

Differential promoter methylation may be a key molecular mechanism in regulating BubR1 expression in cancer cells

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Abbreviations: 5-Aza-2-DC, deoxycytidine; APC/C, anaphase-promoting complex/cyclosome; ChIP, chromatin immunoprecipitation assay; CIN, chromosome instability

Abstract

The BubR1 mitotic-checkpoint protein monitors proper attachment of microtubules to kinetochores, and links regulation of chromosome-spindle attachment to mitotic-checkpoint signaling. Thus, disruption of BubR1 activity results in a loss of checkpoint control, chromosomal instability caused by a premature anaphase, and/or the early onset of tumorigenesis. The mechanisms by which deregulation and/or abnormalities of BubR1 expression operate, however, remain to be elucidated. In this study, we demonstrate that levels of BubR1 expression are significantly increased by demethylation. Bisulfite sequencing analysis revealed that the methylation status of two CpG sites in the essential BubR1 promoter appear to be associated with BubR1 expression levels. Associations of MBD2 and HDAC1 with the BubR1 promoter were significantly relieved by addition of 5-aza-2'-deoxycytidine, an irreversible DNA methyltransferase inhibitor. However, genomic DNA isolated from 31 patients with colorectal carcinomas exhibited a +84A/G polymorphic change

in approximately 60% of patients, but this polymorphism had no effect on promoter activity. Our findings indicate that differential regulation of BubR1 expression is associated with changes in BubR1 promoter hypermethylation patterns, but not with promoter polymorphisms, thus providing a novel insight into the molecular regulation of BubR1 expression in human cancer cells.

Keywords: Bub1 spindle checkpoint protein; colorectal neoplasms; DNA methylation; polymorphism, genetic

Introduction

Mitotic-checkpoint controls trigger cell cycle arrest and/or elimination of cells harboring cell cycle defects. Mitotic-checkpoint proteins, such as Bub1, Bub3, BubR1 (a homolog of yeast Bub1 and Mad3), Mad1, and Mad2 preferentially localize to the kinetochores of unaligned chromosomes where they produce a diffusible "wait anaphase" signal (Chen *et al.*, 1996; Taylor and McKeon, 1997; Taylor *et al.*, 1998; Waters *et al.*, 1998; Chan *et al.*, 1999; Abrieu *et al.*, 2000). This signal delays the onset of anaphase by inhibiting the anaphase-promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase required for degradation of securin and the subsequent activation of separase, leading to separation of sister chromatids (Sudakin *et al.*, 1995; Nasmyth *et al.*, 2000; Yu, 2002). Defects in the mitotic-checkpoint function, therefore, result in premature mitosis and mis-segregation of chromosomes, leading to chromosome instability (CIN) and the aneuploidy that is observed in many human cancers (Cahill *et al.*, 1998).

The results of recent studies indicate that human cancer cells contain mutations in the mitotic-checkpoint genes that encode Bub1, BubR1, Mad1, Mad2 and others (Kops *et al.*, 2005). The effect of these mutations on mitotic-checkpoint signaling and/or the oncogenic process, however, has not been examined, with the exception of the mutant Mad1 allele isolated from a lymphoma (Tsukasaka *et al.*, 2001). The first evidence demonstrating mutations in a checkpoint-gene associated with a human disease was reported to involve five families with the rare recessive condition termed mosaic variegated aneuploidy (MVA). A total of nine mutations were found in

the BUB1B gene that encodes the BubR1 protein (Hanks *et al.*, 2004). It has not been determined whether these mutant alleles affect checkpoint signaling. Nevertheless, it seems that defects in the expression of these checkpoint genes leads to chromosome mis-segregation, causes CIN and aneuploidy, and facilitates tumorigenesis, particularly in animal models. BubR1 has been shown to inhibit APC/C through direct binding to Cdc20, and thus defects in BubR1-mediated signaling also abolish checkpoint control. BubR1 monitors proper attachment of microtubules to kinetochores and links regulation of chromosome-spindle attachment to mitotic-checkpoint signaling (Taylor *et al.*, 1998; Mao and Abrieu, 2003; Lampson and Kapoor, 2005). Recent observations show that deletion of one BubR1 allele in mice, which express nearly 30% of normal protein levels, results in enhanced genomic instability and tumor formation (Dai *et al.*, 2004; Wang *et al.*, 2004). Our recent results demonstrate that the apoptotic potential of BubR1 appears to monitor abnormalities in cells that have aberrantly exited from mitotic arrest, and that expression of BubR1 suppresses the development of tumors established with adapted aneuploid cells (Shin *et al.*, 2003).

