[CANCER RESEARCH 59, 2029–2033, May 1, 1999]

Advances in Brief

Methylation of CpG in a Small Region of the hMLH1 Promoter Invariably Correlates with the Absence of Gene Expression[1]

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Abstract

Microsatellite instability (MSI) has been described in tumors from patients with hereditary nonpolyposis colorectal cancer, sporadic colorectal cancer, and other types of cancers. MSI is caused by the dysfunction of mismatch repairs genes. Loss of expression and mutation in one of the major mismatch repair genes, hMLH1, and the methylation of CpG sites in its promoter occur frequently in primary tumors and cell lines of colorectal cancer with MSI. To understand the mechanisms involved in the silencing of hMLH1 expression by methylation, we examined the methylation status of all CpG sites in the hMLH1 promoter in 24 colorectal cancer cell lines by the $NaHSO_3$-sequencing method. We identified a small proximal region (−248 to −178, relative to the transcription start site) in the promoter in which the methylation status invariably correlates with the lack of hMLH1 expression. This correlation was further supported by the observation that cell lines that showed methylation-suppressed hMLH1 expression can be induced to reexpress hMLH1 by a methyl transferase inhibitor, 5-aza-2′-deoxycytidine, and the small region that we identified exhibited significant demethylation in all cell lines examined.

Introduction

MMR[3] is an important mechanism by which cells correct errors in DNA replication during proliferation to maintain the fidelity of the genome. Cells with MMR defects show mutation rates up to 1000-fold greater than those observed in normal cells. The mutator phenotype, which can be measured by MSI analysis, has been detected in tumors from patients with hereditary nonpolyposis colorectal cancer (1, 2), sporadic colorectal cancer (1–4), and other types of cancers. Thus far, two major genes, *hMLH1* and *hMSH2*, as well as *hMSH3*, *hMSH6*, and *hPMS2*, have been cloned and demonstrated to participate in DNA MMR (5, 6). Germline mutations or somatic mutations in these genes give rise to nonfunctional gene products, leading to the mutator phenotype (7, 8). More recently, immunohistochemical analysis has demonstrated that loss of expression of MMR genes (mainly *hMLH1*) occurs frequently in sporadic colon cancers with MSI (9, 10). The loss of hMLH1 expression was further shown to correlate with cytosine methylation of CpG sites in its promoter in colon cancer cell lines and tissues (11–14). The epigenetic silencing of hMLH1 by methylation has been shown to be a common route leading to the genesis of MSI in colon cancers. Methylation-sensitive enzyme (*e.g.*, *Hpa*II) digestion and MSP have been successfully applied to determine the methylation status of the hMLH1 promoter (11–14). However, methylation-sensitive enzyme digestion can identify only those CpG sites in enzyme-recognition-sequences and, therefore, fails to recognize most CpG sites. Similarly, the MSP method can analyze only two CpG sites at the 3′ ends of each of the two PCR primers. To characterize precisely the regions involved in the epigenetic silencing of hMLH1, we have conducted the $NaHSO_3$ treatment-sequencing method for methylation analysis of hMLH1 promoter. In our study, we examined MSI, mutation and expression of *hMLH1* gene, and methylation status of hMLH1 promoter in 24 colorectal cancer cell lines. By looking at hMLH1 expression and methylation status of all CpG sites in the promoter, we identified a small proximal region (−248 to −178 relative to the transcription start site) in the promoter in which the methylation status invariably correlates with the lack of hMLH1 expression. This correlation was further supported by the observation that cell lines that showed methylation-suppressed hMLH1 expression can be induced to reexpress hMLH1 by a methyl transferase inhibitor, 5-aza-2′-deoxycytidine. The small region we identified was demethylated by this treatment, as well. Methylation of a more upstream region seemed not to be critical for gene silencing because partial methylation was detected in this area in cell lines that showed normal hMLH1 expression.

