

Constitutively Methylated CpG Dinucleotides as Mutation Hot Spots in the Retinoblastoma Gene (RB1)

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Summary

A wide spectrum of mutations, ranging from point mutations to large deletions, have been described in the retinoblastoma gene (RB1). Mutations have been found throughout the gene; however, these genetic alterations do not appear to be homogeneously distributed. In particular, a significant proportion of disease-causing mutations result in the premature termination of protein synthesis, and the majority of these mutations occur as C→T transitions at CpG dinucleotides (CpGs). Such recurrent CpG mutations, including those found in RB1, are likely the result of the deamination of 5-methylcytosine within these CpGs. In the present study, we used the sodium-bisulfite conversion method to detect cytosine methylation in representative exons of RB1. We analyzed DNA from a variety of tissues and specifically targeted CGA codons in RB1, where recurrent premature termination mutations have been reported. We found that DNA methylation within RB1 exons 8, 14, 25, and 27 appeared to be restricted to CpGs, including six CGA codons. Other codons containing methylated cytosines have not been reported to be mutated. Therefore, disease-causing mutations at CpGs in RB1 appear to be determined by several factors, including the constitutive presence of DNA methylation at cytosines within CpGs, the specific codon within which the methylated cytosine is located, and the particular region of the gene within which that codon resides.

Introduction

The methylation of mammalian DNA occurs at cytosine residues within CpG dinucleotides (CpGs). CpGs are distributed unevenly in the genome, and there is considerable evidence for the involvement of CpG methylation in the regulation of gene expression (Bird 1987; Razin

and Cedar 1991; Laird and Jaenisch 1994) and in the process of mutagenesis (Cooper and Krawczak 1989; Magewu and Jones 1994; Tornaletti and Pfeifer 1995; Andrews et al. 1996). The presence of DNA methylation at specific transcription-factor binding motifs within gene regulatory regions is correlated with gene silencing and contributes to temporal and tissue-specific patterns of gene expression (Holler et al. 1988; Iguchi-Ariga and Schaffner 1989; Comb and Goodman 1990). In contrast, methylation within gene coding regions generally is not associated with the regulation of gene expression. Rather, DNA methylation of exonic CpGs is potentially a major contributor of point mutations leading to human genetic disease. One-third of germ-line point mutations leading to human genetic diseases occur at CpGs; most of these mutations are C→T transitions (Cooper and Youssoufian 1988; Magewu and Jones 1994; Tornaletti and Pfeifer 1995). This is because 5-methylcytosine (m5C) is highly mutable by deamination, resulting in transitional mutations (i.e., C→T) at CpGs. In view of the symmetry of these CpG motifs, the methylcytosine on the opposite strand also may be affected, leading to G→A changes. As a consequence, CpGs are hot spots for mutations, in a variety of genes (Cooper and Krawczak 1989; Tasheva and Roufa 1993; Ory et al. 1994; Tornaletti and Pfeifer 1995; Rodenhiser et al. 1996, 1997).

Retinoblastoma is a childhood tumor of the eye that results from mutations inactivating both alleles of RB1. A wide spectrum of mutations, ranging from point mutations to large deletions, have been described in this gene (Canning and Dryja 1989; Cowell et al. 1994; Lohmann et al. 1996; Van Orsouw et al. 1996). These mutations have been found throughout the gene; however, such genetic alterations do not appear to be homogeneously distributed. In retinoblastoma tumors, the most common point mutation found in RB1 is a C→T transition, most of which occur at CGA (arginine) codons and result in premature termination. In fact, in hereditary cases of retinoblastoma, 10%–15% of all mutations occur at the CGA codons found in exons 8 and 17 (Cowell et al. 1994). However, the mechanisms responsible for RB1 mutations at these motifs are unclear.

