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Methylation of CpG Loci in 5′-Flanking Region Alters Steady-State Expression of Adenomatous Polyposis Coli Gene in Colon Cancer Cell Lines

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Abstract The APC genetic locus has been linked to the tumorigenesis and progression of colorectal cancer, although the precise mechanism of its involvement in this disease remains unknown. We used high sensitivity mapping of the methylated cytosine, Northern blot analysis and immunocytochemical staining in six colorectal cancer cell lines (DLD-1, SW480, Colo320, HT29, WiDr, and Colo201) to examine the relationship between the methylation status of the CpG loci in the 5′-flanking region of the APC gene and its expression. APC mRNA expression levels determined by Northern blot analysis correlated well with APC protein levels visualized by immunocytochemistry. In these colorectal cancer cell lines, no major genetic alterations of the APC gene, such as amplification or deletion, were detected. Analysis of the epigenetic control of APC gene expression in these lines revealed that methylation of the CpG loci in the 5′-untranslated region of APC mRNA repressed steady-state expression of the gene. Furthermore, epigenetic alteration of the APC gene was independent of the APC protein truncation and CpG methylation of the hMLH1 promoter. Although less eminent than protein truncation by point mutation within the coding region of the APC gene, epigenetic alteration suppressing APC gene expression may significantly contribute to oncogenesis and the progression of colorectal cancer. J. Cell. Biochem. 80:415–423, 2001.

Key words: APC; hMLH1; methylation; bisulfite modification; MSP

The human adenomatous polyposis coli (APC) gene, located at 5q21 [Nishisho et al., 1991; Kinzler et al., 1991], encodes a large 300 kD protein that interacts with β-catenin [Munemitsu et al., 1995]. APC is mutated in the germline of patients with familial adenomatous polyposis (FAP). APC mutations are also present in more than 85% of sporadic (non-familial) colorectal cancer [Otori et al., 1998]. The close association of the mutations with the early stages of both familial and non-familial colorectal tumorigenesis makes APC a strong candidate for a tumor suppressor gene in colorectal cancer [Powell et al., 1992]. The APC gene product is part of a tetrameric complex, composed of conductin, a negative regulator of Wnt signaling, APC, GSK-3β (serine-threonine kinase glycogen synthase kinase 3β) and β-catenin. This complex regulates the level of β-catenin by direct binding [Behrens et al., 1998]. Thus, deranged functioning of APC is a crucial step leading to carcinogenesis and tumor progression.

Aberrant methylation of CpG loci within 5′-regulatory regions affects the interactions of DNA with chromatin proteins and transcription factors [Kitazawa et al., 1999; Kass et al., 1997; Bester, 1998] to facilitate tissue-specific expression of genes. The mechanism of gene-silencing by methylated cytosine, however, is varied among promoters. The most general mechanism is transcriptional repression by methyl-CpG-binding proteins (MeCP1 and MeCP2) that bind DNA in a sequence-independent manner [Nan et al., 1998; Jones and Laird 1999]. The binding of methyl-CpG-binding proteins results in alterations of chromatin structure, preventing transcription factors

Abbreviations used: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; GSK-3β, serine-threonine kinase glycogen synthase kinase 3β; Rb, retinoblastoma; VHL, von Hippel-Lindau.

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such as Sp1 from binding to DNA. Preceding tumor progression, aberrant methylation of CpG loci in the 5′-flanking region of tumor suppressor genes Rb (retinoblastoma) [Greger et al., 1994], $p15^{INK4b}$ [Herman et al., 1996a], $p16^{INK4a}$ [Herman et al., 1995], p53 [Greenblatt et al., 1994], BRCA1, E-Cadherin [Yoshiura et al., 1995], VHL (von Hippel-Lindau) [Herman et al., 1994] and hMLH1 [Fleisher et al., 1999] is associated with gene repression [Bird and Wolffe, 1999]. Methylation of the APC promoter region in colorectal cancer has also been reported although its relation to the expression level of the APC gene remains unknown [Tsuchiya et al., 2000].

To investigate the relationship between the CpG methylation status of the promoter and the expression of the APC gene in colon cancer cell lines, we employed the sodium bisulfite-modified genomic sequencing technique to map the methylation status of 31 CpG loci in a 603-bp promoter region of the APC gene.

