA1 does not require normal cellular p53 function.

To further determine the transactivation of the *GADD45* promoter by BRCA1 is a specific effect rather than a general effect on the stress-responsive genes, c-*Jun* and c-*fos* promoter CAT reporter constructs were cotransfected with BRCA1 expression vector and the CAT assay was followed to measure CAT activity. In Figure 2c, overexpression of BRCA1 protein did not activate the c-*Jun* and c-*fos* promoter although these two genes are induced in response to a

variety of genotoxic stress agents that also activate *GADD45* expression. In contrast, the c-*fos*-CAT and *Jun*-CAT reporter constructs were activated by protein expression of MEKK1, an activator of JNK and p38 kinase. Again, BRCA1 had no effect on the actin promoter. Therefore, BRCA1 expression is not generally associated with the activation of other stress-induced gene promoters, such as c-*Jun* and c-*fos*.

Mapping of BRCA1-responsive region in the GADD45 *promoter*

In order to localize the control elements involved in activation of the *GADD45* promoter by BRCA1, analysis of the human *GADD45* promoters with different 5′-deletions was undertaken. As shown in Figure 3a, the longest promoter, pHG45-CAT1, was strongly responsive to expression of BRCA1. With progressive 5′ deletions, induction of the *GADD45* promoter did not change substantially until the last construct, pHG45-CAT3, which extended 5′ to −74 relative to the transcription start site. With this minimal promoter, expression of BRCA1 had little effect. Since the pHG45-CAT11, which contains the promoter region spanning from −121 to +144, still showed strong induction by BRCA1, the region between −121 and −75 may contain the major controlling elements required for responsiveness to the expression of BRCA1. Interestingly, all *GADD45* promoter deletion constructs presented similar patterns of induction by BRCA1 in both MCF7 and H1299 cell lines. To further determine if the region between −121 and −75 was required for induction of the *GADD45* promoter by BRCA1, similar experiments were carried out with a reporter construct containing a deletion from −121 to −75. When this region was deleted in pHG45-CAT2ma, BRCA1-induction of the *GADD45* promoter was markedly reduced and more than 70% of the effect was lost compared to that of pHG45-CAT2 (Figure 3b).

We next used a labeled double-strand oligonucleotide corresponding to the sequence between −121 and −71 of the *GADD45* promoter for EMSAs. Although one specific prominent DNA-protein complex was observed, we were unable to demonstrate BRCA1 protein directly binding to the promoter using a supershift assay (results not shown). These results suggest that BRCA1 regulation of the GADD45 promoter might not involve a direct binding of BRCA1 protein to the promoter.

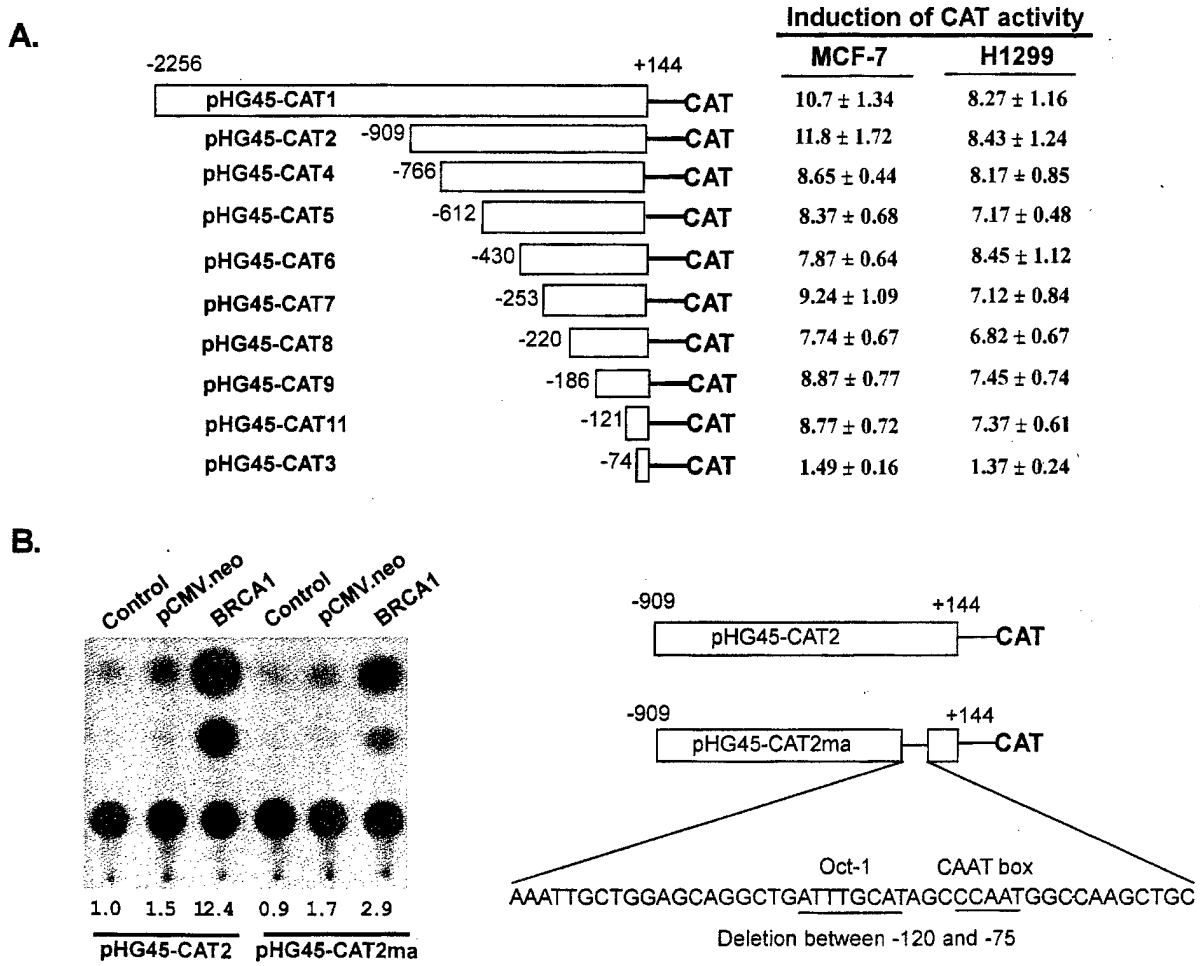

Figure 3 Mapping of the promoter region that contains the BRCA1-responsive elements. (**a**) Localization of the BRCA1-responsive element in the *GADD45* promoter. 4 μg of the CAT reporter constructs containing the indicated regions of the *GADD45* promoter were cotransfected with pCR·BRCA1 into MCF-7 and H1299 cell lines. The CAT assay was carried out as in Figure 1a. (**b**) pHG45-CAT2ma, in which the promoter sequence from −121 to −75 was deleted, was cotransfected with the BRCA1 expression vector and the CAT activities were measured as in Figure 1a. The assays were at least conducted three times but only the representative experiment is shown here

Discussion

In this report, we have analysed the effect of BRCA1 on activation of the human *GADD45* promoter in several human cell lines. We found that expression of wild type BRCA1 strongly activates the human *GADD45* promoter, while the BRCA1 mutants of lacking transcriptional activity failed to induce the *GADD45* promoter. Activation of *GADD45* by BRCA1 is in a p53-independent manner and this activation is not due to the BRCA1-induced general effect on the stress-inducible genes since BRCA1 does not activate c-*jun* and c-*fos* promoters. Using the 5′-deletion analyses, the region of the *GADD45* promoter between −121 to −75 was found to associate with the BRCA1-regulatory elements. Deletion of this region substantially abrogated the BRCA1-activation of the *GADD45* promoter. Therefore, our findings have demonstrated that the mechanism by which BRCA1 induces *GADD45* mRNA is through the transactivation of the *GADD45* promoter. Theses evidence further support the observation that *GADD45* may act as one of the BRCA1-downstream genes.