We assessed the contribution of methylation in generating the recurrent germ-line mutations found within the CGA codons of RB1, in order to determine whether

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selective methylation of particular codons could account for the mutability of CGA codons in particular regions of RB1. We analyzed tissue- and allele-specific DNA methylation in RB1, using the sodium-bisulfite conversion method (Clark et al. 1994; Andrews et al. 1996), and we established a precise map of DNA methylation at all cytosine residues in four exons (8, 14, 25, and 27). This includes the sites of recurrent mutations in exon 8 (codons 251 and 255) and in exon 14 (codon 445). We determined that, in all four exons examined, methylation was restricted to CpGs and included cytosines that were involved in recurrent mutations, as well as those that were not reported to be mutated. We did not identify any site-specific methylation that could account for differences in the occurrence or retention of mutations at CpGs. Our data suggest that methylation is an important contributor to, but not the only factor responsible for, transition mutations in RB1.

Material and Methods

DNA Isolation and Modification

Total genomic DNA was isolated (Sambrook et al. 1989) from normal human-tissue samples (muscle, placental, and retinal) and from tumor-tissue samples (breast carcinoma), as well as from cultured fibroblasts. Lymphocyte DNA was isolated from healthy blood donors. To determine the methylation status of cytosines within RB1, DNA was modified by use of the sodium-bisulfite protocol (Frommer et al. 1992; Clark et al. 1994; Andrews et al. 1996; Rodenhiser et al. 1996). In brief, 10 μ g of genomic DNA was digested with *Eco*RI at 37°C for 6 h and subsequently was denatured with 1 M fresh NaOH (final concentration of 0.3 M) at 37°C for 15 min. The denatured template was treated with 3.6 M NaHSO₃ (pH 5) and 100 mM hydroquinone (to a final concentration of 3.1 M and 0.5 mM, respectively) and was incubated under mineral oil, at 55°C for 16 h. This incubation included 5-min soak periods at 94°C every 3 h, to ensure that the template remained denatured (Tasheva and Roufa 1993; Clark et al. 1994). Following this treatment, the template was desalted with the Wizard DNA-Cleanup System (Promega) and was treated with 1 M NaOH (at 37°C for 15 min) to remove the remaining sodium bisulfite. The DNA was precipitated with 6 M ammonium acetate (pH 7) and with 2 volumes of 95% ethanol; then the pellet was washed in 75% ethanol and was dissolved in 50 μ l double-distilled H₂O.

PCR

In the subsequent PCR amplifications, the following specific primers were constructed, to amplify the sense strand of the bisulfite-converted genomic DNA: exon 8—forward, 5'-GTTAGAATATTTTATTATTTTATA-

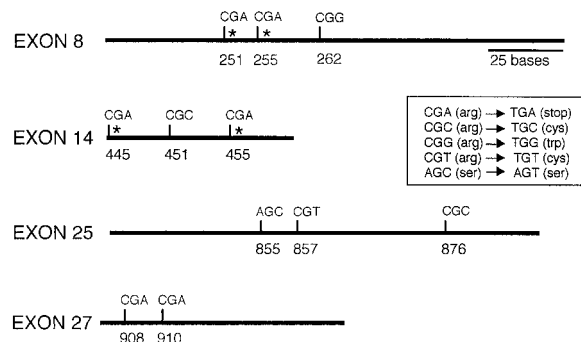


Figure 1 RB1 exons evaluated in this study. CpGs and sites of recurrent mutation (indicated by an asterisk [*]), in the RB1 coding region, are depicted. *Inset*, Representation of changes to codon identities, as a result of C→T transition mutations.

TGATGG-3', and reverse, 5'-AATATTTTACTACTA-CAAAAAAATTAACAC-3'; exon 14—forward, 5'-TGAGTTTtaggAGTGTGAAGGTTAGTTTGGG-3', and reverse, 5'-AACCAAATAATCTTAATACCTTA-ACCTCC-3'; exon 25—forward, 5'-TTTTGATAT-ATTTTAAATTATAATTTGAGG-3', and reverse, 5'-CAAAAACAATACTAAACTCTAAATTCCCC-3'; and exon 27—forward, 5'-ATTAGTTTGATATGAGTATAATATATATGG-3', and reverse, 5'-CTAACATTTCAAATAACTTAAAAATCACCC-3'. These primers amplified RB1 exon fragments of 348 bp, 306 bp, 309 bp, and 350 bp, which included all or most of each of these RB1 exons. PCR was performed in 25- μ l reactions containing 50–150 ng bisulfite-treated genomic DNA. The amplification conditions were 5 cycles at 94°C (1 min), 60°C (2 min), and 72°C (3 min), followed by 30 cycles at 94°C (1 min), 60°C (2 min), and 72°C (1 min). The PCR reactions were “hot started” by adding 1 μ l of the reverse primer to each of the individual samples, during an initial 94°C incubation period. A final 16 min soak (at 72°C) also was performed.