Additionally, mutant mice with compromised BubR1 expression exhibit aneuploidy, early onset of malignancies, and premature cellular senescence (Baker *et al.*, 2004). These observations indicate that the BubR1 mitotic-checkpoint protein plays an essential role in the maintenance of genomic integrity, and that defects in BubR1-mediated signaling not only eliminate checkpoint control, but are also linked to certain human diseases. Aberrant expression of the BubR1 protein has been frequently observed in human cancer cells (Shin *et al.*, 2003), despite the rarity of genetic alterations of the BUB1B gene in such cells (Saeki *et al.*, 2002). Thus, it is possible that aberrant expression of BubR1 in human cancers is modulated by mechanisms other than mutational inactivation.

In this report we show that differential expressions of BubR1 in human cancers are associated with the status of BubR1 promoter hypermethylation, but not with the status of promoter polymorphisms.

Materials and Methods

Luciferase assay and immunoblot analysis

HeLa cells were transfected with the 5 µg of promoter construct DNA by electroporation (Bio-Rad) and were harvested using report lysis buffer (Promega) 48 h later. All transfections were normalized against an internal control (pCMV-βgal). For immunoblot

analysis, cells were harvested by scraping, washed twice in cold PBS, and lysed in a buffer containing 50 mM HEPES (pH 7.2), 250 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 µg/ml of aprotinin, 1 µg/ml of leupeptin, and 50 µg/ml of PMSF. Equal amounts of protein from each sample, as determined using the Bio-Rad protein assay, were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with anti-BubR1 (BD Biosciences Pharmingen), and anti-Actin (Sigma) antibodies.

Cell lines and subjects

HeLa, Beas2B, THP-1, and 293 cells were purchased from the ATCC. LL86, L132, Calu-3, HCC95, SK-MES-1, HCC1171, HCC1833, HCC2108, NCI-H596 and SK-LU-1 cells were purchased from the Korean Cell Line Bank. Surgically resected colorectal carcinoma tissues were obtained from Seoul National University Hospital. All study protocols were approved by the Institutional Review Board of the hospital, and informed consent was obtained as described previously (Chang *et al.*, 2005).

Identification of the promoter sequences and transcription start sites

A SMART RACE cDNA amplification kit (BD Biosciences Pharmingen) was used to identify the transcription start sites in the BubR1 promoter. First strand cDNA was synthesized from the total RNA of Jurkat T cells, and 5'RACE was performed using universal primer A and a primer specific for BubR1 cDNA (5'-TGTTGTGCAGTCTCTCCACATATGG-3'). Amplified PCR products were cloned into the pGEM-T-easy (Promega) vector, and 20 clones were then sequenced. To identify the minimal BubR1 promoter region, deletion constructs were progressively generated by PCR and cloned into the pXP2 vector.

PCR and Sodium bisulfite DNA sequencing

Genomic DNAs were digested with *Hind*III restriction enzymes and the isolated DNA fragments were denatured with sodium hydroxide (0.3 N) at 39°C for 30 min, then treated with hydroquinone (5.5 mM) and sodium bisulfite (pH 5, 2.5 M). Samples were incubated in a Thermocycler at 55°C for a total of 16 h and punctuated every 3 h by a 5 min denaturation at 95°C. Converted DNAs were desalted using a Wizard DNA clean up kit (Promega), denatured with sodium hydroxide (0.3 N) at 37°C for 15 min, and purified by ethanol precipitation. The bisulfite-modified DNAs were amplified by PCR using BubR1 promoter-specific primers (5'-GTAGTT-

TGTTTAAGGTTGTATATTTGGTATG-3' and 5'-CCA-TCCTACATTCCCTAACTCAAAT-3'). Products from nested PCR amplifications (5'-TAAGGGTGGGGT-AGGAAATAGTTAGG-3' and 5'-ACTACAAACCTT-TAATCTAAACCAC-3') were cloned into the pGEM-T vector (Promega), and 10 clones of each PCR product were sequenced using an M13 reverse primer.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the manufacturer's instructions (Upstate). Antibodies against MBD2 (Upstate) and HDAC1 (Santa Cruz) were used for immunoprecipitation. Precipitated protein-DNA complexes were washed twice sequentially with a low salt buffer (20 mM Tris-HCl [pH 8.1], 2 mM EDTA, 150 mM NaCl, 0.1% SDS and 1% Triton-X100), a high salt buffer (20 mM Tris-HCl [pH 8.1], 2 mM EDTA, 500 mM NaCl, 0.1% SDS and 1% Triton-X100), an LiCl buffer (10 mM Tris-HCl [pH 8.1], 1 mM EDTA, 250 mM LiCl, 1% NP40, 1% sodium deoxycholate), and 1 × TE buffer. Precipitated proteins and any DNA were dissolved in 1 × TE buffer and incubated at 65°C overnight to reverse crosslinks. Following RNase A, proteinase K, phenol/chloroform extraction, and ethanol precipitation, samples were subjected to 40 cycles of PCR amplification. The primers for detection of the BubR1 promoter were 5'-GATAAGCTTGAATTCAAGGGT-GGGCAGGAAAC-3' and 5'-GATTGATCTTCA-AGCCACACCCCTAGCCGCC-3'.