MATERIALS AND METHODS

Culture and Maintenance of Cell Lines

The six human colon cancer cell lines, DLD-1, SW480, Colo320, Ht29, WiDr, and Colo201 were cultured in RPMI 1640 (ICN Biomedicals, Cleveland, OH) supplemented with 10% FBS (ICN Biomedicals), 100 U/ml penicillin and 100 μ/ml streptomycin (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO_2.

Immunocytochemical Staining

Exponentially growing floating cells (SW480, Colo320, and Colo201) were collected by centrifugation at 1500 rpm onto glass slides. Cells showing adherent phenotypes (DLD-1, Ht29, and WiDr) were cultured in a chamber slide (Nunc Inc., Naperville, IL). Cells were fixed in 100% methanol for 20 min at room temperature. Specimens were washed with PBS, treated with 3% hydrogen peroxide to block endogenous peroxidase activity, incubated with 2% non-fat dry milk, then incubated for 12 h at 4°C with either region-specific antibody Ab3, recognizing the N-terminal portion, or Ab4, recognizing the non-truncated C-terminal portion of the APC protein (Oncogene Research Products, Cambridge, MA). The sections were washed three times with PBS for 10 min each and incubated with biotin-labeled antirabbit IgG for 15 min at room temperature. After three washes with PBS for 10 min each, specimens were incubated at room temperature with 10 μg/ml peroxidase-conjugated streptavidin for 15 min, washed twice with PBS for a total of 20 min, and incubated with 0.2 mg/ml 3,3′-diaminobenzidine–4HCl, 0.1% hydrogen peroxide, 0.01% cobalt chloride (Sigma) and 0.01% $NiSO_4(NH_4)_2SO_4$ (Sigma) for 15 min. The colorimeric reaction was stopped with H_2O. Specimens were observed under a Hoffman Modulation contrast microscope without counterstaining. The intensity of the immunocytochemical staining was scored as strongly positive + + (more than 80% of cells were strongly immunoreactive); positive + (60–80% of cells were strongly positive); weakly positive ± (40–60% of cells were strongly immunoreactive); and negative − (less than 30% of cells were positive or of weak immunoreactivity).

Northern Blot Analysis

Total RNA, extracted from cells by RNAzol (Tel-Test, Inc., Friendswood, TX), was separated by denaturing electrophoresis in formaldehyde-agarose gels and transferred onto high bond N + nylon membranes (Amersham, UK). Full length APC cDNA (kindly provided by Dr. Akiyama, Institute of Molecular and Cellular Bioscience, Tokyo University), radioactively labeled by random primer technique, was used as a probe. Membranes were hybridized at 65°C for 16 h, washed twice in 2 × SSPE containing 0.1% SDS, twice in 1 × SSPE containing 0.1% SDS, and finally once in 0.1 × SSPE containing 0.1% SDS at 65°C. Blots were analyzed using image analyzer BAS-EWS 4075 (FUJIX, Tokyo, Japan). Relative expression was estimated by the optical density of the APC mRNA band normalized to the 28S ribosomal band stained with ethidium bromide.

RT-PCR (Reverse Transcription-PCR) to Detect Promoter Usage

One microgram of total RNA, isolated from each of the colon cancer cell lines, was reverse-transcribed to produce cDNA. cDNAs were amplified by 30 cycles of PCR according to the following regime; 95°C for 1 min, 53°C for 1 min, 72°C for 1 min. Primer sets for RT-PCR were for 5′ nontranslation region, 5′-GGAGACAGAATGGAGGTGC-3′ (NA), 5′-GCGAGCAGGAGCTGCGT-3′ (NB) and 5′-CAAGAATTCATATGAAGCTGCAGCCAT-3′ (APC5), located in exons 1A, 1B, and 2, respectively (Fig. 6). The

concentration of primer utilized was 1 μM for primers NA and NB, 3 μM for APC5.