Cloning of PCR Product

PCR product was cloned into the PCRII-TA cloning vector (TA Cloning Kit, Invitrogen). Individual positive clones were identified, and plasmid DNA was isolated by alkaline lysis (Sambrook et al. 1989) and was sequenced with the T7 sequencing system (Pharmacia) by use of the forward primer (Andrews et al. 1996). For each exon, a minimum of four different types of tissue were examined, and at least 10 clones were sequenced for each type of tissue, to determine the methylation status of all cytosine residues within the region analyzed. CpG-dinucleotide motifs were found to be present at codons 251, 255, and 262 (exon 8); at codons 445, 451, and 455 (exon 14); at codons 855, 857, and 876 (exon 25); and at codons 908 and 910 (exon 27) (fig. 1). For each type of tissue, bisulfite-converted template was com-

pared with unconverted genomic DNA, to allow the methylation status of all cytosines to be determined. The methylation status of each of these CpGs was determined for every clone in all tissue samples. Unmethylated cytosines were characterized as those bands that were absent in the C lane but that were present in the T lane, of the reactions from the converted template. Any bands remaining in the C lane of the sequenced converted DNA would indicate methylated cytosines, which subsequently were amplified as cytosines (Clark et al. 1994; Andrews et al. 1996; Rodenhiser et al. 1996).

Results

In the present study, we used a bisulfite sequencing method to evaluate DNA methylation at cytosines within CpGs in RB1. In this method, the difference between cytosines and m5C is identified by differences in their reactivity to sodium bisulfite. This highly specific chemical reaction permits the methylation status to be determined for each cytosine residue on individual DNA strands that have been amplified from the target template. Total genomic DNA is denatured and then treated with sodium bisulfite, causing the deamination of cytosines and creating uracil residues. Any m5Cs remain unreacted. PCR amplification of the treated genomic DNA yields a fragment of interest in which all the uracil (formally the unmethylated cytosine) residues are amplified as thymine, whereas only the m5C residues are amplified as cytosines. The amplified, converted DNA fragments then can be cloned and sequenced, and the methylation status of all the CpGs within the DNA fragment can be established. The results can be used to evaluate tissue-specific or allele-specific DNA methylation at mutational hot spots.

We found that all CpGs within exons 8, 14, 25, and 27 of RB1 were consistently methylated, with a few

exceptions. Methylation at sites other than a CpG was observed rarely. The methylation status of CpNpG trinucleotides also was assessed, in view of the capacity of the methylating machinery in mammals to modify CpNpG sites through maintenance and de novo methylation pathways (Clark et al. 1995). However, no cytosines within CpNpG motifs in these RB1 exons were found to be methylated.

Cumulative data of methylation status for all cytosines contained within each of these RB1 exons is presented in table 1. First, in exon 8 of RB1, we evaluated the methylation status of three CpGs and of 48 non-CpG cytosines, for each clone sequenced (fig. 2). In total, we analyzed 162 CpGs and 2,592 non-CpGs, within five different tissue types (breast carcinoma, fibroblast, lymphocyte, muscle, and retinal). By comparing the T and C lanes of the converted DNA sequences with those of the genomic DNA sequences, we found that 93% of the CpGs contained within the exon 8 clones were methylated. None of the non-CpG cytosines were found to be methylated.