Inherited mutations in the BRCA1 gene are associated with high risk of developing breast cancer (Easton *et al*., 1995; Ford and Easton, 1995; Miki *et al*., 1994). A number of studies have linked BRCA1 to tumor growth suppression (Rao *et al*., 1996), apoptosis, DNA repair, transcriptional regulation and the control of cell cycle progression (Anderson *et al*., 1998; Chapman and Verma, 1996; Gowen *et al*., 1998; Scully *et al*., 1997; Somasundaram *et al*., 1997). Inactivation of BRCA1 has been found to correlate with genomic instability (Shen *et al*., 1998). Much effort has been put on exploring the mechanism(s) of how BRCA1 participates in these important biological events. Recent report by Harkin *et al*. (1999) demonstrated that expression of BRCA1 induces *GADD45* mRNA. This indicated that the biological roles for BRCA1 as a tumor suppressor might be mediated at least in part through its downstream genes, such as p21 and *GADD45*. It has been shown that *GADD45* can be induced by a variety of DNA damaging agents and is regulated by the tumor suppressor p53. A number of studies have suggested that *GADD45* be involved in the processes of apoptosis, DNA repair and signaling pathway (Smith *et al*., 1994, 1996; Takekawa & Saito, 1998). Recently, Gadd45 protein has implicated in the control of cell cycle G2-M checkpoint via the inhibition of Cdc2/cyclin B1 kinase activity (Wang *et al*., 1999; Zhan *et al*., 1999). Therefore, it is postulated that *GADD45* might be also able to mediate the role of BRCA1 in the control of cell cycle progression, whose disruption leads to genomic instability and tumorigenesis.

In the initial promoter-mapping work, the promoters employed in our CAT reporter constructs extend 3′ to +144 relative to the transcription start site. However, in Harkin's report (Harkin *et al*., 1999), the authors did not observe activation of the *GADD45* promoter by BRCA1 when using the promoter region from −994 to +1. For this reason, we further constructed two more promoter reporters, which contain the regions from −994 to +1 and −994 to +144. Both reporters showed strong activation following the expression of BRCA1 in four different human cell lines. This could be due to the difference in cell types tested because we also did not observe significant activation of the GADD45 promoter by BRCA1 in U2OS cells, which were used in Harkin's report (results not shown). Therefore, it can be assumed that there might be certain inhibitory factors existing in U2OS cells or lack of some mediating-factors, which are required for BRCA1 transactivation, in those cells. We have also identified that the 5′ region from −121 to −75 is required for the BRCA1-mediated activation of the *GADD45* promoter. Deletion of this region remarkably reduced the induction of *GADD45* by BRCA1. Interestingly, this region has been recently found to contain the important DNA-damage responsive elements (paper in preparation). Therefore, the results indicate that the region between −121 and −75 plays a crucial role in the regulation of the *GADD45* gene in response to either DNA damage or growth arrest signals. Analysis of DNA sequence shows that there are two known transcriptional binding sites (Oct-1 and CAAT box) in this region. To our knowledge there is currently no report to demonstrate that BRCA1 can physically associate with the proteins binding to these two sites. The future study will be focused on the dissection of this region in order to characterize the specific responsive elements regulated by BRCA1.

The precise molecular and biochemical mechanisms of how BRCA1 activates the *GADD45* promoter require further investigation. At the current time, BRCA1 has not been well characterized as a DNA-binding transcription factor. In our EMSA experiments, we were unable to demonstrate BRCA1 directly binding to the *GADD45*, but we were able to detect Oct-1 protein binding to this region in a supershift assay (results not shown). Therefore, it can be speculated that BRCA1 might activate the *GADD45* promoter probably through its interaction with another protein(s) that directly binds to the *GADD45* promoter. The interactions between the BRCA1 and the third protein(s) might also involve more complex signal transduction cascade. The similar observation has been obtained in our previous report. In it, p53 has been found to participate in the transcriptional induction of the *GADD45* promoter through its interaction with WT1 protein that is a transcription factor and directly binds to *GADD45* promoter (Zhan *et al*., 1998). In addition, the post-translational modification, such as phophorylation and acetylation, of BRCA1 and its interactions with transcriptional co-activators might also be involved in the regulation of *GADD45* promoter. Taken all together, this study has further confirmed the BRCA1-*GADD45* pathway and provided the insight into the understanding of how BRCA1 regulates it downstream genes as well.

Materials and methods

Plasmid clones

The following plasmid constructs were used: pHG45-CAT1, constructed by inserting the *Sal*I-*Sma*I fragment of *GADD45* promoter spanning −2256 to +144 relative to the transcription start site into pCAT-Basic (promega); pHG45-CAT2, similarly constructed by inserting the *Hind*III-*Sma*I fragment

of *GADD45* promoter from −909 to +144 into pCAT-Basic (Zhan *et al.*, 1998). pHG-CAT23 was constructed by inserting the fragment of the *GADD45* promoter from 994 to +1 into pCAT-Basic. Dr Weber provided pCR3-BRCA1, a construct expressing wt human BRCA1 protein, and BRCA1 mutants including pCR3-BRCA1$_{1749R}$, pCR3-BRCA1$_{1853insA}$ and pCR3-BRCA1$_{\Delta 500-1863}$ (Somasundaram *et al.*, 1997; Thakur *et al.*, 1997). Dr Vogelstein provided pC53-SCX3, which expresses a dominant-negative mutant p53 protein containing a substitution of Ala for Val-143 and PG-13, which contains 13 repeats of a p53-binding site inserted 5′ to the polyomavirus promoter linked to a CAT report construct (Kern *et al.*, 1992). pCMV-E6 expresses human HPVE6 protein and has been shown to block p53 function (Kessis *et al.*, 1993). c-fos-CAT was described as previously (Zhan *et al.*, 1998). Dr Ashwell provided Tac the Tac expression vector pCI.

Cell lines, tissue culture and transfection

The human breast carcinoma MCF-7 line and its subline MCF7-E6, the human lung carcinoma line H1299 and the human colorectal carcinoma line HCT116 were grown in F12 medium supplemented with 10% fetal bovine serum. The cells were transfected by the calcium phosphate (Zhan *et al.*, 1993). In these experiments, 4 μg of the *GADD45* promoter reporter constructs and 4 μg of indicated expression vectors were cotransfected into human cells. Forty hours later, cells were collected for the CAT assay. In addition, 4 μg of pCMV-GFP plasmid (which contains green fluorescence protein) was included in each experiment. After transfection, expression of GFP protein is detected by Western blotting assay to determine transfection efficiency.

For RNase protection analysis, 10 μg of either pCR3-BRCA1 or pCMV.neo were co-transfected into MCF-7 with 1.0 μg of pCI Tac, a cell surface protein expression vector, by the lipofectamine method. Forty-eight hours after transfection, cells were harvested and washed once with cold medium. Dynabeads (Dyna, Lake Success, NY, USA) coated with anti-Tac antibody were added to the cells and incubated with gentle rotation at 4°C for 2 h. The Tac-positive cells were isolated magnetically, washed once with buffer saline, and then lysed for the RNA protection assay.

CAT assay

Measurement of CAT activity was carried out as described previously (Zhan *et al.*, 1993). Cells were collected and resuspended in 0.25 M TRIS (pH 7.8). Cells were disrupted by three cycles of freeze-thaw. The equal amounts of protein were used for each CAT assay. The CAT reaction mixture was incubated at 37°C overnight and the CAT activity was determined by measuring the acetylation of ^{14}C-labeled chloramphenicol by the thin-layer chromatography. Radioactivity was measured directed with Betascope analyser. The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated. The relative CAT activity was determined by normalizing the activity of the treated samples to that of the untreated sample. Each value represented the average of at least three separate determinations.

RNA isolation and RNAase protection assay

Cells were lysed in 4 M guanidine thiocynate and total cellular RNA was isolated by the acid phenol method. For RNAase protection assay, the plasmids pRibo-Hg45 and pGAPD were linearized with *Hind*III or *Bam*H1 and *in vitro* transcription was carried out at 4°C with T3 or T7 RNA polymerases. *GADD45* and *GAPD* riboprobes were labeled with [α-^{32}P]UTP. Ten μg of whole cellular RNA was hybridized with both riboprobes simultaneously in the same test tube at 53°C for 15 h and then digested with RNAase. Following protease K digestion and pheno-chloroform extraction, the samples were analysed on an 8 M urea, 5% acrylamide gel. Protected bands were visualized by autoradiography and were quantitated with a Phosphoimager analyser.

