Journal Title: **Oncogene**

Volume: 8 **Issue:** 4
Month/Year: 1993
Pages: 1063-1067

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Article Title: CPG METHYLATION INACTIVATES THE PROMOTER ACTIVITY OF THE HUMAN RETINOBLASTOMA TUMOR-SUPPRESSOR GENE

ISSN: 09509232

English only!

Notes/Alternate Delivery:

Oncogene (1993), **8**, 1063–1067

CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene

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Cytosine methylation of CpG sites in the promoter region of eucaryotic genes is involved in the inactivation of expression of certain genes. Given that methylation can lead to reduced transcription, it is possible that expression of tumor-suppressor genes is also inactivated by hypermethylation, thereby contributing to the etiology of cancer. Recently we found five sporadic retinoblastoma tumors (16% of all unilateral cases) with hypermethylation of the 5′ end of the retinoblastoma gene without detecting any structural abnormalities. However, it is unclear whether the promoter of the retinoblastoma gene is actually inactivated by its hypermethylation. Here we show that specific hypermethylation in the promoter region of the retinoblastoma gene reduces its expression to only 8% of the unmethylated control. Furthermore, we have found that two transcription factors important for the promoter activity, an activating transcription factor (ATF)-like factor and the retinoblastoma binding factor 1, do not bind when their recognition sequences are CpG methylated. These results *in vitro* strongly support the hypothesis that CpG methylation of the human tumor-suppressor gene can result in the inactivation of the gene and thus lead to oncogenesis.

Introduction

In cancer cells, an alteration in the level of CpG methylation seems to be a frequent event, but its effect on oncogenesis remains unclear (Jones, 1986; Jones & Buckley, 1990). Generally, DNA hypomethylation is thought to be common in malignant cells (Feinberg & Vogelstein, 1983a; Goelz *et al.*, 1985). Also oncogenes, such as *ras* (Feinberg & Vogelstein, 1983b) and c-*myc* (Cheah *et al.*, 1984), are reported to be hypomethylated. This hypomethylation in cancer cells has been proposed to result in the activation of oncogenes, leading to oncogenesis and promoting the degree of malignancy (Jones, 1986; Jones & Buckley, 1990). In contrast, recent studies have indicated that hypermethylation of tumor-suppressor genes is involved in malignant change (de Bustros *et al.*, 1988; Greger *et al.*, 1989; Sakai *et al.*, 1991a). Inactivation of tumor-suppressor genes by hypermethylation was first suggested by the fact that the short arm of chromosome 11 in certain neoplastic cells is regionally hypermethylated (de Bustros *et al.*, 1988). Several tumor-suppressor genes are thought to be clustered in that region (Saxon *et al.*, 1986; Weissman *et al.*, 1987; de Bustros *et al.*, 1988). Moreover, Greger *et al.* (1989) and Sakai *et al.* (1991a) have found hypermethylation in the 5′ region of the retinoblastoma (Rb) gene in Rb tumors. In our analysis (Sakai *et al.*, 1991a), 16% of unilateral cases were hypermethylated in the Rb promoter region without any deletion or mutation. We also found an allele-specific hypermethylation, which is consistent with the general idea that hypermethylation could be one mechanism of inactivation of the Rb gene (Sakai *et al.*, 1991a).

As to the mechanism of the gene inactivation by hypermethylation, it is supposed that CpG methylation alters chromatin structures and/or inhibits the binding of transcription factors (Cedar, 1988; Adams, 1990; Boyes & Bird, 1991). The binding of certain transcription factors has been reported to be inhibited by CpG methylation in their binding sites. For example, the binding of cAMP response element-binding protein (CREB) (Iguchi-Ariga & Schaffner, 1989), AP-2 (Comb & Goodman, 1990) and E2F (Kovesdi *et al.*, 1987) is inhibited by CpG methylation, while Sp1 (Harrington *et al.*, 1877; Höller *et al.*, 1988) and CCAAT transcription factor (CTF) (Ben-Hattar *et al.*, 1989) are reported to be unaffected by methylation.