Similar results were found for exon 14 of RB1 (fig. 3). For each clone, we analyzed 3 CpGs and 43 non-CpG cytosines. We found that most (95%) of the 123 CpGs analyzed were methylated, in each of the four types of tissue (fibroblast, lymphocyte, muscle, and retinal) analyzed. The remaining 1,763 non-CpGs were consistently unmethylated. Furthermore, the region under investigation also included a portion of intron 14, which contained four additional CpGs. These cytosines also were found to be consistently methylated (>95%).

In exon 27 (fig. 4), two CpGs are present. Of the 110 CpGs analyzed for this exon, 94% were found to be methylated, in the five types of tissue (fibroblast, lymphocyte, muscle, placental, and retinal) examined. All 3,850 non-CpG cytosines were determined to be unmethylated.

Table 1

Frequency of Methylated CpGs in RB1

	EXON 8 [n = 54]			EXON 14 [n = 41]			EXON 25 [n = 82]			EXON 27 [n = 55]	
	CGA	CGA	CGG	CGA	CGC	CGA	GCG	CGT	CGC	CGA	CGA
Position	251	255	262	445	451	455	855	857	876	908	910
No. (%) of methylated CpGs	48 (89%)	52 (96%)	51 (94%)	38 (93%)	40 (98%)	39 (95%)	59 (72%)	55 (67%)	72 (88%)	51 (93%)	52 (95%)

NOTE.—Cumulative data of methylation status of cytosines and CpGs in RB1 exons 8, 14, 25, and 27. PCR product was amplified from the bisulfite-converted DNA and was cloned into the PCRII-TA cloning vector, and individual positive clones were identified and were sequenced. The DNA analyzed was from the following tissues: retinal (exons 8, 14, 25, and 27), fibroblast (exons 8, 14, 25, and 27), lymphocyte (exons 8, 14, 25, and 27), breast carcinoma (exon 8), muscle (exons 8, 14, 25, and 27), and placental (exons 25 and 27). The number and frequency of methylated CpGs and non-CpG cytosines are presented for each CpG within each exon. DNA methylation appeared to be consistent throughout RB1 exons 8, 14, and 27 (>90%). However, within exon 25, DNA methylation was less prevalent, with the rates of methylation averaging 76%. Within muscle tissue in particular, only 50% (21/42) of the CpGs analyzed in exon 25 were found to be methylated.

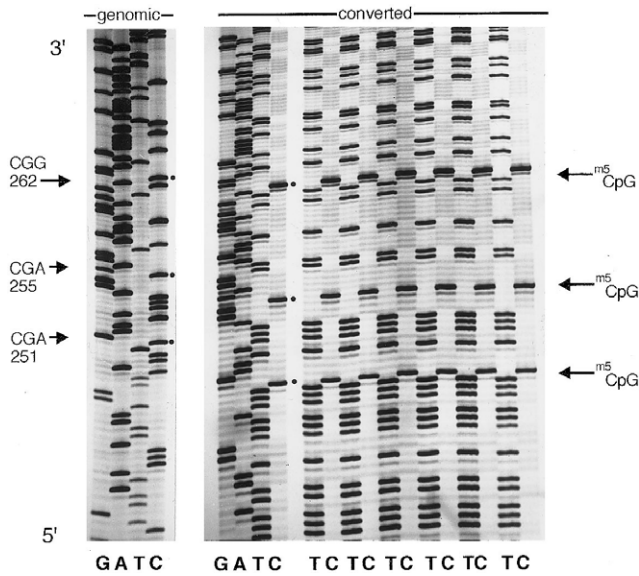


Figure 2 Bisulfite-converted template compared with unconverted genomic DNA, to allow for the determination of the methylation status of all cytosines. Unmethylated cytosines are characterized as those bands that were absent in the C lane but that were present in the T lane, of the reactions from the converted template. Bands remaining in the C lane of the sequenced converted DNA indicate methylated cytosines, which subsequently were amplified as cytosines. Results of DNA sequencing of genomic DNA (*left*) and of cloned PCR product from exon 8 of RB1 (*right*), following sodium-bisulfite conversion, are shown. The PCR primer pair used in this experiment amplified a 348-bp fragment that included exon 8 of RB1. The locations of CpGs are indicated in the genomic sequence and in the converted sequence. The occurrence of DNA methylation at these CpGs is indicated (“m⁵”). The CpGs identified in this figure occur within codons 251, 255, and 262 of RB1. DNA methylation was restricted to the cytosines in these CpGs and was found in all tissues analyzed.