Results

BubR1 expression is regulated by DNA hypermethylation

Changes in promoter hypermethylation patterns represent an alternative to genetic lesions as causative factors for the aberrant expression of genes. To examine the possibility that DNA methylation is integral to the regulation of BubR1 expression, Beas2B, HeLa, Jurkat, THP-1, and 293 cells were treated with the irreversible DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-Aza-2-DC), and the BubR1 levels were determined by immunoblotting (Figure 1A and 1B). It is of interest that 4 of the 5 cell lines tested (Beas2B, HeLa, Jurkat, and 293), but not actin, contained markedly increased BubR1 protein levels after treatment with 5-Aza-2-DC, indicating that BubR1 expression is controlled by promoter hypermethylation. The defined BubR1 gene promoter (GenBank accession no. DQ206625) and its flanking regions contain approximately 34 methylatable CG pairs (Figure 2A). To examine the methylation patterns of these potential substrate sites, DNA isolated from each of the Beas2B and HeLa cell lines was treated with sodium bisulfite, PCR fragments were cloned, and 10 independent clones per cell line were sequenced. We determined that three CG pairs (at -346, -342, and -293) among seven methylatable residues in the 5' region of the minimal BubR1 promoter sequences were highly methylated in both cell lines (Figure 2B). We then assessed whether three of the CG residues were

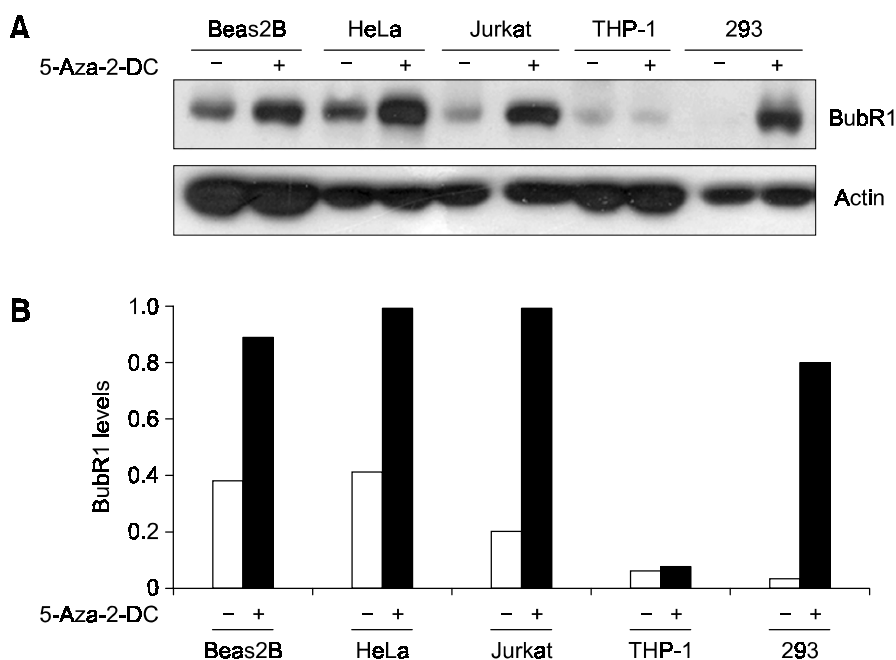


Figure 1. Effect of a demethylating agent on expression of BubR1. (A) Beas2B, HeLa, Jurkat, THP-1 and 293 cells were cultured in the absence (-) or presence (+) of 5-Aza-2-DC (4 μ mole/l) for 48 h. Protein extracts (100 μ g) were processed and immunoblotted with anti-BubR1 and anti-Actin antibodies. (B) Gel densities of relative BubR1 levels in each cell line after demethylation were quantified by densitometry. Results are representative of three independent experiments.