Southern Blot Analysis

High-molecular-weight genomic DNA was isolated by standard protocol. Eight micrograms of each genomic DNA were digested for 24 h at 37°C with 12 U of *Eco*RI and 20 U of *Hin*dIII, electrophoresed and transferred onto high bond N + nylon membranes (Amersham). A full length APC cDNA was used as a probe. Membranes were hybridized at 65°C for 12 h and then washed twice in 2 × SSPE containing 0.1% SDS, twice in 1 × SSPE containing 0.1% SDS and finally once in 0.1 × SSPE containing 0.1% SDS at 65°C. The blots were then analyzed with an image analyzer BAS-EWS 4075 (FUJIX).

DNA Extraction and Bisulfite Modification

The bisulfite reaction was carried out as described [Frommer et al., 1992, Kitazawa et al., 2000]. Five micrograms of genomic DNA was denatured by NaOH (final concentration, 0.3 M) in a volume of 100 μl of TE. Sixty microliters of freshly prepared 10 mM hydroquinone and 1040 μl of 3 M sodium bisulfite (pH 5) were added. The samples were then incubated for 20 min at 37°C followed by 16 h at 50°C under mineral oil. Bisulfite-modified DNA was purified using Wizard DNA purification resin according to the manufacturer's recommended protocol (Promega, Madison, WI) and eluted into 100 μl of H_2O. Modification was completed by treatment with NaOH (final concentration, 0.3 M) for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in 20 μl of H_2O and stored at −20°C until PCR amplification.

PCR Amplification of Bisulfite-Modified Human APC Gene Promoter Region

This procedure was carried out as described [Hiltunen et al., 1997]. Bisulfite-modified DNA (100 ng) was subjected to a preliminary round of PCR followed by nested PCR using the following sets of primers:

5′-GTGTTGTAAAAATTATAGTAATC/TGAGATGTAA-3′: sense
5′-CAACTTTTCTCAACAAACTCCCCT-3′: antisense
5′-ATTATTTTTTTTTTTTATTTTC/TGGTATTTTG-3′: nested sense
5′-CTACCCCTCCCCCTTCTCCTTCTTCTTC-3′: nested antisense

The condition of the first PCR was as follows: 94°C for 2 min for denaturation, 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 90 s, with a final elongation step of 5 min at 72°C. The PCR mixture contained 1 × buffer (Takara, Kyoto, Japan) with 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs and 100 ng of bisulfite-modified DNA in a final volume of 50 μl. One microliter of the first PCR reaction was used for the template for the nested PCR. The conditions for the nested PCR were as follows: 94°C for 2 min for denaturation, 34 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 90 s, with a final elongation step of 5 min at 72°C. The PCR mixture contained 1 × buffer (Takara) with 1.5 mM $MgCl_2$, 5 pmol of each primer, 0.2 mM dNTPs in a final volume of 50 μl. Each PCR product was loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The nested PCR products were purified from agarose gels and subjected to the sequencing reaction by the dideoxy nucleotide termination method with a DNA sequencing kit (Perkin Elmer, Foster City, CA). Samples were analyzed on a 310 Genetic Analyzer (Perkin Elmer).

Methylation-Specific PCR (MSP) for APC Gene

The DNA methylation pattern in the CpG island of the APC gene was determined by treatment with sodium bisulfite and subsequent MSP as described [Herman et al., 1996b]. The primer sets were:

5′-TGTTTTGCGGATTTTTTTC-3′: sense, 1AMf
5′-GCAATAAAACACAAAACCCCG-3′: antisense, 1AMr for methylated DNA sequences of promoter 1A, (P1 in Fig. 6)
5′-GTGTTTTATTGTGGAGTGTGGGTT-3′: sense, 1AUf
5′-CCAATCAACAAACTCCCAACAA-3′: antisense, 1AUr for unmethylated DNA sequences of promoter 1A,
5′-TGTTTAGGTAGTAATGGTTTAC-3′: sense, 1BMf
5′-TAAAACCTATTATACGCAAACG-3′: antisense, 1BMr for methylated DNA sequences of promoter 1B, (P2 in Fig. 6)

5′-GGTTGTTTAGGTAGTAATGGTTTAT-3′: sense, 1BUf
5′-AAACTAAAACCTATTATACACAAACA-3′: antisense, 1BUr for unmethylated DNA sequences of promoter 1B.