In exon 25 (fig. 5), we found that the methylation status of the three cytosines at CpGs within each clone was significantly more variable than in the other three exons. We assessed methylation in a total of 246 CpGs and 4,264 non-CpG cytosines, within five different types of tissue (fibroblast, placental, muscle, retinal, and lymphocyte). The most consistently methylated CpG of the three was found in codon 876, which was methylated 88% of the time. The other two CpGs, in codons 855 and 857, were methylated an average of 72% and 67% of the time, respectively. It was apparent that the rate of methylation dropped significantly for exon 25, as compared with that for the other exons, most notably those in the muscle clones. In contrast to the data previously collected for the exons of RB1 and of other genes (NF1 [Tornaletti and Pfeifer 1995], p53 [Andrews et al. 1996], and BRCA1 [Rodenhiser et al. 1996]) in which exonic CpGs are predominantly methylated, we have demonstrated that, in muscle clones, for exon 25 only ~50% of the CpGs were methylated. More specifically, codons 876, 855, and 857 were methylated in muscle

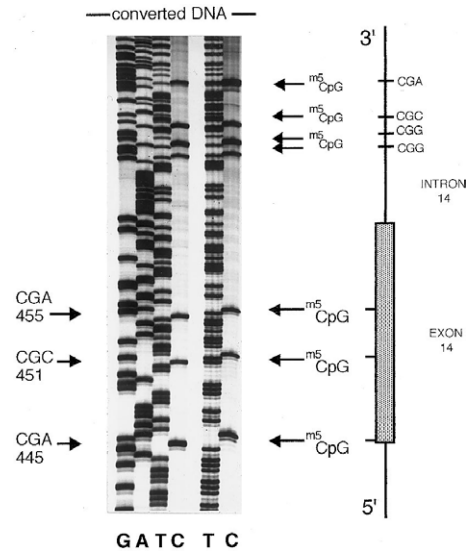


Figure 3 Results of DNA sequencing of cloned PCR product from exon 14 of RB1, following sodium-bisulfite conversion. The PCR primer pair used in this experiment amplified a 306-bp fragment that included exon 14 of RB1 and an intronic sequence flanking this exon. The locations of CpGs are indicated on the converted sequence. The occurrence of DNA methylation at these CpGs is indicated (“m⁵”). The CpGs identified in this figure occur within codons 445, 451, and 455 of RB1. DNA methylation was restricted to the cytosines in these CpGs and was found in all tissues analyzed. DNA methylation also was detected at five CpGs within intron 14, showing that methylation extends throughout the RB1 region.

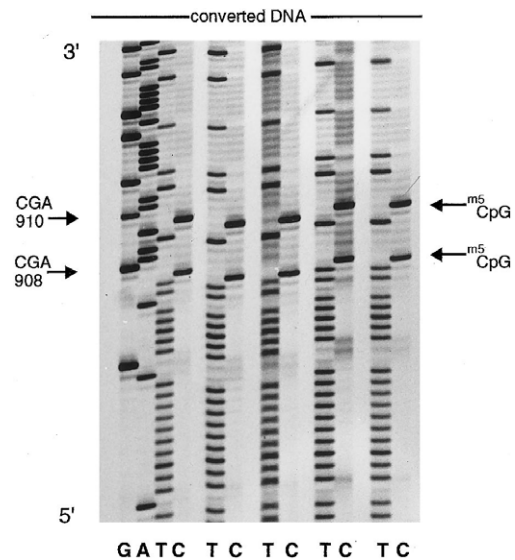


Figure 4 DNA sequencing of cloned PCR product from exon 27 of RB1, following sodium-bisulfite conversion. The PCR primer pair used in this experiment amplified a 350-bp fragment that included exon 27 of RB1. The locations of CpGs are indicated on the converted sequence. The occurrence of DNA methylation at these CpGs is indicated (“m⁵”). The CpGs identified in this figure occur within codons 908 and 910 of RB1. DNA methylation was restricted to the cytosines in these CpGs and was found in all tissues analyzed.