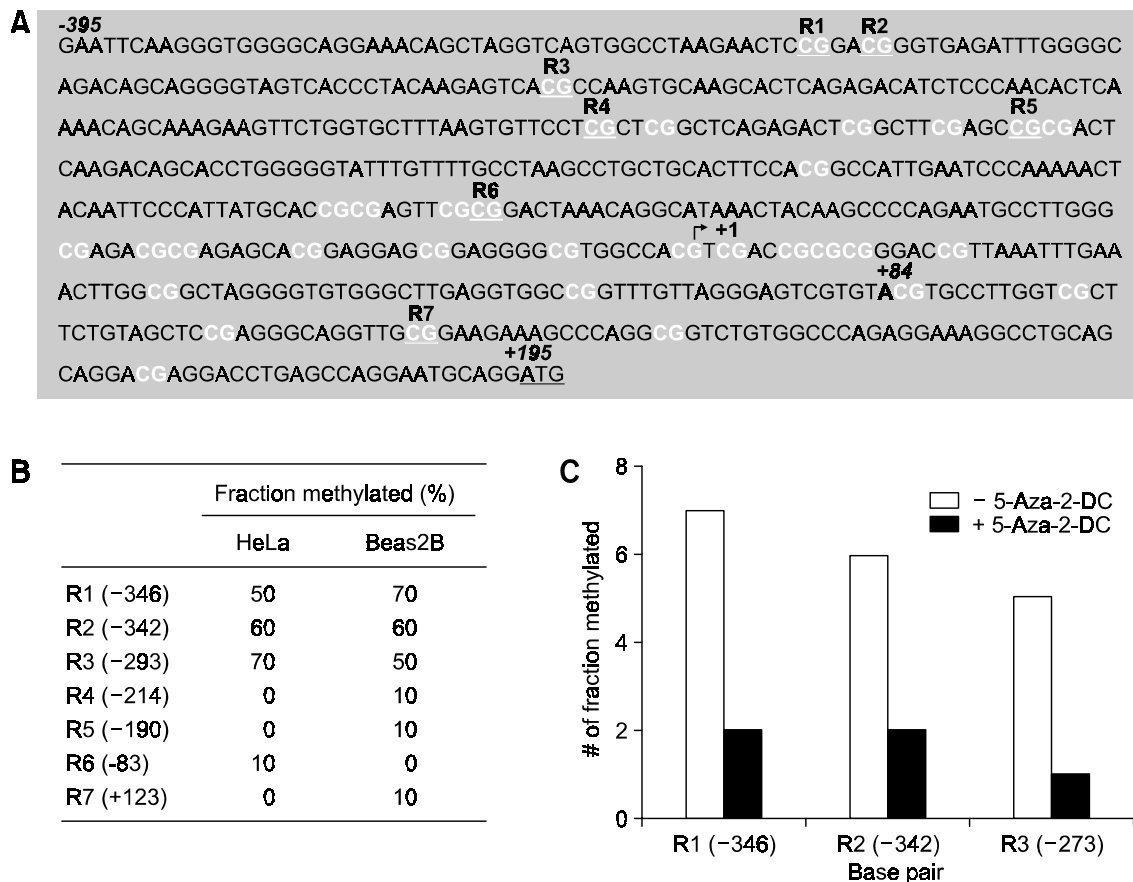


Figure 2. Methylation CG residues in BubR1 promoter. (A) Based on a BLAST homology search and progressive deletion analyses we identified and amplified a 590 nucleotide DNA sequence upstream of the BubR1 coding exon. The BubR1 promoter of parts of this sequence exhibited a nearly 200-fold higher luciferase activity compared with background activity from the promoterless backbone vector (data not shown). The major transcription start site (arrow) was determined by 5'RACE. CG pairs are marked in white with capital letters. (B) DNAs were isolated from HeLa and Beas2B cells and treated with sodium bisulfite. Amplified PCR fragments (-395 to +195) were cloned into the universal T-vector (pGEM-T) and 10 independent clones were sequenced per PCR fragment using M13 reverse primer. The positions of 7 methylatable CG residues in both cells are presented. (C) HeLa cells were cultured in the absence (-) or presence (+) of 5-Aza-2-DC. DNA was isolated, treated with sodium bisulfite, and used for PCR as described in (B). Ten independent clones were sequenced per PCR fragment. The fractions of methylated CG residues are presented.

demethylated following 5-Aza-2-DC treatment, resulting in enhanced BubR1 protein levels due to demethylation. DNA isolated from Beas2B cells cultured in the absence or presence of 5-Aza-2-DC was treated with sodium bisulfite, cloned, and sequenced. As shown in Figure 2C, these methylated residues were significantly demethylated by 5-Aza-2-DC exposure. These findings indicate that hypermethylation of the BubR1 promoter directly affects BubR1 expression.

The status of promoter hypermethylation is associated with aberrant expression of BubR1 in lung cancer cells

Based on DNA methylation regulation of BubR1 expression, we hypothesized that aberrant expres-

sion of the BubR1 protein in cancer cells is correlated with hypermethylation. To examine this possibility, we monitored the levels of the BubR1 protein in lung cell lines (immortalized nontumor, LL86, and L132; squamous cell carcinoma, Calu-3, HCC95 and SK-MES-1; adenocarcinoma, HCC1171, HCC1833, HCC2108, NCI-H596, and SK-LU-1). We previously examined the expression profiles of the BubR1 protein using human cancer specimens, and our staining results showed that BubR1 is significantly down-regulated in colon (Shin *et al.*, 2003), liver, and lung cancers (data not shown). As shown in Figure 3A, 7 of 10 lung cell lines (LL86, L132, Calu-3, HCC95, SK-MES-1, HCC1171, HCC1833, HCC2108, NCI-H596 and SK-LU-1) exhibited reduced BubR1 protein levels. Therefore, we examined whether the different levels of BubR1 expression