Recently we identified two important *cis*- elements binding to retinoblastoma binding factor 1 (RBF-1) and an ATF-like factor in the Rb promoter (Sakai *et al.*, 1991b). We found naturally occurring point mutations in these binding sites in the DNAs from Rb patients. Each mutation results in the binding inhibition of the transcription factors and a remarkable reduction in the promoter activity (Sakai *et al.*, 1991b).

These studies raise the possibility that CpG methylation as well as a point mutation could cause the inactivation of the Rb promoter, thereby resulting in oncogenesis. To address the effect of methylation on Rb promoter activity, we specifically methylated the promoter *in vitro*, and examined its activity in transfection assays. Our results demonstrate that methylation results in a significant reduction in promoter activity. Moreover, we have shown that CpG methylation at the RBF-1 and ATF-like binding sites in the Rb promoter reduces the binding of the transcription factors, contributing to the reduction in Rb promoter activity. These results are consistent with a model in which hypermethylation of the tumor-suppressor gene reduces its expression, thereby contributing to the pathogenesis of certain types of cancers (de Bustros *et al.*, 1988; Baylin *et al.*, 1991).

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Received 4 September 1992; accepted in revised form 27 October 1992

Results

Specific methylation in the promoter of the Rb gene abolishes its expression

To measure the effect of methylation on promoter activity of the Rb gene, we selectively methylated the Rb promoter region in an Rb promoter–luciferase expression plasmid. To specifically methylate only the Rb promoter region, we used the method described in Materials and methods. Briefly, pXRP1, a plasmid with the Rb promoter fused to the luciferase reporter gene, was digested with BamHI and XhoI, and the two DNA fragments were purified by gel electrophoresis. The shorter fragment, containing the Rb promoter, was methylated using specific methylation enzymes. Methylation in the fragment was confirmed by digestion with methylation-sensitive enzymes, and this fragment was then ligated to the larger fragment, which contains the luciferase reporter gene. After this procedure, methylation in the Rb promoter sequences was again confirmed using methylation-sensitive enzymes. The control plasmid was generated using the same method without methylase treatment. This procedure results in a plasmid construction in which only the Rb promoter region is selectively methylated. To assay the effect of specific methylation on Rb promoter activity, the ligated plasmids were then transfected into B104 cells (rat neuroblastoma cell line) and the promoter activity was measured using a luciferase assay. B104 cells were used because Rb promoter activity is high in this cell line.

The map of the methylation enzyme sites used is shown in Figure 1a. The CpG sites in the ATF-binding sequence (TGACGT) and the RBF-1-binding sequence, which overlaps with the Sp1 recognition sequence (GGGCGG) (Sakai *et al.*, 1991b), are not methylated by treatment with HpaII methylase, but are methylated by treatment with CpG methylase (Figure 1a). When the Rb promoter was methylated by HpaII methylase, the promoter activity was 67% of the unmethylated control. When the promoter was treated with FnuDII methylase, the activity was significantly reduced to 24% of the control value ($P<0.025$), suggesting that the sites methylated by FnuDII methylase are important for activating the Rb promoter. The activity of the Rb promoter fully methyated by CpG methylase at CpG sites, including the ATF and RBF-1 binding sites, was only 8% of the control promoter ($P<0.025$). Furthermore, the activity of the Rb promoter methylated by CpG methylase was significantly lower than the activity of the promoter treated with FnuDII methylase ($P<0.005$) (Figure 1b).

CpG methylation in the ATF-like and RBF-1 sites in the Rb promoter inhibits binding of the transcription factors

To examine whether the reduction in promoter activity is the result of binding inhibition of the two presumed transcription factors by CpG methylation, a gel-shift assay was utilized. The oligonucleotides for gel-shift assays were synthesized as shown in Figure 2 with the specific cytosine residue methylated. Both binding sites have one CpG site, which was associated with the mutations we previously found in the DNAs derived from Rb patients (Sakai *et al.*, 1991b). To further determine which cytosine in the RBF-1 site causes the binding inhibition by CpG methylation, the RBF-1 oligomer DNA was also methylated *in vitro* with the FnuDII methylase (–C^{m}GCG–, RBF-1-Met2). A nuclear extract prepared from B104 cells was used in the gel-shift analysis.