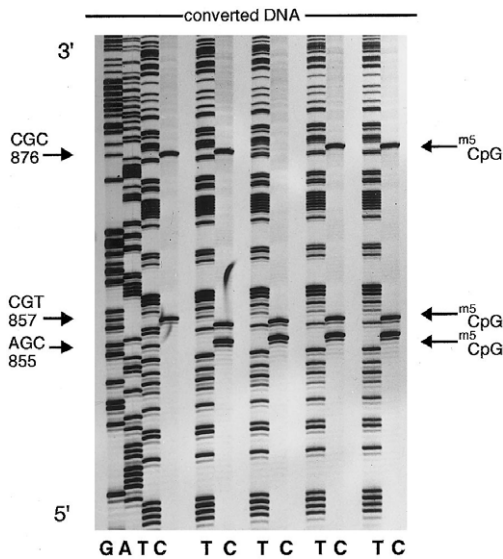


Figure 5 DNA sequencing of cloned PCR product from exon 25 of RB1, following sodium-bisulfite conversion. The PCR primer pair used in this experiment amplified a 309-bp fragment that included exon 25 of RB1. The locations of CpGs are indicated on the converted sequence. The occurrence of DNA methylation at these CpGs is indicated (“m5”). The CpGs identified in this figure occur within codons 855, 857, and 876 of RB1. DNA methylation was restricted to the cytosines in these CpGs and was found in all tissues analyzed. However, methylation at these sites was less consistent than that in other tissues.

only 36%, 43%, and 71% of the time, respectively. Although the rates of methylation in this particular tissue were comparatively low for all the exons analyzed, this general phenomenon of relative hypomethylation seems to have been restricted to exon 25.

Discussion

Approximately 35% of mutations associated with human genetic diseases are known to occur at CpGs (Cooper and Youssoufian 1988; Magewu and Jones 1994; Tornaletti and Pfeifer 1995). The hypermutability of these sites is thought to be related to the methylation status of the cytosines found in CG motifs and is attributed to the spontaneous deamination of m5C (Cooper and Krawczak 1989; Laird and Jaenisch 1994). Given that CpGs are underrepresented ~5-fold and that m5C represents <1% of all nucleotides, in the mammalian genome (Laird and Jaenisch 1994), the proportion of mutations attributed to CpGs is quite significant. In fact, the incidence of transition mutations is thought to be 20 × higher at CpGs than at non-CpG sites (Tornaletti and Pfeifer 1995). Lohmann et al. (1996) recently identified recurrent mutations at most of the 14 CGA codons in RB1, with the exception of exons 25–27, despite the presence of two CGA codons within this 3' region of

RB1. They demonstrated an overrepresentation (~19-fold) of transitions at CpGs, in RB1 germ-line mutations, and determined that 69% of the single-base substitutions found in 119 patients occurred at CGA codons and led to premature terminations. This group of researchers reported that no mutations were found at the CGA codon in exon 1 of RB1. Since this codon is found within the unmethylated CpG island, it was suggested that it is precisely this absence of methylation that correlates to the lack of mutations found in this exon (Greger et al. 1989; Lohmann et al. 1996).

We determined that the methylation status of exonic CpGs in RB1 indeed may contribute to the hypermutability of these sites. We have established a precise map of DNA methylation at all cytosine residues in four exons (8, 14, 25, and 27) of RB1. This includes the sites of recurrent mutations in exon 8 (codons 251 and 255) and in exon 14 (codon 445) (Cowell et al. 1994; Blanquet et al. 1995; Lohmann et al. 1996). By also analyzing the methylation status of cytosines found within exons 25–27, we attempted to determine whether the lack of mutations associated with this region can be attributed to an absence of methylation within these exons. We observed that exonic CpGs in RB1 appear to be consistently methylated, including those CpGs at which mutations have not been described. In at least one exon (exon 14), methylation also was apparent at the intronic CpGs that were examined.