EMSA

Nuclear extracts were prepared and an electrophoretic mobility shift assay (EMSA) was carried out as described previously (Zhan *et al.*, 1998). DNA binding reactions were performed for 10 min at room temperature in a binding buffer containing 20 mM HEPES (pH 7.8), 150 mM NaCl, 1 mM dithiothreitol, 1 μg of poly(dIdC), 10% glycerol and 20 μg of nuclear protein. 4×10^4 d.p.m. of labeled probe. The probe was a 50-mer double stranded synthetic oligonucleotide containing the region spanning −121 to −71 of the *GADD45* promoter. Each strand was labeled separately and the strands were annealed, then purified by G-25 column. The samples were analysed on a 4% non-denaturing acrylamide gel (Zhan *et al.*, 1998).

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BRCA1 regulates GADD45 through its interaction with the OCT-1 and CAAT motifs

Keywords: BRCA1, *GADD45*, OCT-1 motif, CAAT box and gene regulation

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ABSTRACT

The implication of BRCA1 in gene regulation can greatly account for its role as a tumor suppressor. The BRCA1-targeted genes are though to mediate BRCA1' s biological function. Previous studies by our and other demonstrate that BRCA1 induces GADD45, a p53-regulated and stress-inducible gene that plays an important role in cellular response to DNA damage. However, the molecular mechanism(s) by which BRCA1 regulates GADD45 remains to be elucidated. In this report, we have shown that BRCA1 strongly activates the GADD45 promoter in a p53-independent manner and the BRCA1 activation of the GADD45 promoter is mediated through the OCT-1 and CAAT motifs located at the GADD45 promoter region between –107 and –57. Site-directed mutations of both OCT-1 and CAAT motifs abrogate induction of the GADD45 by BRCA1. When several repeats of OCT-1 or CAAT are cloned upstream of a minimal polyomavirus promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene, both OCT-1 and CAAT motifs confer BRCA1 inducibility on the non-related minimal promoter. Physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, are established by biotin-streptavidin pull-down and coimmunoprecipitation assays. Such protein interactions are required for interaction of BRCA1 with the GADD45 promoter since either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT1and CAAT motifs disrupt BRCA1 binding to the GADD45 promoter. These findings indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.

INTRODUCTION

Mutations of the breast cancer susceptibility gene, BRCA1, are associated with more than half of the hereditary breast cancer (1-3). A number of observations have implicated BRCA1 in cellular response to DNA damage. BRCA1 associates with and colocalizes with Rad51 protein and may be involved in DNA recombination. Following DNA damage, BRCA1 become hyperphosphorylated by ATM (4) and hCds1/Chk2 (5) and dynamically redistributes to complex containing proliferating cell nuclear antigene (PCNA) (6). Additionally, BRCA1 was found to play an important role in the transcription-coupled repair (7) and in the control of cell cycle arrest following DNA damage (8, 9). Recently, multiple reports have suggested that BRCA1 might also play a role in apoptosis (10-12). Therefore, through its functions in DNA repair process, apoptosis and cell cycle arrest, BRCA1 plays an important role in the maintenance of genomic integrity. This is strongly supported by the demonstration that murine embryos carrying a BRCA1 null mutation exhibit hypersensitivity to DNA damage and chromosomal abnormalities, probably due to defective G2/M checkpoint control and improper centrosome duplication (13).

GADD45 is a DNA damage-responsive gene and induced by a wide spectrum of genotoxic stress agents, including ionizing (IR), UV radiation (UV), methyl methanesulfonate (MMS) and medium starvation (14-16). It has been shown that induction of GADD45 is mediated via both p53-dependent (17, 18) and –independent pathways (19). Expression of Gadd45 protein suppresses cell growth (20, 21). It has been shown that Gadd45 protein associates with multiple important cellular proteins, including PCNA (22), p12 (23, 24), Cdc2 (25), core histone (26) and MTK/MEKK4 (27). A significant body of evidence suggests that GADD45 be involved in the control of cell cycle checkpoint (28) and apoptosis (27, 29). This argument is further support by

the finding that GADD45-null mice exhibit significant genomic instability, which is exemplified by aneuploidy, chromosomal aberrations and gene amplification, and increased carcinogenesis after DNA damage (30). Therefore, GADD45 appears to be an important player in the maintenance of genomic stability.

Several lines of evidence strongly support a role for BRCA1 in regulation of transcription. BRCA1 has an N-terminal ring finger domain and a C-terminal transcription activation domain that activates transcription when fused to a DNA-binding domain (31). It has been shown that BRCA1 interacts the transcriptional regulators p53 (32, 33), c-Myc (34), STAT1 (35) and estrogen receptor (36) and proteins involved in chromatin remodeling including p300/CBP (37) and RBAP46/48-HDAC (38). Expression of BRCA1 activates or suppresses expression of several important cellular proteins, such as p21waf1/CIP1 (9) and cyclin B1 (39). Most recently, studies from our group and others have demonstrated that BRCA1 strongly activates GADD45 in a p53-indendent manner [Harkin, 1999 #27; (39, 40). Activation of the GADD45 promoter requires normal transcription function of BRCA1 since the tumor-derived BRCA1 mutants (1749R and Y1853insA), which lack transcription activity, are unable to activate the GADD45 promoter (40). However, the molecular mechanism by which BRCA1 upregulates GADD45 is complex and may involve the regulatory elements located at either the third intron or the promoter region of GADD45. BRCA1 is also reported to repress GADD45 expression through its interaction with ZBRK1 transcription factor (41). In spite of the discrepancy of the BRCA1's effect on GADD45 transcription, it has been well accepted that GADD45 is one of the BRCA1 downstream effortors, and probably mediates BRCA1's role in maintenance of genomic stability.

The transcription factor Oct-1, a member of the POU homeodomain family, expresses ubiquitously and binds to the consensus sequence (AGTCAAAA) through its DNA-binding POU domain (42). High affinity Oct-1 binding sites are found in a number of cellular promoters (43). Binding of Oct-1 factor to its consensus motif normally activates its-regulated genes (44-48). NF-Y is also a ubiquitous transcription factor consisted of three subunits A, B, C. NF-Y specifically recognizes CAAT box motif, which is one of the most ubiquitous elements presenting in 30% of eukaryotic promoters (49, 50). Recently, both Oct-1 and NF-YA, but not NF-YB and NF-YC, are found to be induced following genotoxic agents, indicating that these two transcription factors may participate in cellular response to DNA damage (51, 52).

In this manuscript, we identified OCT-1 and CAAT as the BRCA1-regulatory elements required for BRCA1 activation of the GADD45 promoter. Disruption of the OCT-1 and CAAT motifs abolish activation of the GADD45 promoter by BRCA1. Moreover, BRCA1 exhibits physically association with Oct-1 and NF-YA proteins. Therefore, these results demonstrate one of the molecular mechanisms by which BRCA1 regulates GADD45.

METERIALS AND METHODS

Plasmid clones. The following GADD45 promoter reporter constructs were used: pHG45-CAT1, pHG45-CAT2, pHG45-CAT5, pHG45-CAT7, pHG45-CAT11, pHG-CAT12 and pHG45-CAT13, which were described previously ((52, 53). The mutants of the *GADD45* promoter reporters that contain mutations in either Oct-1 or CATT box motifs (pHg45-CAT11m1, pHg45-CAT11m2, pHg45-CAT11m3, pHg45-CAT11m4, pHg45-CAT11m5, pHg45-CAT11m6 and pHg45-CAT11m7) were constructed by PCR cloning as described previously (52). pCR3-BRCA1, a construct expressing wt human BRCA1 protein, was provided by Barbara Weber (9). pC53-SN3 expressing wild-type p53 protein was provided by Bert Vogelstein (54). PG-CAT-107/-57, was constructed by

cloning the HindIII-Pst1 DNA fragment corresponding to –107 and –57 of the GADD45 promoter upstream of a minimal polyomavirus early promoter linked to a CAT gene, which was derived from PG-13 CAT (). Similarly, PG-OCT-1wt or PG-OCT-1mut was constructed by cloning direct 5 repeats of the intact OCT-1 motif (TGATTTGCATAGCCCTGTGG) or mutated OCT-1 motif (TGGCCTGCATAGCCCTGTGG) upstream of a minimal polyomavirus early promoter linked to a CAT gene via HindIII and Pst1 cloning sites. In the case of PG-CAATwt or PG-CAATmut, 3 repeats of the intact CAAT motif (TTAACCAATCAC) or mutated CAAT box (TTAACGTATCAC) was cloned into same reporter plasmids as described above.