To analyse the binding of the ATF-like factor, we used both the wild-type oligonucleotide (Rb-ATF-WT) and the ATF oligonucleotide with CpG methylation (Rb-ATF-Met) as cold competitors (Figure 2). The Rb-ATF-Met oligonucleotide with CpG methylation did not compete for binding, whereas the wild-type Rb-ATF competed effectively (Figure 3a). The same result was obtained with CV-1 (monkey kidney cell line) nuclear extract (data not shown). Furthermore, purified CREB was able to bind to the Rb-ATF-WT oligonucleotide, but not to the Rb-ATF-Met oligonu-

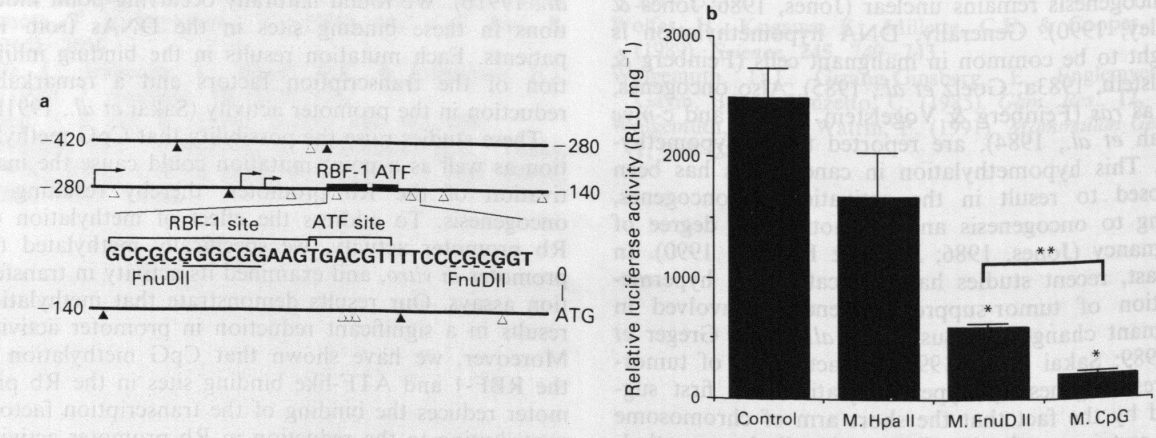

Figure 1 The methylated promoter activity of the Rb gene. (**a**) The sites of HpaII methylase and FnuDII methylase in the Rb promoter. ▲, HpaII methylase (–CC^{m}GG–); △, FnuDII methylase (–C^{m}GCG–); arrows, transcriptional start sites (Hong *et al.*, 1989); underline, GGGCGG sequence, which is the same as consensus Sp1 site, broken line; FnuDII site. (**b**) Relative activity of the Rb promoter in B104 cells using a luciferase reporter gene. Relative luciferase activity is shown by raw light units (RLU) per milligram of protein of cell lysate. Data are shown by means ± s.e. ($n=3$): $*P<0.025$; $**P<0.005$. M. HpaII, HpaII methylase; M. FnuDII, FnuDII methylase; M. CpG, CpG methylase