Materials and Methods

Cell Lines. Colorectal carcinoma cell lines SW1116, HCT8, Colo201, Colo320, CaCo2, SW1463, HRT18, HT29, SW620, LS123, LS174T, HCT116, SW48, Lovo, and H498 were obtained from American Type Culture Collection (Manassas, VA). Cell lines VACO5, VACO6, VACO411, and VACO10P were kindly provided by Dr. Sanford D. Markowitz. Cell lines RW2982 and RW7213 were from Dr. Lance M. Tibbetts. Cell line C1a was derived from 5583s, provided by Dr. Fred T. Bosman. RKO and C cells were from Dr. Michael Brattain. Cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO_2 atmosphere.

5-Aza-2′-Deoxycytidine Treatment. Cells were seeded at 2×10^5 cells/T75 flask on day 0. The cells were treated with 5, 10, or 15 μM 5-aza-2′-deoxycytidine for 24 h on days 2 and 5. The medium was changed 24 h after adding 5-aza-2′-deoxycytidine. Cells were harvested on day 8 for analysis of hMLH1 expression and methylation status of promoter.

MSI Analysis. The MSI status of each cell line was determined through analysis of the *BAT25* and *BAT26* loci, as described by Thibodeau *et al.* (3). The DNA patterns were compared with those from an unaffected normal tissue (control). Because *BAT25* and *BAT26* patterns are essentially monomorphic within the human population, any difference reflects MSI (15). Thus, a cell line that showed variation with either marker was scored as MSI.

Sequencing of *hMLH1* Gene. Total RNA (1 μg) isolated from cell lines was reverse-transcribed using random hexanucleotides (Boehringer Mannheim) and SuperScript II reverse transcriptase (Life Technologies, Inc.) in a volume of 50 μl. After reverse transcription, 1 μl of cDNA was amplified separately by PCR using five primer sets (F1/R1, F2/R2, F3/R3, F4/R4, and F5/R5) that are designed to amplify five overlapping cDNA fragments covering the entire coding sequence of hMLH1. The sequences of the primers are: F1: 5′CTTGGCTCTTCTGGCGCCAA, R1: 5′CTCCTCGTGGCTATGTTGTAA; F2: 5′AGATCACGGTGGAGGACCTT, R2: 5′TCCTCGTGCAGGAAGTGAAC; F3: 5′GTGCACCCCACAAAGCATGA, R3: 5′TTCCCGATGTCTCTTTCTGG; F4: 5′AGAGGACCTACTTCCAGCAA, R4: 5′TCT-

Received 12/18/98; accepted 3/19/99.

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[1] Supported by Theodora Betz Foundation Fund and Veterans Administration Medical Research Service.

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[3] The abbreviations used are: MMR, mismatch repair; MSI, microsatellite instability; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR.

CAGCCTTCTTCTTCAGAA; F5: 5′GAAGGACTTGCTGAATACATT, R5: 5′CCCACAGTGCATAAATAACCAT. The RT-PCR products were purified by electrophoresis on a 1.5% agarose gel and eluted with QIAquick gel extraction kit (Qiagen). One-third of the eluted DNA was mixed with 5 pmol of the corresponding primer and sequenced on an ABI sequencer with dye terminators (Applied Biosystem).

Determination of hMLH1 mRNA Expression. RNA isolated from each cell line was reverse-transcribed as described above. cDNA (1 μl) was amplified by PCR, together with two primer sets. The first set was used for amplifying a 196-bp fragment spanning exons 1–3 of the *hMLH1* gene (F: 5′CAGCGGCCAGCTAATGCTAT, R: 5′AATCCTCAAAGGACTGCAGTT). The second primer set was for amplifying a β-actin fragment, with the size of 242 bp, as an internal control (F: 5′TCACCAACTGGGACGACATG, R: 5′ACCGGAGTCCATCACGATG). The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining. The amount of each fragment was determined with a densitometer. The RNA expression level was represented as the ratio of the amount of hMLH1 fragment over β-actin.