Relative location of each primer is indicated by arrows in Figure 6. The condition of the MSP was as follows: 94°C for 2 min for denaturation, 7 cycles of 95°C for 30 s, 72°C for 30 s, and 25 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s, with a final elongation step of 5 min at 72°C. The PCR mixture contained 1 × buffer (Takara) with 1.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM dNTPs and 100 ng of bisulfite-modified DNA in a final volume of 25 μl. PCR products were analyzed by 3% agarose gel electrophoresis.

MSP for hMLH1 Gene

The primer sets were 5′-ACGTAGACGTTT-TATTAGGGTCGC-3′ (sense) and 5′-CCTCA-TCGTAACTACCCGCG-3′ (antisense) for methylated DNA sequences, and 5′-TTTTGA-TGTAGATGTTTTATTAGGGTTGT-3′ (sense) and 5′-ACCACCTCATCATAACTACCCACA-3′ (antisense) for unmethylated DNA sequences. hMLH1 MSP utilized the same conditions as those employed for APC with the exception of an annealing temperature of 63°C. PCR products were analyzed by 3% agarose gel electrophoresis.

RESULTS

Immunocytochemical Staining

Immunocytochemistry with Ab3, recognizing the N-terminal portion of the APC protein, strongly stained Colo320 (Fig. 1E) and WiDr (Fig. 1I) and weakly stained DLD-1 (Fig. 1A) and HT29 (Fig. 1G) (summarized in Table I). SW480 (Fig. 1C) and Colo201 (Fig. 1K) were not significantly labeled by Ab3. Staining with Ab4 for the non-truncated C-terminal portion showed that all cell lines were negative. Colo320 and WiDr, therefore, express high levels of truncated APC protein. SW480 and Colo210 express low levels of APC, with or without truncation. DLD-1 and HT29 express moderate levels of the APC protein with (DLD-1) and without (HT29) truncation.

Northern Blot Analysis

Mirroring the immunocytochemical analysis with Ab3, Colo320 and WiDr expressed high levels of a 8.5-kb APC mRNA (Fig. 2). The expression levels of APC mRNA normalized to HT29 expression were 0.45 for DLD-1, 0.95 for SW480, 4.8 for Colo320, 2.3 for WiDr, and 0.20 for Colo201.