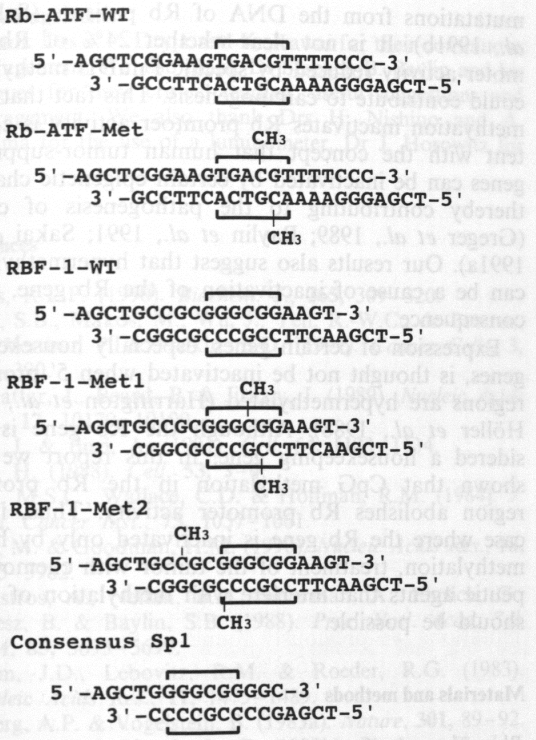

Figure 2 Synthetic oligonucleotides used for gel-shift assays. Oligonucleotides were synthesized for wild-type ATF (Rb-ATF-WT), ATF with CpG methylation (Rb-ATF-Met), wild-type RBF-1 (RBF-1-WT), RBF-1 with CpG methylation in the GGGCGG sequence (RBF-1-Met1), RBF-1 that was methylated by FnuDII methylase (RBF-1-Met2) and consensus Sp1. All the oligonucleotides have flanking partial HindIII and SalI recognition sequences. The parentheses in Rb-ATF-WT and Rb-ATF-Met show the core sequence of ATF. The parentheses in the other oligonucleotides show the core sequence of Sp1

cleotide or to the Rb-ATF oligonucleotide with a naturally occurring mutation (–TGACTT–) (Sakai *et al.*, 1991b) (data not shown). Although we have not identified the specific ATF-like factor that binds to the Rb promoter, our results suggest that Rb promoter activity is reduced by either methylation or mutation of the site.

We next examined the ability of RBF-1 to bind to the RBF-1 site. As cold competitors, we used the wild-type RBF-1 (RBF-1-WT), RBF-1 with CpG methylation in the GGGCGG sequence (RBF-1-Met1) and RBF-1 methylated by FnuDII methylase (RBF-1-Met 2) oligonucleotides as well as a consensus Sp1 oligonucleotide (Kadonaga *et al.*, 1988) (Figure 2). As shown in Figure 3b, RBF-1-Met1 did not compete for RBF-1 binding, whereas RBF-1-Met2 competed with the same efficiency as RBF-1-WT. Thus CpG methylation in the GGGCGG sequence in the RBF-1 site inhibited the binding of RBF-1, but methylation by FnuDII methylase did not. Therefore, the reduction of Rb promoter activity by FnuDII methylase to 24% was not caused by the inhibition of the binding of RBF-1. We speculate that the reason why 24% of Rb promoter activity still remains is that FnuDII methylation affects the binding of additional factors that are necessary, but not essential, to maintain complete Rb promoter activity. The consensus Sp1 oligonucleotide did not compete with the wild-type RBF-1 as we