DNA Methylation Analysis. The $NaHSO_3$ treatment-sequencing procedure, as described by Clark *et al.* (16), was applied to determine the methylation status of all CpG sites in the hMLH1 promoter (bases −711 to +15, relative to the start of transcription). DNA (1 μg) was diluted in 50 μl of 10 mM Tris-HCl (pH7.6), 1 mM EDTA, and 0.3 N NaOH and incubated at 37°C for 15 min. Hydroquinone (30 μl of 10 mM) and 520 μl of 3.6 M $NaHSO_3$ (pH5.05) were added to the denatured DNA solution. The tube was incubated at 55°C for 16 h. The $NaHSO_3$-treated DNA was purified using the Wizard DNA clean-up system (Promega), denatured by 0.3 N NaOH, precipitated with ethanol, and dissolved in 20 μl of 10 mM Tris-HCl (pH7.6) and 0.1 mM EDTA. DNA (1 μl) was amplified by PCR separately in 50 μl with three primer sets, PF1/PR1, PF2/PR2, and PF3/PR3, which can amplify three overlapping fragments covering the region from −766 to +15. The sequences of primers are: PF1: 5′TTTTAGTTGTGATTTTTTAAGGTT, PR1: 5′AAAACAATAAAACCCTATACCTAA; PF2: 5′GTGATAGATTAGGTATAGGGTT, PR2: 5′AATATCCAACCAATAAAAACAAAAATA; PF3: 5′ATTATTTTAGTAGAGGTATATAAGT, PR3: 5′CCCTACCACAAACAACATTTTAA. The amplified fragments were separated on a 1.5% agarose gel and eluted using a QIAquick gel extraction kit (Qiagen). One-third of the DNA was mixed with 5 pmol of primer and sequenced on an ABI sequencer with dye terminators (Applied Biosystem). This procedure results in the conversion of unmethylated cytosine to thymine, whereas the methylated cytosine was not affected. Thus, the ratio of peak height of C to T at a CpG site indicates the ratio of methylated to unmethylated cytosine. The quotient of C over C+T indicates the percentage of methylation. DNA methylation was also measured by *Hpa*II digestion and MSP, as described (12, 13).

Results and Discussion

MSI, hMLH1 Expression, and Mutation Analysis of Colorectal Cancer Cell Lines. Twenty-four colorectal cancer cell lines were analyzed for MSI, hMLH1 expression, and hMLH1 mutations (Table 1). Fifteen of 24 cell lines (63%) were microsatellite stable. The other nine cell lines showed MSI. hMLH1 was expressed in 18 of 24 cell lines (75%), with 6 cell lines not expressing message, as determined using the sensitive RT-PCR method. hMLH1 mutation data were obtained by sequencing or collected from published data. In all 15 cell lines that were microsatellite stable, hMLH1 was expressed and no mutation in hMLH1 was detected. Mutations in hMLH1 or hMSH2 and lack of hMLH1 expression were observed in all of the nine cell lines that showed MSI. Cell lines LS174T, C1a, and HCT116 showed hMLH1 mutations (the mutations in LS174T and Cla were determined in this study). Missense mutations were observed in LS174T and C1a, whereas a nonsense mutation was detected in HCT116. hMLH1 expression was observed in LS174T and HCT116, but not in C1a. In other six cell lines with MSI, five cell lines (SW48, VACO5, VACO6, RKO, and C) showed wild-type hMLH1, but did not express hMLH1. Cell line Lovo, showing MSI, had wild-type hMLH1 and expressed hMLH1. But a truncated *hMSH2* gene (exons 4–8) was observed in this cell line (17). Thus, the absence of hMLH1 expression observed

Table 1 *MSI, hMLH1 mutations, expression, and methylation status in human colorectal cancer cell lines*