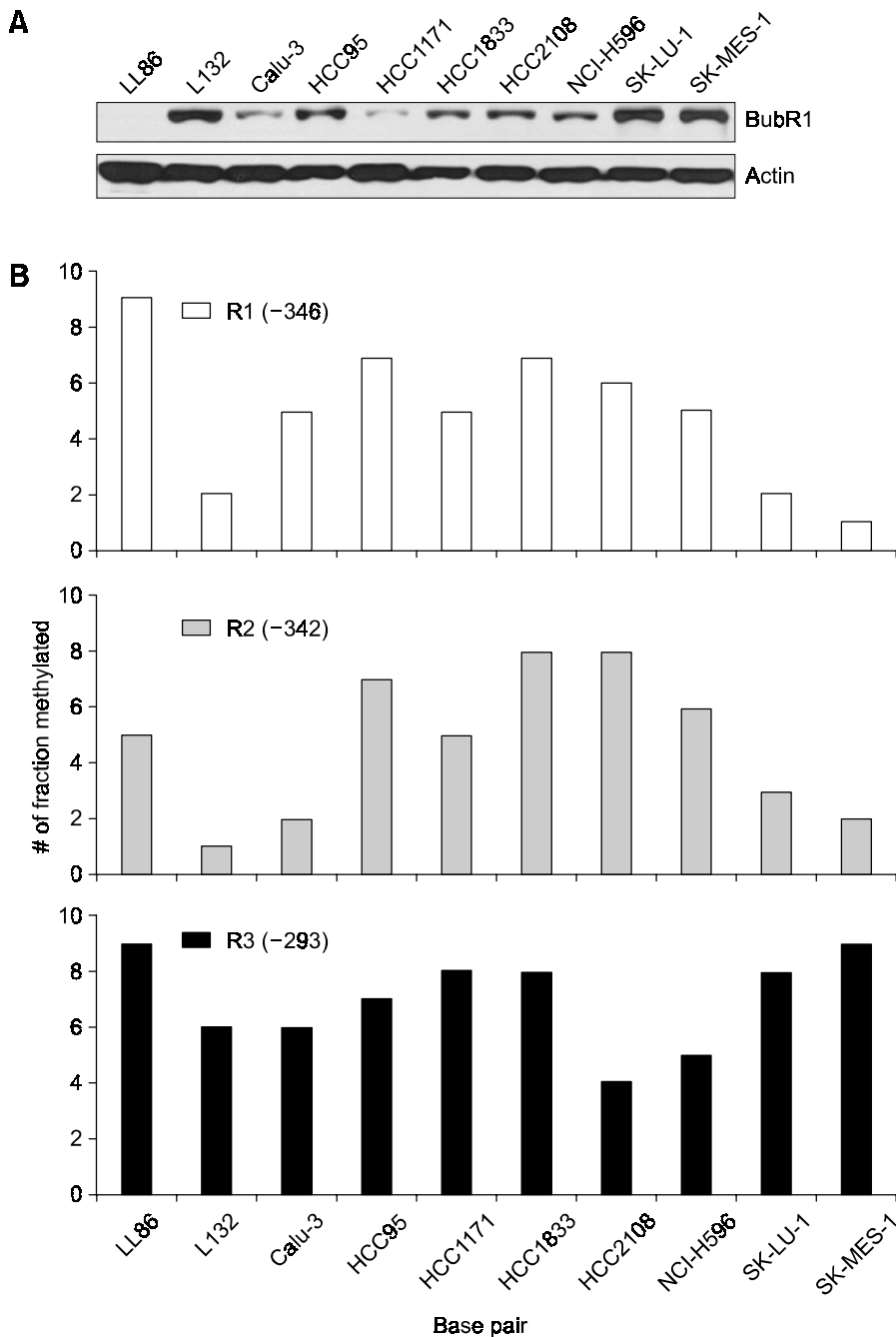


Figure 3. Aberrant expression of BubR1 in lung cancer cells associated with promoter hypermethylation. (A) Ten lung cell lines (immortalized non-tumor, LL86 and L132; squamous cell carcinoma, Calu-3, HCC95 and SK-MES-1; adenocarcinoma, HCC1171, HCC1833, HCC2108, NCI-H596 and SK-LU-1) were maintained, and protein extracts were immunoblotted with anti-BubR1 and anti-Actin antibodies. (B) DNA was isolated from 10 lung cell lines and treated with sodium bisulfite. Amplified PCR fragments (-395 to +195) were cloned into the universal T-vector (pGEM-T), and 10 independent clones were sequenced per PCR fragment. Fractions methylated at 3 CG residues -346 (R1), -342 (R2), and -293 (R3) are shown.