We also determined that all the examined CpGs that are associated with recurrent mutations are found within CGA codons. Both exons 8 and 14 contain three CpGs (fig. 1). For each exon, two of these CpGs are located within CGA codons, and the remaining CpGs are located within CGG and CGC codons. However, only those cytosines found within the CGA codons are associated with recurrent mutations in retinoblastoma patients. It would appear that, although the majority of exonic CpGs are consistently methylated, not all of these cytosines are associated with mutations and that the hypermutability of CpGs cannot be attributed to methylation status alone. The mutability of particular cytosines also seems to be dependent on the particular amino acid changes resulting from C→T or G→A transition mutations. In the case of RB1, such changes at CGA codons are particularly significant, since these transitions lead to the formation of TGA codons, resulting in premature termination of the protein product.

Similar observations have been made by Tornaletti and Pfeifer (1995), who evaluated the contribution of methylation to mutagenesis in the p53 tumor-suppressor gene. In p53, five of six mutational hot spots are found at CpGs, and each of these cytosines were methylated in all the tissues that were examined. These data for the p53 gene, as well as data from previous work from our own lab, on DNA methylation within the NF1 and

BRCA1 genes (Andrews et al. 1996; Rodenhiser et al. 1996, 1997), lead us to conclude that the occurrence of mutational hot spots is not the result of selective methylation at a subset of CpGs. Rather, other factors modulate the mutagenicity of methylated CpGs in the coding regions of these tumor-suppressor genes. These factors may cause regional differences in the degree of methylation at specific regions of the coding sequence, such as those we have observed in exon 25 of RB1.

Therefore, at least two features contribute to mutations at CpGs in RB1—(1) the constitutive presence of DNA methylation at cytosines within CpGs and (2) the specific codon within which that methylated cytosine is located (Ory et al. 1994; Tornaletti and Pfeifer 1995). We feel that it is particularly relevant that these recurrent mutations at CGA codons in RB1, p53, and NF1 lead to premature-termination mutations, and we suggest that the clinical disease phenotype selects for mutations at this codon. These mutations no doubt have a profound effect on the expression of these tumor-suppressor genes, since the inactivation of such genes likely confers a proliferative advantage resulting from the release of growth constraints normally enforced by these genes. Therefore, it may be beneficial for the individual cell to maintain such mutations in order to outcompete surrounding cells, despite the obvious detriment to the whole organism. In this way, any individual tumor cell that has been able to restore at least one RB1 allele, by the removal or prevention of methylation, would be overtaken nonetheless by those cells that lack any restrictions on proliferation (Sakai et al. 1991; Greger et al. 1994).

Limited differences in the specificity of methylation found within the various regions of RB1 also are apparent. The relative hypomethylation demonstrated within all the tissues analyzed for exon 25, as compared with the incidence of methylation found in exons 8, 14, and 27, may suggest that this is why no methylation-mediated mutations have been associated with the exon 25 region of RB1. However, exon 27 (also devoid of known mutations) has been shown to possess rates of methylation comparable to that of the other two exons (8 and 14) analyzed, within which recurrent mutations have been reported. Lohmann et al. (1996) have suggested that mutations at the 3'-terminal region of RB1 may not be oncogenic. A distinguishing feature of exon 25, however, is that it contains a nuclear-localization signal (NLS), to direct the protein product of RB1 (p110RB1) through the nuclear membrane into the nucleus (Zacksenhaus et al. 1993). The 3' end of this consensus sequence for the NLS motif contains an arginine codon (CGC at position 876), which was one of the CpGs under analysis in this study and which was found to be consistently methylated. It is possible that this NLS is controlled partially by methylation status and is regu-

lated in a cell-cycle-specific manner, much like phosphorylation differentiates between active and inactive forms of the RB1 protein product. Further studies are required to determine whether cell-stage-specific methylation indeed does occur.