Cell line	MSI	*hMLH1* mutation	*hMLH1* expression	Methylation in different regions (%)[a]			
				A[b]	B[c]	C[d]	D[e]
SW1116	−	wild type[f]	+	62	0	0	0
HCT8	−		+	57	0	0	0
Colo201	−	wild type[f]	+	60	0	0	0
Colo320	−	wild type[f]	+	55	0	0	0
RW2982	−		+	61	0	0	0
RW7213	−		+	57	0	0	0
H498	−		+	59	0	0	0
VACO411	−		+	64	0	0	0
VACO10P	−		+	60	0	0	0
CaCo2	−	wild type[f]	+	67	0	0	0
SW1463	−		+	40	0	0	0
HRT18	−		+	54	0	0	0
HT29	−	wild type[f]	+	35	37	0	0
SW620	−	wild type[f]	+	24	10	0	0
LS123	−		+	36	24	0	0
LS174T	+	exon 4, codon 117, ACG to ATG, Thr to Met[g]	+	45	11	0	0
HCT116	+	exon 9, codon 252, TCA to TAA, Ser to Stop[h,i]	+	62	0	0	0
C1a	+	exon 5, codon 129, AGT to GGT, Ser to Gly[g]	−	95	82	100	89
SW48	+	wild type[f,j]	−	98	96	100	93
VACO5	+	wild type[f,i,j]	−	98	96	100	100
VACO6	+	wild type[h,i]	−	95	80	100	38
RKO	+	wild type[i]	−	79	82	94	76
C	+	wild type[g]	−	90	85	98	79
Lovo[k]	+	wild type[f]	+	60	0	0	0

[a] The average of methylation percentage of all CpG sites in each region.
[b] From −711 to −577, containing 23 CpG sites.
[c] From −552 to −266, containing 19 CpG sites.
[d] From −248 to −178, containing eight CpG sites.
[e] From −109 to +15, containing seven CpG sites.
[f] Ref. 17.
[g] Determined in this study.
[h] Ref. 6.
[i] Ref. 14.
[j] Ref. 8.
[k] Lovo shows the expression of truncated RNA (exons 4–8 deletion) of *hMSH2* gene.

in six of nine cell lines with MSI indicates that the silencing of hMLH1 is an important mechanism leading to MSI.

Methylation of CpG Sites in −248 to −178 Region of hMLH1 Promoter Correlates with Loss of hMLH1 Expression. Methylation status in the region from −711 to +15 of hMLH1 promoter in each of the 24 colorectal cancer cell lines was determined by the $NaHSO_3$-sequencing technique. Of all 18 cell lines that expressed hMLH1, 14 cell lines showed no methylation of CpG sites in the region from −552 to +15 and partial methylation in the region from −711 to −577 (Fig. 1*A*). In the other four cell lines showing normal hMLH1 expression (HT29, SW620, LS123, and LS174T), there was no methylation in the proximal area either, but the partially methyl-