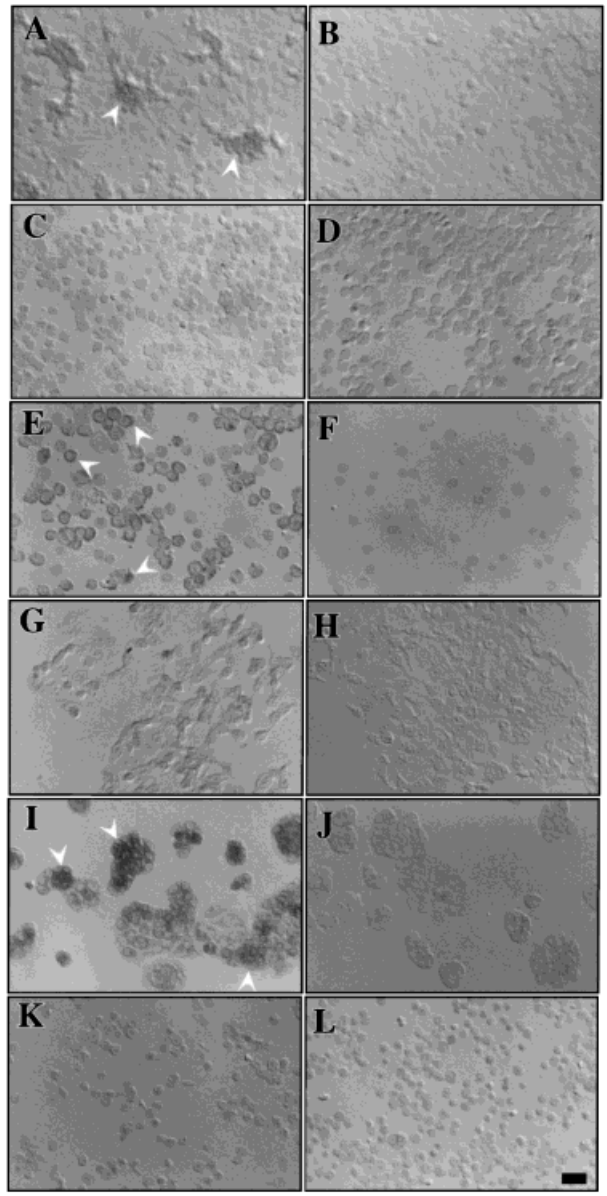

Fig. 1. Truncated and non-truncated APC protein expression in colorectal cancer cell lines by immunohistochemistry. Immunostaining with Ab3 recognizing the N-terminal portion of the APC protein demonstrates high expression in Colo320 (E) and WiDr (I), and weaker expression in DLD-1 (A) and HT29 (G). SW480 (C) and Colo201 (K) express little protein. In contrast, all cell lines (B, D, F, H, J and L) appear almost negative by immunostaining with Ab4 recognizing the non-truncated form of the APC protein (×200, Hoffman modulation contrast microscope).

TABLE I. Summary of Immunocytochemical Staining

	Immunocytochemical Staining	
	Ab3	Ab4
DLD-1	+	–
SW480	–	–
Colo320	++	–
HT29	+	±
WiDr	++	–
Colo201	–	–

The intensity of staining was scored as strongly positive + + where more than 80% of cells were positive with strong immunoreactivity; positive + where 80–60% of cells were positive with strong immunoreactivity; weakly positive ± where 60–40% of cells were positive with strong immunoreactivity; and negative—where less than 30% of cells were positive or of weak immunoreactivity.

Southern Blot Analysis

Genomic DNA from the six cell lines revealed two major bands of equal intensity at 4.5 and 2.0 kb (Fig. 3), indicating that neither gene amplification nor a major rearrangement occurred in the six cell lines.

RT-PCR

The APC gene has two promoters, yielding two transcripts exhibiting alternative usage of the non-translated exons, termed exon 1A and exon 1B (Fig. 6) [Horii et al., 1993]. To determine which promoter was utilized in the transcription of the APC gene, we performed RT-PCR using RNA isolated from each of the six colon cancer cell lines. As shown in Figure 4A, all cell lines showed positive RT-PCR products from the primer pair for exon 1A, and no products from the primer pair for exon 1B, indicating that transcriptional initiation occurred at exon 1A in all six cell lines.

MSP for APC Gene Promoter

DNA methylation patterns in the APC promoters were determined by MSP. In all six cell lines, an unmethylated pattern was observed at the promoter for exon 1A and hypermethylation pattern at that for exon 1B (Fig. 4B).

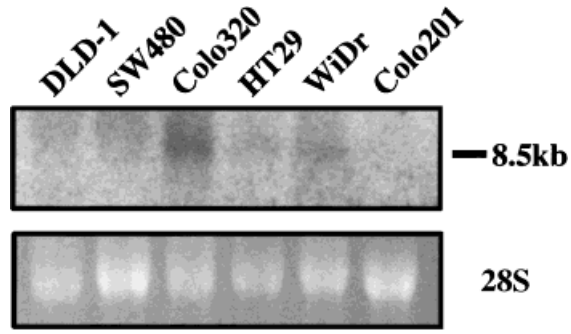

Fig. 2. APC mRNA expression in the six colorectal cancer cell lines by Northern blot analysis. Using a full length cDNA probe, APC transcripts of 8.5 kb are observed. Strong expression of APC gene is observed in the Colo320 cell line. The expression level relative to HT29 is 0.45 for DLD-1, 0.95 for SW480, 4.8 for Colo320, 2.3 for WiDr and 0.20 for Colo201.