in each lung cell line were associated with the status of promoter hypermethylation. Evaluation of 10 individual clones from each cell line using the bisulfite sequencing analysis method revealed that the methylation status of two CpG sites at R1 (-346) and R2 (-342) varied depending on the cell line, while the high methylation profile in R3 (-293) was relatively constant throughout (Figure 3B). Three cell lines (L132, SK-LU-1 and SK-MES-1) that showed

high levels of BubR1 expression were less methylated at R1 (-346) and R2 (-342). In contrast, cell lines with reduced levels of BubR1 expression (LL86, HCC95, HCC1171, HCC2108, and NCI-H596), except Calu-3 cells, were densely methylated at these sites. These results indicate that the methylation status of two CpG sites in the BubR1 promoter appear to be inversely correlated with the expression levels of the BubR1 gene. Apparently,

differential promoter methylation contributes to silencing or inactivation of BubR1 expression.

Effects of a demethylating agent on the recruitment of DNA methylation-associated factors to the BubR1 promoter

Recent evidence that methyl-CpG binding proteins (e.g., MBD2 and MeCP2) interact with HDAC1 and HDAC2 and recruit the Sin3 corepressor protein supports a molecular mechanism involving DNA methylation. This mechanism negatively regulates target gene expression either by interfering with transcription factor binding or by facilitating formation of an unfavorable chromatin structure (Bird, 2002). The possibility that silencing or repression of the BubR1 gene is coordinated with the binding of DNA methylation-associated factors, such as MBD2 and HDACs, in the context of promoter methylation status was investigated by ChIP analyses using primers that encompassed the BubR1 gene promoter region (Figure 4). Comparable binding of MBD2 and HDAC1 to the BubR1 promoter was revealed in HeLa cells treated with and without 5-Aza-2-DC. Association of the BubR1 promoter with MBD2 was present prominently in HeLa cells, but was significantly decreased by exposure to 5-Aza-2-DC (compare tracks 3 and 7). Similarly, low levels of association of HDAC1 with the BubR1 promoter were observed in non-treated HeLa cells,

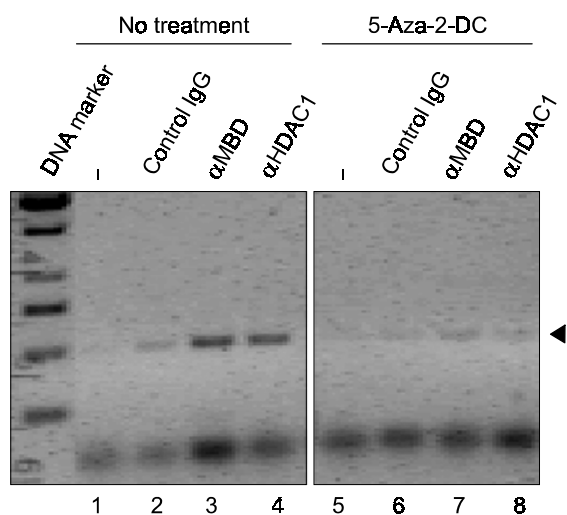


Figure 4. Recruitment of MBD2 and HDAC1 to the BubR1 promoter. ChIP analyses of the association of MBD2 and HDAC1 with the BubR1 promoter region in HeLa cells treated with or without 5-Aza-2-DC. Crosslinked cell lysates were prepared from HeLa cells. Proteins immunoprecipitated with antibodies against MBD2 and HDAC1 or control IgG were decrosslinked. Each DNA extracted from immunoprecipitated chromatin samples was amplified by PCR using BubR1 promoter-specific primers for the region from -395 to +56.

while the association was absent in cells treated with 5-Aza-2-DC (compare tracks 4 and 8). These findings indicate that the methylation states of the BubR1 promoter likely affect the recruitment of DNA methylation-associated factors.

Potential correlation between BubR1 expression and promoter polymorphisms

To determine whether polymorphic base changes in the BubR1 promoter region affect BubR1 transcription, we sequenced this region in genomic DNA isolated from surgically resected colorectal carcinoma tissues of 31 patients. We found an allelic variation (+84A) in the defined BubR1 essential promoter region (Figure 5A and B). Direct sequencing analysis revealed that the heterozygous (A/G) allele was present in approximately 35% of the colorectal carcinomas and that the mutant homozygous allele (G/G) was present in 23% of these specimens (Figure 5C). Therefore, to examine any potential correlation between allelic variation and BubR1 transcriptional activity, we performed luciferase report gene assays using BubR1 promoter constructs (-395 to +195) containing either the wild type (A/A) or the mutant homozygous (G/G) alleles. We observed no significant difference in transcriptional activity (Figure 5D), suggesting that this promoter polymorphism is not integrated into the regulation of BubR1 expression.