Our group and others have established that CpGs in the coding regions of many genes are consistently methylated (Tornaletti and Pfeifer 1995; Andrews et al. 1996; Rodenhiser et al. 1996). In view of the propensity of methylated cytosines to be deaminated, leading to the formation of mutations, as well as the expense of maintaining the methylating machinery, what is the possible benefit of coding-region methylation to the mammalian genome? Certainly the functional importance of methylation must outweigh its apparent deleterious effects. DNA methylation is thought to play a role both in the regulation of gene expression and in the maintenance of the structural integrity and organization of chromatin (Antequera et al. 1990). Several studies have suggested a role for methylation in maintaining a heterochromatinized state. Methylation may play a role in the formation of an inactive chromatin structure, which inhibits access to transcription factors and/or particular nucleases, such as DNase I and *MspI*. This inactivated state is thought to involve the binding of proteins specific to methylated DNA. One group of proteins, known as "methyl-CpG binding proteins" ("MeCP 1" and "MeCP 2"), have been demonstrated to be associated with centromeric heterochromatin (Nan et al. 1996; Tate et al. 1996). Another group of proteins, the methylated DNA binding proteins, have been shown to possess some sequence homology with histone H1. Several studies suggest that histone H1 preferentially binds to CpG-methylated DNA (McArthur and Thomas 1996).

Methylation also may serve as a protective measure to preserve the integrity of the mammalian genome, against foreign (viral) invasion or against attack by nucleases (Barlow 1993; Keshet et al. 1986). In the promoter regions of genes, hypomethylation permits the transcription of genes, through increased access of transcription factors to their requisite DNA-sequence elements. However, this same lack of methylation, when present within gene-coding regions, has been associated with genomic instability, leading to deletion mutations. These events may occur when hypomethylation leads to an increased accessibility of sequences to DNA-damaging agents (such as DNase I) (Keshet et al. 1986), as well as an increased accessibility to oxidants and/or enzyme-induced DNA-strand breakage (Leteurtre et al. 1994; Pogribny et al. 1995). Furthermore, hypomethylation may decrease binding sites for methyl-specific binding proteins (Pogribny et al. 1995) or may alter DNA-histone interactions. In fact, deletions have been detected in the regions surrounding exon 25 of RB1 (i.e., in exons 23 and 24 and in intron 24) (Hogg et al. 1993; Henson et

al. 1994; Kato et al. 1994; Lohmann et al. 1994). Further work is required to determine whether the apparent relative decrease in constitutive methylation that we have described, in this region of RB1, is related to specific deletion events in RB1.

Although the methylation status of specific CpGs within particular codons appears to vary slightly among clones, we have found that, for each exon, one CpG site tends to be more consistently methylated across all clones than the others. Magewu and Jones (1994) have shown that in p53, despite extensive treatment, *in vitro*, with 5-aza-2'-deoxycytidine, some CpG sites (including, in this instance, codon 248) could not be demethylated below a level of 80%. They therefore suggest that these resistant sites are part of a so-called methylation center that serves as an initiation point for the spread of methylation to nearby CpGs (Magewu and Jones 1994). Such sites of consistent methylation also could serve as targets for methyl-binding proteins and thereby could ensure proper chromatin packaging (Graessman and Graessman 1993; Weng et al. 1995).

In conclusion, we have shown that methylation is an important contributor to the manifestation of mutations within RB1. However, methylation is not an exclusive determinant of recurrent DNA lesions. Although we detected limited differences in methylation patterns in exon 25 of RB1, we have not identified any site-specific methylation of particular codons, which may account for differences in the occurrence or the retention of mutations at cytosines across RB1 or even within the same exon. Rather, deamination of cytosines contained within CGA codons (leading to a truncated protein product) seems to be a critical target for mutagenesis. Disease-causing mutations at CpGs in RB1 therefore appear to be determined by several factors, including the constitutive presence of DNA methylation at cytosines within CpGs, the specific codon within which the methylated cytosine is located, and the specific region of the gene within which the particular RB1 codon resides. Nonetheless, elucidation of the role of DNA methylation in mutagenesis, in the regulation of gene expression, and in the maintenance of chromatin structure will clarify how epigenetic alterations in tumor-suppressor genes lead to carcinogenesis.

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