Cell culture and treatment. The human breast carcinoma MCF-7 line, the human lung carcinoma line H1299 and the human colorectal carcinoma line HCT116 were grown in F12 medium supplemented with 10% fetal bovine serum as described previously. For MMS treatment, cells were exposed in medium to MMS (Aldrich) at 100 μg/ml for 4 hr and then the medium was replaced with fresh medium. For UV radiation, cells in 100-mm dishes were rinsed with PBS and irradiated to a dose of 10 Jm^{-2}. Cells treated with MMS and UV were collected 16 hour posttreatment for CAT assay (19, Zhan, 1998 #176).

Transfection and CAT assay. 4 μg of the *GADD45* promoter reporter constructs and 4 μg of indicated expression vectors were cotransfected into human cells via the method of calcium phosphate precipitation. 40 hours later, cells were collected for the CAT assay. Measurement of CAT activity was carried out as described previously. Induction of transcriptional activity was determined by normalizing the CAT activity of the treated samples to that of the untreated sample. Each value represented the average of at least three separate determinations (53, 55).

Antibodies, preparation of nuclear protein, immunoprecipitation and immunoblotting analysis. Antibodies against BRCA1, Oct-1, NF-YA and Actin were commercially provided by

Santa Cruz Biotechnology (Santa Cruz biotechnology, Santa Cruz, CA, USA). Nuclear protein was prepared for exponentially growing HCT116 cells as described previously (55, Zhan, 1998 #176). For immunoprecipitation and immunoblotting analysis, 300 μg of nuclear protein was immunoprecipitated with anti-BRCA1, Oct-1, NF-YA or Actin antibodies and protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4 ^{0}C. The immunoprecipitated protein complexes were washed three times with lysis buffer as described previously (). After electrophoresis, the protein was transferred to Protran membranes. Membranes were blocked in 5% milk, washed with PBST (PBS with 0.1% tween), and incubated with anti-Oct-1, NF-YA and BRCA1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Following washing and incubation with HRP-conjugated anti-rabbit or anti-mouse antibody at 1:4000 in 5% milk, membranes were washed and detected by ECL (Amersham, Arlington Height, IL) and exposed to X-ray film.

Biotin-streptavidin pull-down assay. 51-bp oligonucleotides correspond to position –107 to –57 of the human GADD45 promoter were used in the assay. Following annealing to their respective complementary oligonucleotides and gel purification, one microgram of each double-strand oligonucleotide was incubated with 300 μg of nuclear protein for 20 minutes at room temperature in binding buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 4 mM Tris (pH.79), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg of poly(dI-dC) competitor. After reaction, 30 μl of streptavidin-agarose (Sigma) was added to the reaction mixture and incubated at 4 ^{0}C for 4 h. The protein-DNA-streptavidin-agarose complex was washed three times with binding buffer and loaded onto a SDS gel. Detection of BRCA1, Oct-1 and NF-YA proteins was performed as described in Antibodies, preparation of nuclear protein, immunoprecipitation and immunoblotting analysis (53).

RESULTS

Mapping of the BRCA1-regulatory elements in the GADD45 promoter. Recent report from our group has demonstrated that BRCA1 induces expression of GADD45 mRNA and activate the GADD45 promoter (40). As shown in Fig 1A, when pHG45-CAT2, a GADD45 promoter reporter construct that spans –909 to +144 of the GADD45 promoter region, was cotransfected with either pCMV.neo (Neo) or pCR3.BRCA1 (BRCA1) into human breast carcinoma MCF-7 cell line (wt p53), human colorectal carcinoma HCT116 cell line (wt p53) or HCT116 p53-/- cell line (where p53 alleles were deleted via homologue recombination), the GADD45 reporter was strongly activated in all cell lines regardless of p53 status. To determine transfection efficiency, GFP (green fluorescence protein) expression vector was cotransfected with each tested plasmid. The expression of GFP protein detected by immunoblotting analysis indicated that transfection efficiency is similar among different samples and the variations were less than 20%. To map the BRCA1-responsive elements in the GADD45 promoter, a series of the GADD45 CAT reporters that spanning the different regions of the human GADD45 promoter were constructed. Following cotransfection of these GADD45 reporter plasmids with the BRCA1 expression vector into human colorectal carcinoma HCT116 and HCT116 p53-/- cells, CAT assays were conducted and the CAT activities were analyzed. As illustrated in Fig. 1B, most of the GADD45 CAT reporters were strongly activated following expression of BRCA1 protein. With progressive 5' -deletion, pHG45-CAT13 that extended 5' only to –62 relative to the transcription start site exhibited little induction following expression of BRCA1. These observations indicate that the region between –107 and –62 contains the regulatory elements required for the responsiveness of the GADD45 promoter to BRCA1 expression.

To confirm if the region from –107 to –57 is responsible for activation of the GADD45 promoter by BRCA1, we constructed a reporter plasmid designated as PG-CAT-107/-57, where a DNA

fragment corresponding to the GADD45 promoter region between –107 and –57 was cloned upstream of a minimal polyomavirus promoter linked to a CAT reporter gene. This minimal polyomavirus promoter itself is unable to respond to BRCA1 expression or DNA damaging agents (data not shown). When cotransfected with pCR3.BRCA1 (BRCA1) into HCT116 cells, PG-CAT-107/-57 exhibited evident induction (Fig. 1C). In contrast, both pCMV.neo (Neo) and pC53-SN3 (p53) had no effect on this reporter, indicating that the region between –107 and –57 is capable of conferring the BRCA1 inducibility to a non-related promoter reporter. Interestingly, PG-CAT-107/-57 was also shown to be strongly induced by UV radiation and MMS, suggesting that activation of the GADD45 promoter by BRCA1 and DNA damage might share some common regulatory elements. Inspection of DNA sequence exhibits two OCT-1motifs and one CAAT box located at this region of the human GADD45 promoter (Fig. 1D).

BRCA1 activation of the GADD45 promoter is mediated through both OCT1 and CAAT motifs. To define whether the OCT-1 and CAAT box motifs play roles in activation of the GADD45 promoter following expression of BRCA1, we constructed a series of the mutants of the GADD45 promoter CAT reporter constructs, where mutations were made in either OCT-1 or CAAT motifs in different combinations (). It should be noted here that our previous work has demonstrated that there are certain regulatory elements located more upstream of the GADD45 promoter, such as EGR1/WT1 (53). Therefore, to exclude the influence of such responsive elements, we choose pHG45-CAT11, which only contains the region from –121 to +144 of the GADD45 promoter. Following cotransfection of these mutants of the GADD45 promoter reporters into both HCT116 and H1299 cells, where p53 gene is deleted, induction of CAT activity was determined. As shown in Fig 2, pHG45-CAT11 exhibited the strongest activation by BRCA1. Single mutation in either OCT-1 or CAAT1 motifs (pHG45-CAT11m1, pHG45-CAT11m2, pHG45-CAT11m3) reduced little effect

of BRCA1 on the promoter. However, double mutations in OCT-1 and CAAT sites (pHG45-CAT11m4, pHG45-CAT11m5, pHG45-CAT11m6), were shown to evidently affect activation of the GADD45 promoter by BRCA1 and induction of these reporters was reduced by 60%. When all three sites were mutated (pHG45-CAT11m7), the GADD45 promoter reporter did not exhibit any activation following expression of BRCA1. The responsiveness of the pHG45-CAT11m7 to BRCA1 expression was observed to be similar to that seen in pHG45-CAT13 (Fig. 1B), which only contains the GADD45 promoter region from –62 to +144. In addition to HCT116 and H1299, we have also examined the mutants of the GADD45 promoter reporters in MCF-7 (wt 53), HCT116 p53-/-, where the p53 alleles were deleted, and obtained the similar results (data not shown), suggesting that BRCA1 activation of the GADD45 promoter mediated by OCT-1 and CAAT motifs does not require p53. Therefore, these results indicate that both the OCT-1 and CAAT motifs play important role in BRCA1 activation of the GADD45 promoter in a p53-independent manner.

In addition, we also made mutations in all OCT1 and CAAT motifs in pHG45-CAT2, which covers a longer promoter region between –909 and +144 and determined the BRCA1 activation on such construct. The BRCA1 activation of this mutated promoter (pHG45-CAT2ma) was seen to be reduced by 70 % compared the pHG45-CAT2 that contains the intact GADD45 promoter (results not shown). In contrast, BRCA1 activation of the pHG45-CAT11m7 was completely abolished (Fig. 2). This result is in agreement with our previous finding that there are certain regulatory elements (such as EGR1/WT1) (53) at the upstream region of the GADD45 promoter. These upstream responsive elements might also play a role in activation of the GADD45 promoter by BRCA1, even when mutations were made in OCT1 and CAAT1 motifs.