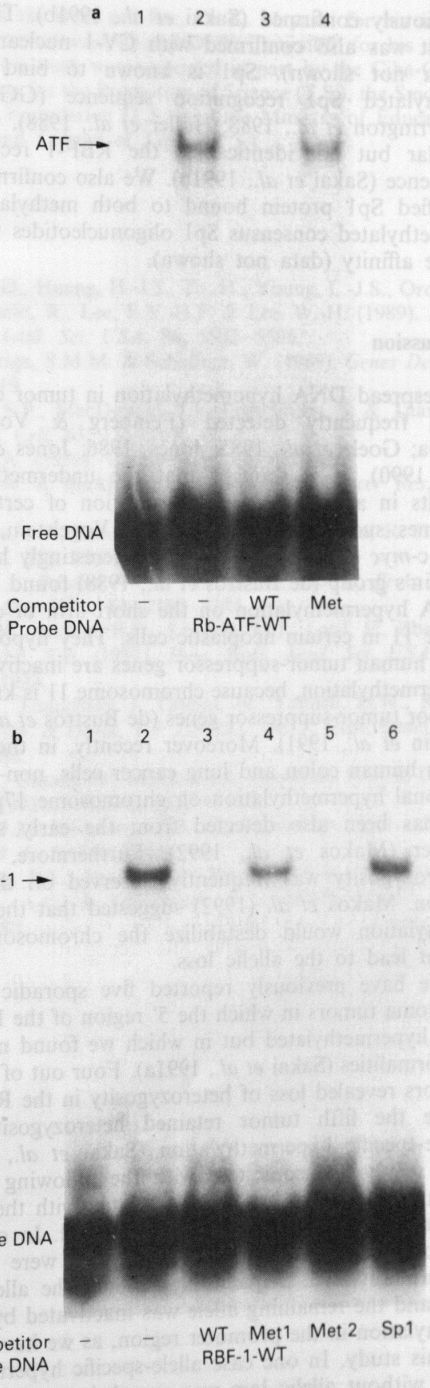

Figure 3 Gel-shift assays on ATF and RBF-1 sites. (**a**) Gel-shift assay of a sequence including the ATF site from the promoter of the Rb gene. Lane 1, radiolabeled wild-type ATF sequence from the Rb gene (Rb-ATF-WT); lane 2, Rb-ATF-WT with nuclear extract from B104 cells; lane 3, same as lane 2 with 20 ng of Rb-ATF-WT as unlabeled cold competitor; lane 4, same as lane 2 with 20 ng of Rb-ATF-Met as unlabeled cold competitor. (**b**) Gel-shift assay of a sequence including RBF-1 site from the promoter of the Rb gene. Lane 1, radiolabeled wild-type RBF-1 sequence from the Rb gene (RBF-1-WT); lane 2, RBF-1-WT with nuclear extract from B104 cells; lane 3, same as lane 2 with 20 ng of RBF-1-WT as unlabeled cold competitor; lane 4, same as lane 2 with 20 ng of RBF-1-Met1 as unlabeled cold competitor; lane 5, same as lane 2 with 20 ng of RBF-1-Met2 as unlabeled cold competitor; lane 6, same as lane 2 with 20 ng of Sp1 consensus recognition sequence as cold competitor

previously confirmed (Sakai *et al.*, 1991b). The same result was also confirmed with CV-1 nuclear extract (data not shown). Sp1 is known to bind to the methylated Sp1 recognition sequence (GGGCGG) (Harrington *et al.*, 1988; Höller *et al.*, 1988), which is similar but not identical to the RBF-1 recognition sequence (Sakai *et al.*, 1991b). We also confirmed that purified Sp1 protein bound to both methylated and unmethylated consensus Sp1 oligonucleotides with the same affinity (data not shown).

Discussion

Widespread DNA hypomethylation in tumor cells has been frequently detected (Feinberg & Vogelstein, 1983a; Goelz *et al.*, 1985; Jones, 1986; Jones & Buckley, 1990). It is thought that the undermethylation results in an increase in transcription of certain oncogenes such as *ras* (Feinberg & Vogelstein, 1983b) and c-*myc* (Cheah *et al.*, 1984). Interestingly however, Baylin's group (de Bustros *et al.*, 1988) found regional DNA hypermethylation on the short arm of chromosome 11 in certain neoplastic cells. They hypothesized that human tumor-suppressor genes are inactivated by hypermethylation, because chromosome 11 is known to harbor tumor-suppressor genes (de Bustros *et al.*, 1988; Baylin *et al.*, 1991). Moreover recently, in the DNAs from human colon and lung cancer cells, non-random regional hypermethylation on chromosome 17p13 and 3p has been also detected from the early stage of cancer (Makos *et al.*, 1992). Furthermore, loss of heterozygosity was frequently observed on the same region. Makos *et al.* (1992) suggested that the hypermethylation would destabilize the chromosome and might lead to the allelic loss.

We have previously reported five sporadic retinoblastoma tumors in which the 5′ region of the Rb gene was hypermethylated but in which we found no other abnormalities (Sakai *et al.*, 1991a). Four out of the five tumors revealed loss of heterozygosity in the Rb gene, while the fifth tumor retained heterozygosity with allele-specific hypermethylation (Sakai *et al.*, 1991a). Our results are consistent with the following mechanism, if the allelic loss was associated with the hypermethylation as Baylin's group suggested. In our four tumors, both alleles of the Rb gene were initially hypermethylated. Sequentially, one of the alleles was lost and the remaining allele was inactivated by hypermethylation in the promoter region, as we have shown in this study. In one case allele-specific hypermethylation without allelic loss was revealed, suggesting that hypermethylation may have only inactivated the methylated allele, but not led to the allelic loss.