Methylation Status of the APC Gene Promoter in the Six Colon Cancer Cell Lines

A schematic overview of the location of CpG sites is outlined in Figure 6. The amplified region contained 31 CpG loci. Each CpG locus was given a number downstream from the transcription start site between the eighth and the ninth CpG loci. Two Sp1 motifs around the 24–27th CpG loci are also shown. Because MSP covers only a part of the total methylated cytosines, we performed high sensitivity mapping of methylated cytosines. By sequence

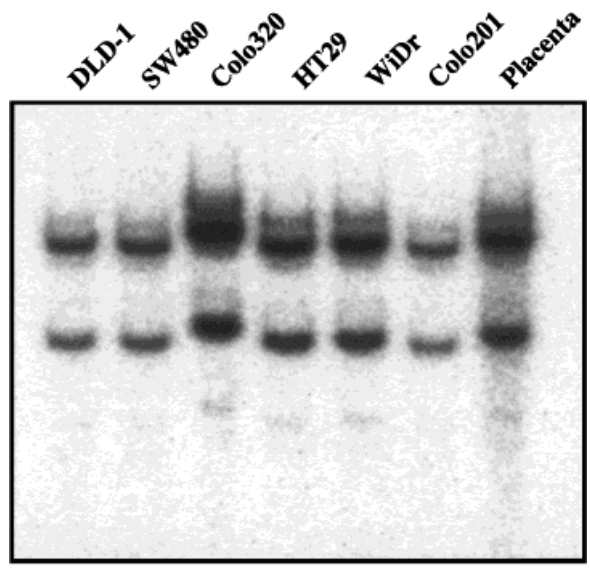

Fig. 3. Southern blot analysis shows that neither gene amplification nor rearrangement has occurred in the six cell lines. Genomic DNA digests from each of the six cell lines shows two major bands at 4.5 and 2.0 kb at equivalent levels.

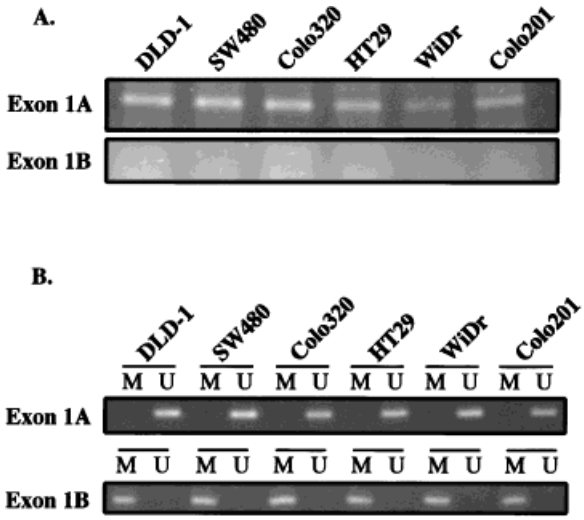

Fig. 4. **A:** RT-PCR of the APC gene promoter. All cell lines generated positive RT-PCR products from primer pair for exon 1A. No products could be detected from the primer pair for exon 1B. **B:** MSP for APC gene promoter. Unmethylation pattern was observed within the promoter for exon 1A, whereas hypermethylation pattern was present within exon 1B.

analysis, no methylated CpG loci were found in Colo320, WiDr or Colo201. On the other hand, DLD-1, HT29, and SW480 possessed methylated cytosines concentrated around the transcriptional start site. DLD-1 also contained methylated cytosines at the sixth and seventh CpG loci (Fig. 5B). SW480 had a methylated cytosine at the 22nd CpG locus and hemimethylation at the second CpG locus (Fig. 6). HT29 possessed a methylated cytosine at the second and third CpG loci (Fig. 5A).

MSP for hMLH1 Gene Promoter

The visible PCR product indicates CpG methylation (lanes marked M) and CpG nonmethylation (lanes marked U) of the hMLH1 gene promoter (Fig. 7). SW480 showed only the methylated pattern of the hMLH1 gene promoter. On the other hand, DLD-1, HT29, WiDr, and Colo201 showed only the unmethylated pattern. Colo320 contained both methylated and unmethylated patterns, indicating hemimethylation of the hMLH1 promoter.

DISCUSSION

In the six colorectal cancer cell lines (DLD-1, SW480, Colo320, HT29, WiDr, and Colo201), the expression level of APC mRNA correlated well with APC protein levels. In addition, as no major genetic alterations, such as amplification or deletion, of the APC gene were found, the major regulation of APC in these cell lines can be attributed to transcriptional machinery. Examination of the epigenetic status of the APC gene by MSP revealed that, in all six cell lines, the P1 promoter did not demonstrate significant methylation at the 13–16th and 21–23rd CpG loci. We, therefore, mapped methylated cytosine around the P1 promoter by sodium bisulfite-modified sequencing, to find that the methylation status of CpG loci of the 5′-untranslated region correlated with steady-state expression of the gene. In addition, MSP for the P2 promoter established a methylated pattern in all cell lines, reinforcing the fact that no transcription occurred from exon 1B by RT-PCR.