To further determine the roles of the OCT-1 and CAAT1 motifs in the BRCA1-mediated transcriptional activation, we constructed both OCT-1 and CAAT reporter plasminds, where the

multiple repeats of either OCT-1 or CAAT motifs were placed upstream of a polyomavirus minimal promoter that is linked to a chloramphenicol acetyl transferase (CAT) gene. In Fig. 3A, PG-OCT-1wt that contains 5 repeats of the intact OCT-1 motifs was transfected with expression vectors for BRCA1, Neo and Oct-1 into HCT116 cells. Evidently, PG-OCT-1wt was activated following expression of BRCA1. As an OCT-1 reporter, this construct was also strongly induced by Oct-1 expression. Interestingly, the OCT-1 reporter was responsive to MMS treatment. In contrast, the PG-OCT-1mut that contains 5 repeats of the mutated OCT-1 sites did not exhibit any responsiveness to expression of BRCA and Oct-1 protein, neither to MMS treatment. Similarly, the PG-CAATwt with 3 repeats of the CAAT motifs demonstrated a clear induction following expression of either BRCA1 or NF-YA, which is one of the subunits of NF-Y transcription factor that binds to CAAT box. In addition, this reporter also exhibited strong activation by MMS. However, PG-CAATmut with mutated CAAT motifs did not respond to expression of BRCA1 and NF-YA or MMS treatment. Collectively, the results presented above further support that the BRCA1 activation of the GADD45 promoter is mediated through the OCT-1 and CAAT motifs.

BRCA1 physically interacts with OCT-1 and CAAT motifs via its physical association with both Oct-1 and NF-YA proteins. Since the OCT-1 and CAAT motifs mediate the transcriptional activation of the GADD45 promoter by BRCA1, effort was made to determine if BRCA1 directly binds to the GADD45 promoter region containing both OCT-1 and CAAT sites. An approach called "biotin-streptavidin pull down assay" was employed to identify the proteins bound to the BRCA1-responsive region of the GADD45 promoter. The biotin-labeled 51-bp double-strand oligonucleotides corresponding to –107 to –57 of the GADD45 promoter was incubated with nuclear extracts form HCT116 cells and pulled down by streptavidin (see Materials and Methods). The protein complexes bound to the oligonucleotides were loaded onto SDD-PAGE gel and analyzed by

immunoblotting assay with antibodies against BRCA1, Oct-1 that binds to OCT-1 motif and NF-YA, which is one of the subunits of NF-Y protein that binds to CAAT box. In Fig. 4A, the Oligo-wt that contains the intact OCT-1 and CAAT motifs was able to pull down the Oct-1, NF-YA and BRCA1 proteins, indicating that all three proteins physically associate with this BRCA1-regulatory region. In Oligo-mut1, where the CAAT box was mutated, both the Oct-1 and BRCA1 proteins but not NF-YA were detected in the precipitated complexes. In the case of Olio-mut2, where two OCT-1 sites were disrupted, BRCA1 and NF-YA proteins were present but no Oct-1. However, when all OCT-1 and CAAT motifs were mutated in the Oligo-mut3, none of the BRCA1, Oct-1 and NY-FA proteins was observed. These results strongly suggest two interpretations: (1). BRCA1 physically associates with the region of the GADD45 promoter between –107 and –57 through its interaction with both OCT-1 and CAAT motifs. (2). BRCA1 interacts with OCT-1 or CAAT motifs independently since single mutation of either motif did not disrupt BRCA1 interaction with the BRCA1-responsive region of the GADD45 promoter.

However, since BRCA1 is not a sequence-specific binding transcription factor, it is most likely that the association of BRCA1 protein with the GADD45 promoter is through its interaction with the Oct-1 and NF-Y factors, which directly bind to these motifs. To address this issue, the Oligo-wt was incubated with the nuclear extracts, which were immunodepletion with anti-Oct-1 or –NF-YA antibodies prior to the pull-down assay. As shown in Fig. 4B, depletion with single antibody to either Oct-1 or NF-YA proteins did not evidently affect binding of BRCA1 to the GADD45 promoter region. However, immunodepletion of both the Oct-1 and NF-YA proteins completely abolished the association of BRCA1 with the GADD45 promoter, indicating that association of BRCA1 with the GADD45 promoter is through its interaction with the Oct-1 and NF-YA proteins, which directly bind to the GADD45 promoter.

Next, we further determined the physical interactions of BRCA1 with Oct-1 and NF-YA proteins. Nuclear extracts isolated from HCT116 cells were incubated with anti-actin, anti-Oct-1, anti-NF-YA and anti-BRCA1 antibodies and immunoprecipitated with protein A/G agarose beads. The immunocomplexes were then analyzed by western blotting assay and the results were shown in Fig. 5. Clearly, NF-YA protein was shown to present in the immunocomplexes precipitated by the antibodies against to Oct-1, NF-YA and BRCA1, suggesting physical interactions of NF-YA with Oct-1 and BRCA1. Oct-1 protein was detected in the immunocomplexes with both anti-Oct-1 and anti-BRCA1 antibodies. Similarly, BRCA1 protein was also observed in the immunocomplexes by antibodies to Oct-1 and BRCA1. In contrast, none of the NF-YA, Oct-1 and BRCA1 proteins s was found present in the actin-immunoprecipitated complex. However, it is somewhat suppressing that we did not see the presence of the Oct-1 and BRCA1 proteins in the immunocomplex with anti-NF-YA antibody. One likely interpretation is that the interacting domains of Oct-1 and BRCA1 in NF-YA protein might share the region with the epitopes of the antibody against NF-YA, which possibly lead to dissociation of the NY-FA/BRCA1 and NF-YA/Oct-1 protein complexes. Taken together, these results indicate an association of BRCA1 with Oct-1 and NF-YA, and an interaction between Oct-1 and NF-YA as well.

DISCUSSION

Studies presented in this report and earlier (Jin et al, 2000) have demonstrated that BRCA1 activates the GADD45 promote. Using 5' -deletion analysis, the BRCA1-regulatory elements have been mapped at the GADD45 promoter region between –107 and –62, where there are two OCT-1 and one CAAT motifs. Disruption of the OCT-1 and CAAT motifs abrogates the activation of the GADD45 promoter following BRCA1 expression, indicating that both OCT-1 and CAAT sites are

required for the BRCA1 activation of the GADD45 promoter. This finding is further supported by the observation that the OCT-1 and CAAT motifs are able to confer BRCA1 inducibility to a non-related minimal polyomavirus promoter, when mutople repeats of these motifs are cloned upstream of the minimal promoter linked to a CAT gene. In the biotin-streptavidin pull down assay, BRCA1 protein exhibits an association with the oligonucleotides corresponding to the GADD45 promoter region from –107 to –57. Mutations of all OCT-1 and CAAT sites in such oligonucleotides disrupt association of BRCA1 with the GADD45 promoter. Importantly, BRCA1 protein is demonstrated to physically interact with both Oct-1 and NF-YA proteins and depletion of Oct-1 and NF-YA proteins results in abrogation of association of BRCA1 with the GADD45 promoter. We conclude that BRCA1 transactivation of the GADD45 promoter is mediated through BRCA1 interaction with Oct-1 and NF-YA proteins.