We have shown that methylation of the Rb promoter results in binding inhibition of both an ATF-like and the RBF-1 transcription factors. Given that mutation in the ATF-like and the RBF-1 binding sites reduces Rb promoter activity (Sakai *et al.*, 1991b), it is likely that specific methylation of these sequences also directly reduces Rb promoter activity. In this report, we have shown that full methylation can reduce Rb promoter activity to a level that could result in oncogenesis. The activity of the Rb promoter with full methylation was only 8% of the control, which is equivalent to the activity of the promoter with specific mutatations from the DNA of Rb patients (Sakai *et al.*, 1991b). It is not clear whether 24% of Rb promoter activity reduced by specific FnuDII methylation could contribute to carcinogenesis. This fact that CpG methyation inactivates Rb promtoer activity is consistent with the concept that human tumor-suppressor genes can be inactivated by certain epigenetic changes, thereby contributing to the pathogenesis of cancer (Greger *et al.*, 1989; Baylin *et al.*, 1991; Sakai *et al.*, 1991a). Our results also suggest that hypermethylation can be a cause of inactivation of the Rb gene, not a consequence.

Expression of certain genes, especially housekeeping genes, is thought not be inactivated when 5′ promoter regions are hypermethylated (Harrington *et al.*, 1988; Höller *et al.*, 1988). Although the Rb gene is considered a housekeeping gene, in this report we have shown that CpG methylation in the Rb promoter region abolishes Rb promoter activity. Thus, in the case where the Rb gene is inactivated only by hypermethylation, treatment of the tumor with chemotherapeutic agents that interfere with methylation of DNA should be possible.

Materials and methods

Plasmid preparation

A plasmid in which the promoter of the Rb gene is fused to the luciferase expression vector (pXRP1) (Sakai *et al.*, 1991b), digested with BamHI and XhoI, results in two fragments (680 bases and 6.5 kb) that were gel purified. The shorter fragment, containing the promoter region of the Rb gene, was methylated by HpaII methylase, FnuDII methylase or CpG methylase. Each of the specifically methylated DNA fragments (0.5 μg) was ligated to 2.5 μg of the larger fragment containing the luciferase reporter gene for 20 h at room temperature. After ensuring complete ligation by gel analysis, the ligation mix was phenol–chloroform extracted, chloroform extracted and ethanol precipitated. The purified ligated DNA was demonstrated to contain only closed circular plasmids by gel electrophoresis. The plasmid (0.5 μg) was used for each transfection.

Transient gene expression and luciferase assay

B104 cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Plasmid DNA (0.5 μg) was transfected into 2×10^5 B104 cells in a 6-cm-diameter dish containing 20 μl of 2 mg ml^{-1} DEAE–dextran. Forty-eight hours after transfection, luciferase activity was measured by a luminometer as described previously (Sakai *et al.*, 1991b).

Gel shift assays

Synthetic oligonucleotides were used for gel-shift assays. To generate the cytosine-methylated oligonucleotides, 5-methylcytidine was used for the syntheses, except for synthesis of the RBF-1-Met2 oligonucleotide. To generate RBF-1-Met2, RBF-1-WT was methylated by FnuDII methylase. All the oligonucleotides have flanking partial HindIII and SalI recognition sequences. Nuclear extract was prepared as described by Dignam *et al.* (1983). The gel-shift assay was carried out according to a modification of the procedure described by Jackson *et al.* (1990). Radiolabeled double-stranded DNA was made by annealing complementary oligomers with 5′ overhangs and then filling in the recessed 3′ ends with ^{32}P-labeled nucleotides using the Klenow fragment of DNA polymerase.

Acknowledgements

We thank Drs T.P. Dryja and K. Kawai for their continuous encouragement. We also thank Dr Stephen B. Baylin and his colleagues for their critical reading, useful suggestions and encouragement. We also thank Drs H. Nishino and A. Iwashima for the use of a luminometer, Dr J. Horowitz for his gift of the B104 cell line, Dr K. Hayashi for his advice about statistical analysis and Dr Y. Tsujimoto for his useful advice. This work was supported in part by the Ciba-Geigy Foundation for the Promotion of Science (T.S.), the Smoking Research Foundation (T.S.) and the Ministry of Education, Science and Culture of Japan (T.S.).

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