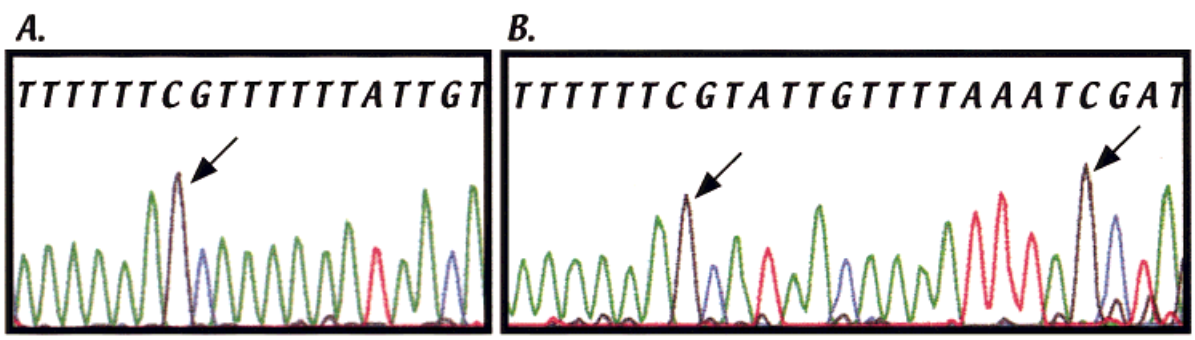

Fig. 5. Processed sequence data for part of the APC gene promoter in two colon cancer cell lines determined by the dideoxy nucleotide termination method. **A:** Total methylation at the second CpG locus in HT29 is specified by the arrow. **B:** Total methylation at the sixth and the seventh CpG loci in DLD-1 is designated by the arrows.

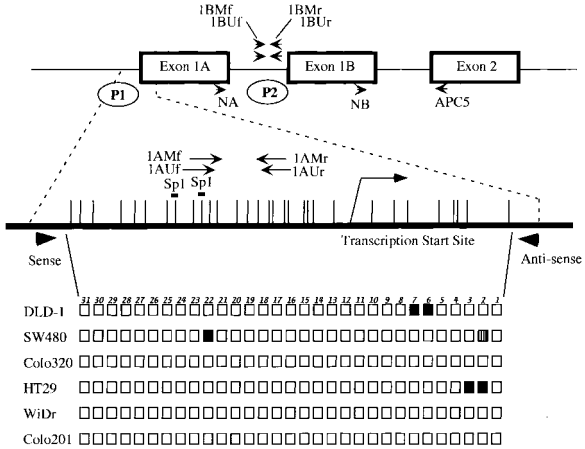

Fig. 6. Schematic overview of the methylated cytosine in the APC gene 5′-flanking region in the six colon cancer cell lines. Two Sp1 motifs are located between the 24th and 27th CpG loci in the amplified APC promoter. A transcriptional start site is located between the eighth and ninth CpG locus. Three cell lines, DLD-1, SW480 and HT29, possessing a small number of APC gene transcripts and little protein by Northern blot and immunocytochemical analysis, have methylated cytosines in the 5′-untranslated region of the APC gene. Methylated cytosine is not frequent in close proximity to the Sp1-binding sites.

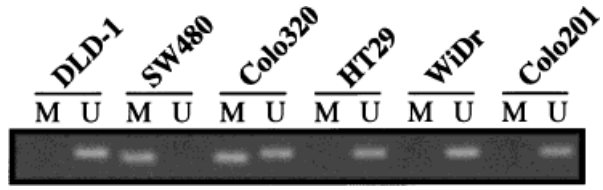

Fig. 7. Methylation status of the CpG island in the hMLH1 gene promoter by MSP. The PCR product visible in lanes marked M indicates methylated hMLH1; products visible in lanes marked U indicate non-methylation. SW480 possesses only the methylated form of the PCR product. DLD-1, HT29, WiDr, and Colo201 exhibit only the unmethylated form of the PCR product. Colo320 produces both methylated and unmethylated forms of the PCR product indicating hemimethylation of the hMLH1 gene promoter.