BRCA1 has been implicated in DNA damage-induced cellular response, including apoptosis, cell cycle arrest and DNA repair (6-12). Inactivation of BRCA1 has been found to correlate with genomic instability (13). Therefore, one of the major roles for BRCA1 is to maintain genomic fidelity. In addition to the direct interactions of BRCA1 with DNA repair or cell cycle controlling proteins, the BRCA1-mediated transcriptional regulation may also greatly contribute to its role in cellular response to DNA damage. For example, both p21and GADD45, which are important players in the control of cell cycle checkpoints (28, 56), are regulated by BRCA1 (9, 40). It has been well accepted that the roles of BRCA1 as a tumor suppressor might be at least in part mediated through its transcriptional properties, given the evidence that tumor-derived mutations within the C-terminus of BRCA1 are defective in transcriptional activation (31, Somasundaram, 1997 #25). In agreement with this point, the tumor-derived BRCA1 mutants (p1749R and Y1853insA) that lack transcriptional activity are unable to activate the GADD45 promoter, demonstrating that BRCA1

activation of the GADD45 promoter requires normal transcriptional property (40). However, the regulation of GADD45 by BRCA1 appears to be complex and might involve differential mechanism(s). The possible reasons for this complex regulation may be due to (b). BRCA1 activation of GADD45 has been shown to involve the BRCA1-responsive elements located at both the intronic or promoter regions of GADD45 (29, 40, 57). (b). Most likely, BRCA1 regulates GADD45 through its interaction with other transcription factors that directly bind to the GADD45 promoter or intronic regions instead of direct binding of BRCA1 to the regulatory-regions. (c). BRCA1 protein might be subject to phosphorylation in the process of DNA damage-induced transcriptional activation (4, 5). (d). BRCA1-mediated transactivation might recruit transcriptional coactivators, such as p300/CBP (37). Therefore, future work will be seeking for detailed biochemical consequence of the interaction between BRCA1 and Oct-1 and NF-YA to determine if Oct-1 and NF-YA are subject to protein stabilization, phosphorylation or acetylation.

GADD45 promoter is strongly activated following genotoxic stress, including UV radiation, MMS and medium starvation (53). Most recently, we have demonstrated that the p53-independent UV induction of the GADD45 promoter is also regulated through both OCT-1 and CAAT motifs located at the same region between –107 and –62 of the GADD45 promoter. Mutation of all OCT-1 and CAAT motifs abolish the induction of the GADD45 promoter by UV radiation and MMS. In addition, protein levels of the transcription factors Oct-1 and NF-YA that binds to those motifs are found to elevate following DNA damage (52). Moreover, MAP kinases (JNK and ERK) are also shown to activate the GADD45 promoter through the OCT-1 and CAAT motifs (paper in press). In the current study, we demonstrate that the OCT-1 and CAAT motifs mediate the BRCA1 activation of the GADD45 promoter. Therefore, it can be speculated that the OCT-1 and CAAT motifs are critical in the regulation in the p53-independent induction of GADD45 in response to growth arrest

signals (such as BRCA1 expression) and a variety of DNA damaging agents. It is worth noting that in the current study, the OCT-1 and CAAT motifs appears to function in an additive but independent manners since single mutation of either OCT-1 or CAAT motifs only exhibited a reduced induction of the GADD45 promoter by BRCA1, but mutations of all OCT-1 and NF-YA resulted in completely disruption of the BRCA1 activation of the GADD45 promoter (Fig. 2).

The finding that BRCA1 regulates the GADD45 through its interaction with transcription factors Oct-1 and NF-YA is of importance, given evidence that both the OCT-1 and CAAT motifs are widely present in the many gene promoters. Oct-1 and NF-YA are ubiquitous transcription factors involved in the development, cell cycle regulation and cellular senescence (49, 50, 58, 59). Recently, we have found that OCT-1 and NF-YA proteins are induced after exposure of cells to multiple DNA damaging agents and therapeutic agents in a p53-independent manner (51, 52). These observations indicate that both Oct-1 and NF-YA proteins are able to participate in cellular response to genotoxic stress. In addition, our current study has shown a physical interaction of NF-YA with Oct-1 protein, suggesting that induction of the GADD45 by BRCA1 might involve a functional interaction between these two proteins. In fact, Oct-1 and NF-YA proteins have previously been reported to synergistically regulated histone H2B gene transcription during Xenopus early development (60). In summary, the study presented here has demonstrated the biochemical mechanism by which BRCA1 regulates the GADD45 promoter and indicated that GADD45 is a BRCA1-downstream effector. Furthermore, identification of the OCT-1 and CAAT1 as BRCA1-responsive elements has greatly broadened the biological roles for BRCA1 in transcriptional regulation.

LEGENDS

Fig. 1. Mapping of the BRCA1-regulatory elements in the GADD45 promoter. *(A)*. 4 μg of the *GADD45* promoter CAT reporter constructs pHG45-CAT2 was cotransfected with 4 μg of either

pCR3.BRCA1 (BRCA1) or pCMV.neo (Neo) expression vectors into MCF-7, HCT116 or HCT116p53-/- cells via the calcium phosphate method. 48 hours later, cells were collected for the CAT assay (see Materials and Methods). To determine transfection efficiency, 4 μg of GFP (green fluorescence protein) expression vector was co-transfected with each tested plasmid and the expression of GFP protein was detected as the internal control of transfection. *(B)*. 4 μg of the CAT reporter constructs containing the indicated regions of the *GADD45* promoter were cotransfected with pCR3.BRCA1 into HCT116 and HCT116 p53-/- cell lines. The CAT assay was carried out as in Fig 1A. *(C)*. 4 μg of PG-CAT-107/-57 plasmid, where the DNA fragment corresponding to –107 to –57 of the GADD45 promoter was cloned upstream of a minimal polyomavirus promoter linked to a CAR gene, was cotransfected with either pCR3.BRCA1 (BRCA1), pCMV.neo (Neo) or pC53-SN3 (p53), The CAT assays were performed and the CAT activities were measured as described in Materials and Methods. In some cases, the cells transfected with PG-CAT-107/-57 alone were treated with UV radiation or MMS, and followed by CAT assay. All experiments presented in (*A*) and (*C*) were repeated at least three times but only representative experiment of CAT assay is shown here. *(D)*. DNA sequence analysis indicates that there are two OCT-1 sites and one CAAT box located at the region of the GADD45 promoter from –107 to –62.

Fig. 2. Mutations of OCT-1 and CAAT motifs abrogate the activation of the GADD45 promoter following expression of BRCA1. 4 μg of the GADD45 promoter reporter constructs containing the indicated mutations either in OCT-1 sites or in CAAT box were cotransfected with pCR3.BRCA1 into either human colorectal carcinoma HCT116cells or human lung carcinoma H1299 cells, which contain deleted p53 gene. 40 hours later, cells were collected for CAT assay as described in Materials and Methods. The values represent the relative induction of the GADD45 promoter CAT reporters by BRCA1 to that of the Neo-cotransfected controls.

Fig. 3. Both OCT-1 and CAAT box motifs confer inducibility of BRCA1 to a non-related minimal promoter. (*A*). 4 μg of PG-OCT-1wt and PG-OCT-1mut constructs, which are OCT-1 reporter plasmids and were constructed by inserting 3 repeats of intact OCT-1 or mutant OCT-1 motifs upstream of the minimal polyomavirus promoter linked to a CAT gene (see Materials and Methods for information on construction), were cotransfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, pCR3.Oct-1) into HCT116 cells. 40 hours later, cells were collected for CAT assay as described in Fig 1A. *(B)*. 4 μg of PG-CAATwt or PG-CAATmut plasmids, which are CAAT reporter constructs (see Materials and Methods), were transfected with 4 μg of the indicated expression vectors (pCMV.neo, pCR3.BRCA1, xxxx.NF-YA) into HCT116 cells. CAT assay was performed as in Fig. 1A.

Fig. 4. Pull-down assay with biotin-labeled oligonucleotides containing the OCT-1 and CAAT1 motifs. (*A*). Nuclear extracts were prepared from HCT116 cells as described in Materials and Methods, and incubated with biotin labeled 51-bp oligonucleotides, which contain either intact or mutated OCT-1 and CAAT sequences. Proteins binding to these nucleotides were isolated with streptavidin-agarose beads and detected for BRCA1, Oct-1 and NF-YA by immunoblotting analysis (see Materials and Methods). (B). The nuclear extracts were immunodepleted with the antibodies against Sp1, Oct-1 and NF-YA prior to incubation with the nucleotide containing intact OCT-1 and CAAT motifs (Oligo-wt).

Fig. 5. Physical association of BRCA with Oct-1 and NF-YA. Nuclear protein from HCT116 cells was prepared (see Materials and Methods) and immunoprecipitated with anti-Sp1, anti-Oct-1, anti-NF-YA and anti-BRCA1 antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA). The immunocomplexes were analyzed by SDS-PAGE gel electrophoresis and immunoblotting with the antibodies against NF-YA, Oct-1 and BRCA1, respectively. The visualized bands are shown; their

estimated massed were 42-46 kDa for NF-YA, 97 kDa for Oct-1 and 220 kDa for BRCA1. IP: immunoprecipitation and IB: immunoblotting analysis.