Our findings provide insight into BubR1 expression and indicate that differential regulation of BubR1 expression is probably associated with DNA hypermethylation, but not with promoter polymorphisms in human cancers.

Discussion

Recent observations show that MEFs, which are heterozygous for the BubR1 allele, express approximately 30% of normal protein levels, resulting in defective mitotic arrest and inducing a high frequency of aneuploidy (Wang *et al.*, 2004). It has been reported that mitotic-checkpoint proteins containing cyclin destruction sequences are specifically degraded by the ubiquitin-proteasome machinery, leading to increased formation of aneuploid cells and eventual tumorigenesis. Recently, we also found that during prolonged mitotic arrest, BubR1 becomes degraded in an ubiquitin-dependent manner and that the subsequent inactivation and/or down-regulation of BubR1 prevents induction of the apoptotic cell death that normally occurs following post-mitotic adaptation (Shin *et al.*, 2003). In addition, deletion of one BubR1 allele in a mouse model not only enhanced susceptibility to carcino-

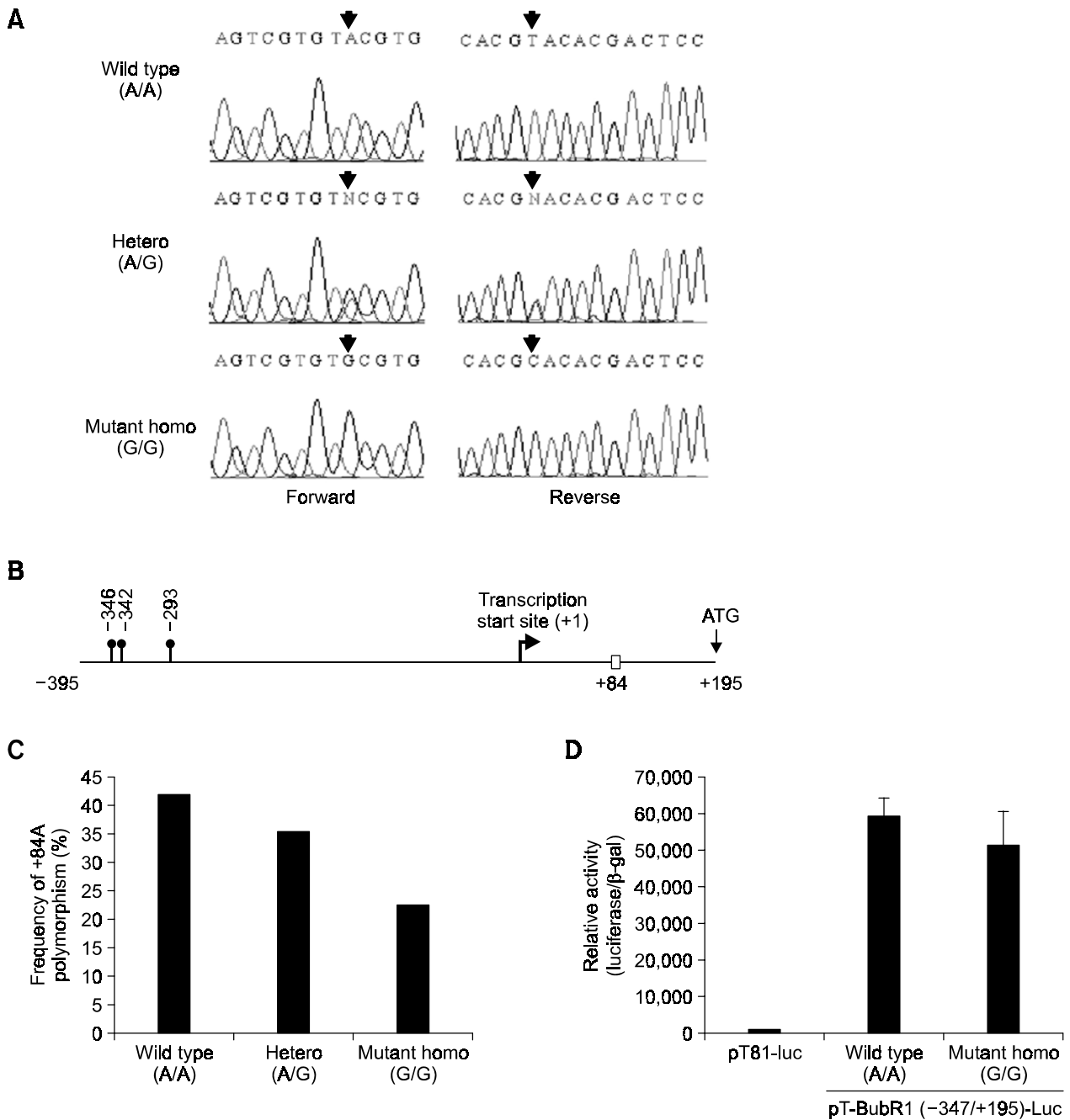


Figure 5. Promoter polymorphism is not involved in regulation of BubR1 transcription. (A) DNA was isolated from surgically resected colorectal adenocarcinoma tissues of 31 patients. The BubR1 promoter region from -395 to +195 was examined by DNA sequencing using forward and reverse primers. Polymorphic changes were observed at +85A in the essential BubR1 promoter region. (B) The positions of 3 methylatable CG pairs, a transcription start site, and a polymorphic base change are depicted. (C) A single polymorphic change was identified with the indicated allele frequencies. (D) The BubR1 promoter sequences (-395 to +195) containing wild-type (A/A) or mutant homozygous (G/G) alleles were PCR amplified and fused to the luciferase gene. The resulting constructs were transiently transfected into HeLa cells, and the luciferase activity was compared between the constructs containing the A/A and G/G genotypes. The relative luciferase to β -galactosidase activity was determined from three independent experiments.

genic induction of tumors, but also resulted in a higher incidence of tumors than in wild-type mice (Dai *et al.*, 2004). Furthermore, reduction of BubR1

levels in *Apc^{Min/+}* mice by generating *Apc^{Min/+} BubR1^{+/-}* mice resulted in a 10-fold increase in the number of high grade tumors (Rao *et al.*, 2005).

These findings indicate that a decrease in the expression level of BubR1 protein is probably a mechanism for the chromosomal instability known to be associated with tumorigenesis. Our results indicate that significant populations of tumor cells with reduced levels of BubR1 expression exhibit restored BubR1 mRNA levels following demethylation, thereby indicating that BubR1 gene promoter hypermethylation might be a major factor in BubR1 expression.