In patients with FAP and sporadic colorectal cancer, germline and somatic mutation of the APC gene causes loss of APC protein function, leading to tumorigenesis [Nakamura, 1993]. Furthermore, the detection of multiple adenomas in the small intestine and colorectum of APC knock-out mice [Oshima et al., 1995; Takaku et al., 1998] clearly links the APC gene to early colorectal tumorigenesis. The intact APC protein normally exerts an antitumor effect by forming a tetramic complex with conductin, GSK-3β and β-catenin, resulting in the accelerated degradation of β-catenin and the inhibition of signal transduction to the LEF-TCF (lymphoid enhancer factor-T cell factor) family of transcription factors [Sakanaka et al., 1998; Kishida et al., 1998]. The truncated APC protein resulting from a point mutation loses the ability to interact with β-catenin [Munemitsu et al., 1995], allowing direct interaction of β-catein with LEF-TCF family transcription factors, promoting cells to enter the cell cycle.

In addition to genetic alteration of oncogenes and tumor suppressors, epigenetical suppression of tumor suppressors such as $p16^{INK4a}$ [Gonzalez-Zulueta et al., 1995], p53, hMLH1, BRCA1 [Rice et al., 1998], E-cadherin and TIMP-3 (tissue inhibitor of metalloproteinase-

TABLE II. Primer Sequences and PCR Conditions for the High-Sensitivity Mapping of Methylated Cytosine

	Condition of First PCR	Condition of Nested PCR
sense primer	5'-GTGTTGTAAAAATTATAGTA ATC/TGAGATGTAA-3'	5'-ATTATTTTTTTTTTTATTTT C/TGGTATTTTG-3'
anti-sense primer	5'-CAACTTTTCTCAACAAACTC CCCT-3'	5'-CTACCCCTCCCCCTTCTCCT TCTTCTTC-3'
hot start	94 C, 2 min.	94 C, 2 min.
cycle	40	34
denaturing	94 C, 15 sec.	94 C, 15 sec.
annealing	60 C, 30 sec.	55 C, 15 sec.
extension	72 C, 90 sec.	72 C, 90 sec.
final extension	72 C, 5 min.	72 C, 5 min.

3) [Bachman et al., 1999] by hypermethylation of the CpG loci within promoters has been found in both colon cancer cell lines and cancer tissues. Hypermethylation of CpG loci, especially around Sp1-binding sites in the APC promoter, has also been reported at a high rate in colorectal cancer tissue [Hiltunen et al., 1997], although the relationship between the promoter methylation status and APC gene expression has not yet been clarified. Two mechanisms of transcriptional suppression by CpG methylation at Sp1 binding sites have been postulated: as a direct inhibitory effect, cytosine methylation at the CpNpG site inhibits Sp1 binding in the CpG island of the retinoblastoma gene [Clark et al., 1997]; as an indirect mechanism, repression of transcription by MeCP2 has been demonstrated in the human leukosialin gene promoter [Kudo, 1998]. In this study, CpG methylation was observed around the transcriptional start site (DLD-1, SW480, and HT29). The only methylated cytosine at the 22nd CpG locus was adjacent to the Sp1 site in the SW480 cells. We therefore speculated that expression of the human APC gene in these cell lines was indirectly repressed through the methylation of its 5′-untranslated region of APC mRNA. In the Colo201 cell line, however, expression of APC mRNA and protein was not enhanced despite the absence of methylation in the 5′-flanking region of the APC gene. Additional factors, like deranged expression of APC regulators or the accelerated degradation of APC mRNA, may influence APC gene expression in the Colo201 cell line.

To determine whether epigenetic changes in the APC gene promoter were selective, we analyzed the methylation status of a non-APC related gene, hMLH1, a major mismatch repair gene promoter. By MSP (Fig. 7), methylation of the hMLH1 gene promoter was detected in SW480 and Colo320 (hemimethylation), independent of the methylation status of the APC gene. We speculated that the epigenetic control of each gene was a selective and specific event during tumorigenesis and cancer progression.

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