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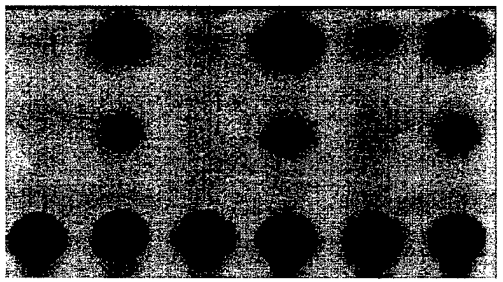
A.

pHG45-CAT2

MCF-7 HCT116 HCT116 p53-/-

Neo BRCA1 Neo BRCA1 Neo BRCA1



GFP

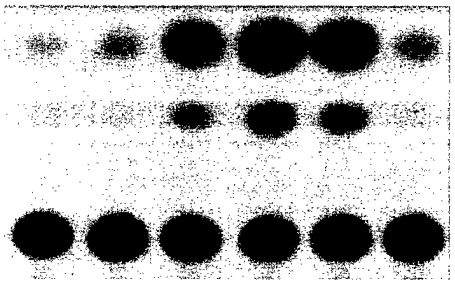
B.

	Induction of CAT activity	
	HCT116	HCT116 p53-/-
-2256 pHG45-CAT1 +144 CAT	10.5 ± 2.01	9.73 ± 1.24
-909 pHG45-CAT2 CAT	10.3 ± 1.44	9.23 ± 2.10
-612 pHG45-CAT5 CAT	10.2 ± 0.88	9.70 ± 0.98
-253 pHG45-CAT7 CAT	9.97 ± 0.84	7.21 ± 1.01
-121 pHG45-CAT11 CAT	12.7 ± 2.21	8.27 ± 0.69
-107 pHG45-CAT12 CAT	10.2 ± 2.25	8.23 ± 0.72
-62 pHG45-CAT13 CAT	1.79 ± 0.21	1.47 ± 0.22

C.

PG-CAT-107/-57

Control Neo BRCA1 UV MMS p53



D.

-107 OCT-1 CAAT box OCT-1 -62

GCAGGCTGATTTGCATAGCCCAATGGCCAAGCTGCATGCAAATGAGG

Figure 1

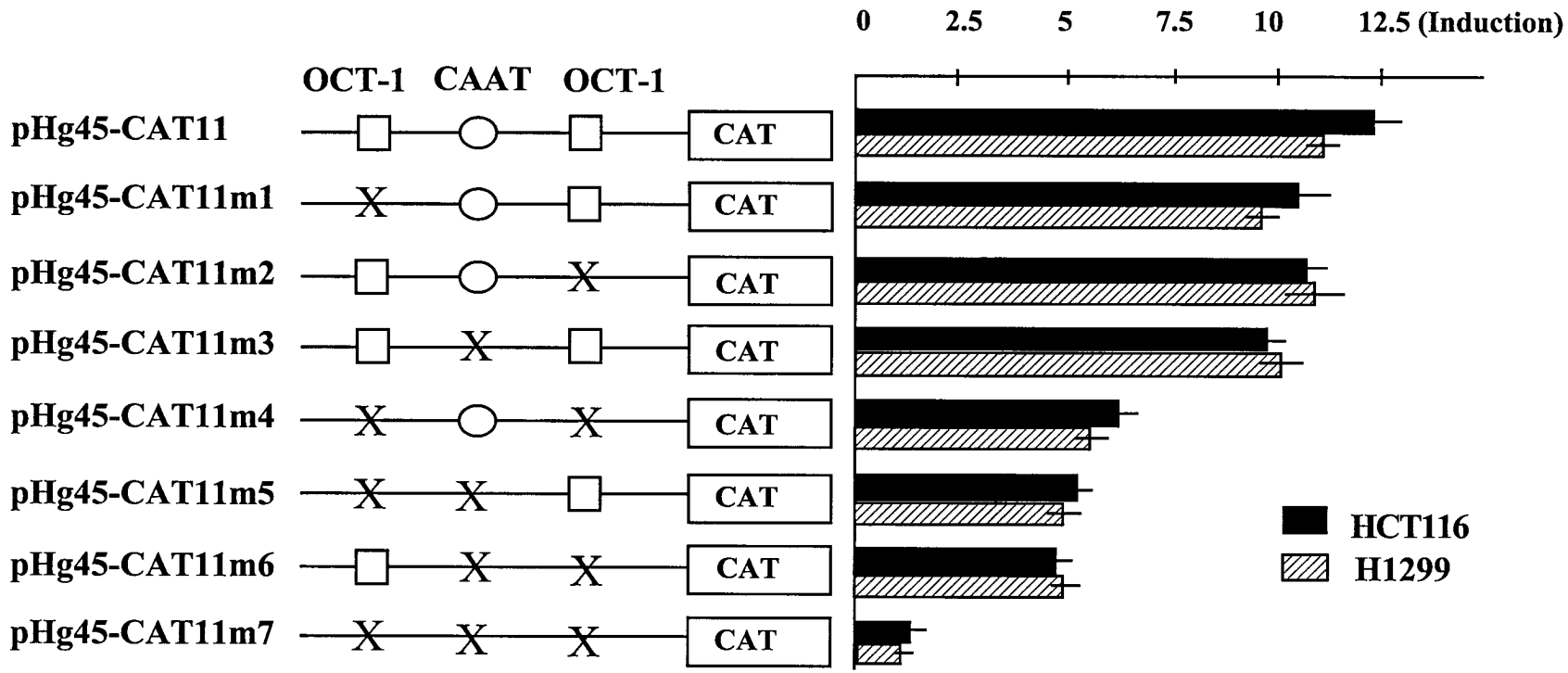

0
2.5
5
7.5
10
12.5 (Induction)
OCT-1
CAAT
OCT-1
pHg45-CAT11
pHg45-CAT11m1
pHg45-CAT11m2
pHg45-CAT11m3
pHg45-CAT11m4
pHg45-CAT11m5
pHg45-CAT11m6
pHg45-CAT11m7
CAT
HCT116
H1299