Although impairment of the mitotic checkpoint is frequently associated with tumorigenesis, mutational inactivation of mitotic-checkpoint genes is only observed in a small proportion of human cancers (Takahashi *et al.*, 1999; Haruki *et al.*, 2001; Hernandez *et al.*, 2001; Jallepalli and Lengauer, 2001; Saeki *et al.*, 2002). For instance, a few mutations in the coding sequence for BubR1 were reported to be present in human cancers (Lengauer *et al.*, 1997; Kops *et al.*, 2005). A recent report has linked MVA to mutations in the BubR1 gene (Hanks *et al.*, 2004), however, it is not known whether these BubR1 mutations actually caused the chromosomal instability and aneuploidy phenotype in the cells. Transcripts of the mitotic-checkpoint genes are maintained at different levels in human cancer cells compared to their neighboring normal cells (Shichiri *et al.*, 2002; Wang *et al.*, 2000, 2002). These observations indicate that transcriptional failure or promoter dysfunction may be responsible for changes in the checkpoint-protein levels causing induction of mitotic-checkpoint dysfunction and chromosomal instability. We recently reported that transcriptional dysfunction of Mad2 is frequently observed in hepatocellular carcinoma cells, and that down-regulation of Mad2 protein expression is correlated with transcriptional silencing of the Mad2 promoter by hypermethylation (Jeong *et al.*, 2004). These results indicate that a relationship between transcriptional abnormality of the mitotic-checkpoint gene and mitotic abnormality exists in human cancers.

Genes may be inactivated under both normal and pathological conditions through promoter hypermethylation, transcription factor dysfunction, and promoter mutation (Herman and Baylin, 2000; Baylin *et al.*, 2001; Rush and Plass, 2002; Yang *et al.*, 2003). Recent studies have demonstrated an association between human cancers and promoter hypermethylation (Lee *et al.*, 2004; Park *et al.*, 2005). Thus, cytosine methylation is highly mutagenic, causing a C to T mutation resulting in loss of the CpG methyl-acceptor site, and aberrant methylation of CpG is characteristic of many human cancers (Egger *et al.*, 2004; Feinberg and Tycko, 2004; Adcock *et al.*, 2006). We have demonstrated that the aberrant expression of BubR1 in cancer cells is associated

with promoter hypermethylation and we have provided evidence that the methylation states of two CpG sites in the BubR1 promoter are integrated into the regulation mechanism of BubR1 expression. Our study provides evidence that promoter hypermethylation of the BubR1 gene may be an important regulator of BubR1 expression. It is also possible that not only promoter hypermethylation but also post-translational degradation and stoichiometric alterations of BubR1 contribute to the dysfunction of BubR1 that abrogates the mitotic-checkpoint resulting in chromosomal instability.

In conclusion, our results indicate that changes in BubR1 promoter hypermethylation patterns are a causative factor for aberrant expression of the BubR1 gene in human cancer cells, and provide insight into the molecular mechanisms that regulate BubR1 expression.

Acknowledgement

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