Figure. 2

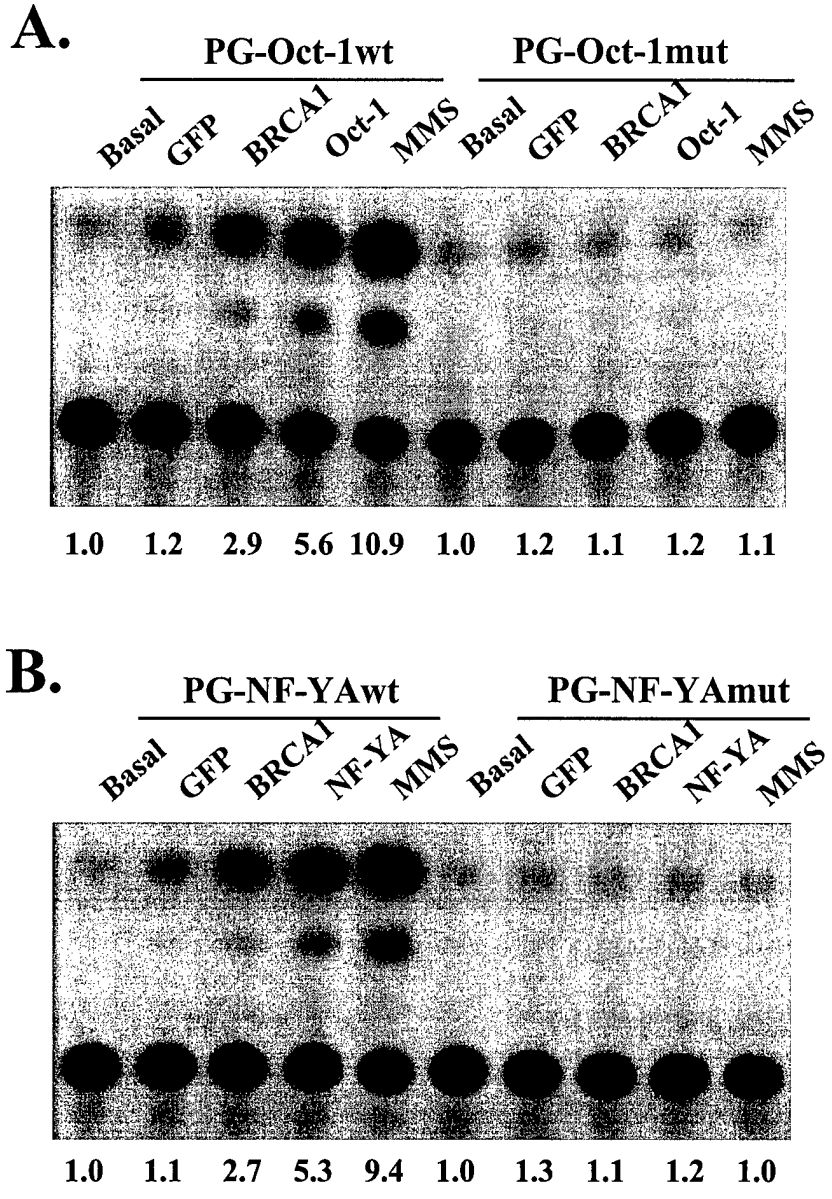

A.
PG-Oct-1wt
PG-Oct-1mut
Basal
GFP
BRCA1
Oct-1
MMS
Basal
GFP
BRCA1
Oct-1
MMS
1.0 1.2 2.9 5.6 10.9 1.0 1.2 1.1 1.2 1.1
B.
PG-NF-YAwt
PG-NF-YAmut
Basal
GFP
BRCA1
NF-YA
MMS
Basal
GFP
BRCA1
NF-YA
MMS
1.0 1.1 2.7 5.3 9.4 1.0 1.3 1.1 1.2 1.0

Figure. 3

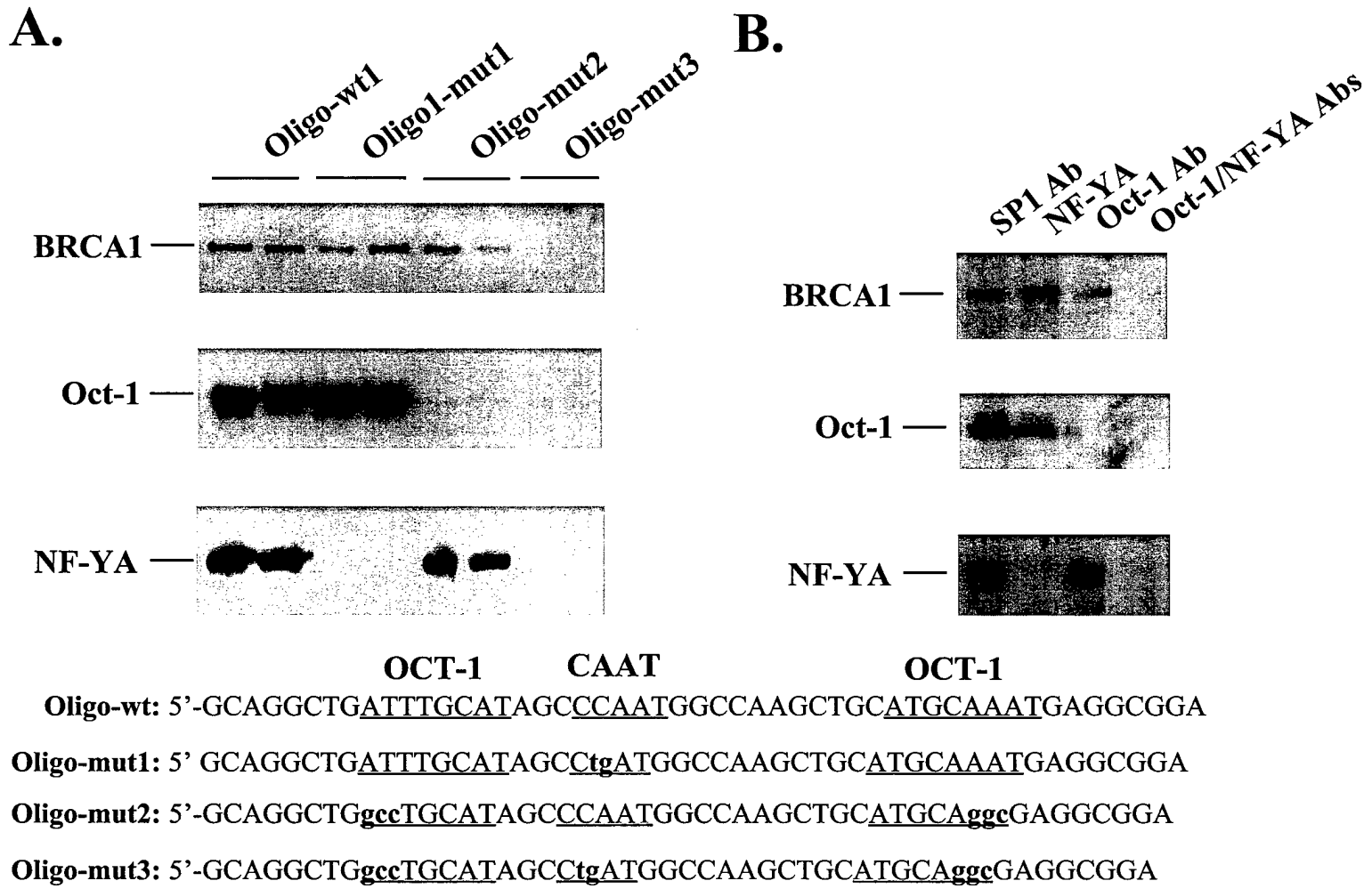

A.
Oligo-wt1
Oligo1-mut1
Oligo-mut2
Oligo-mut3
BRCA1
Oct-1
NF-YA
B.
SP1 Ab
NF-YA
Oct-1 Ab
Oct-1/NF-YA Abs
BRCA1
Oct-1
NF-YA
OCT-1
CAAT
OCT-1
Oligo-wt: 5'-GCAGGCTGATTTGCATAGCCCAATGGCCAAGCTGCATGCAAATGAGGCGGA
Oligo-mut1: 5' GCAGGCTGATTTGCATAGCCtgATGGCCAAGCTGCATGCAAATGAGGCGGA
Oligo-mut2: 5'-GCAGGCTGgccTGCATAGCCCAATGGCCAAGCTGCATGCAggcGAGGCGGA
Oligo-mut3: 5'-GCAGGCTGgccTGCATAGCCtgATGGCCAAGCTGCATGCAggcGAGGCGGA

Figure. 4

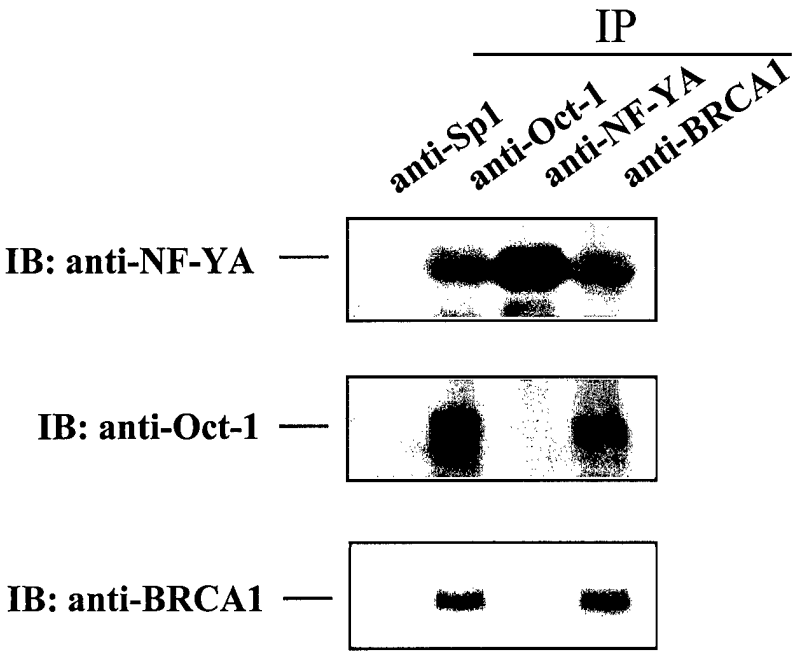

IP
anti-Sp1
anti-Oct-1
anti-NF-YA
anti-BRCA1
IB: anti-NF-YA
IB: anti-Oct-1
IB: anti-BRCA1

Figure. 5