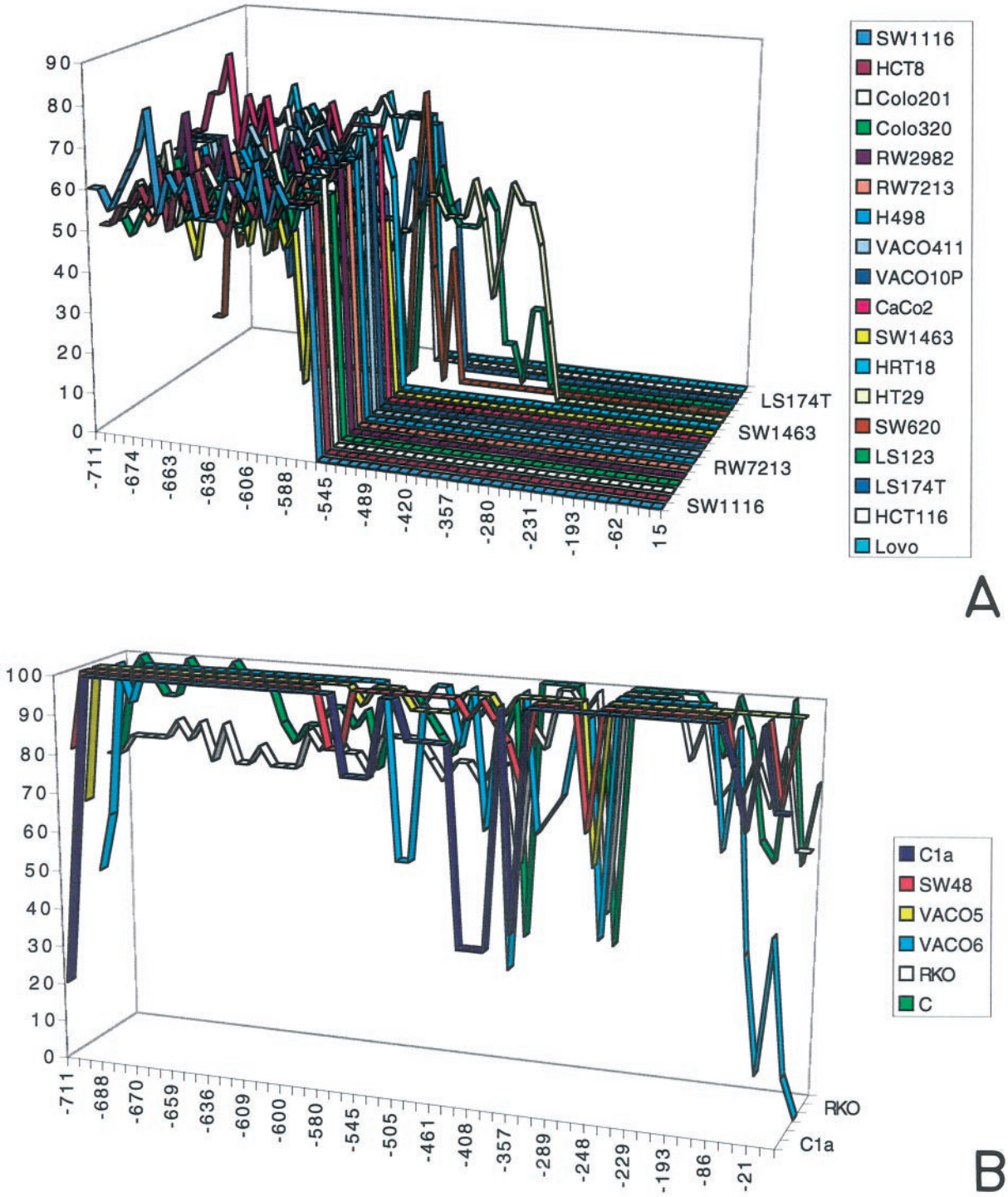

Fig. 1. Methylation status of the hMLH1 promoter between bases −711 and +15 in 24 colorectal cancer cell lines. The percentage of methylation at each CpG site was plotted against its position in the promoter. The CpG sites are at bases −711, −702, −694, −688, −674, −672, −670, −666, −663, −659, −649, −645, −636, −624, −616, −609, −606, −604, −600, −598, −588, −580, −577, −552, −545, −523, −510, −505, −489, −486, −461, −445, −420, −408, −380, −364, −357, −326, −320, −289, −280, −266, −248, −241, −231, −229, −223, −204, −193, −178, −109, −86, −62, −54, −21, −6, and +15. The locations of the CpG sites are not drawn to scale. *A*, methylation status in cell lines SW1116, HCT8, Colo201, Colo320, RW2982, RW7213, H498, VACO411, VACO10P, CaCo2, SW1463, HRT18, HT29, SW620, LS123, LS174T, HCT116, and Lovo. *B*, methylation status in cell lines C1a, SW48, VACO5, VACO6, RKO, and C.

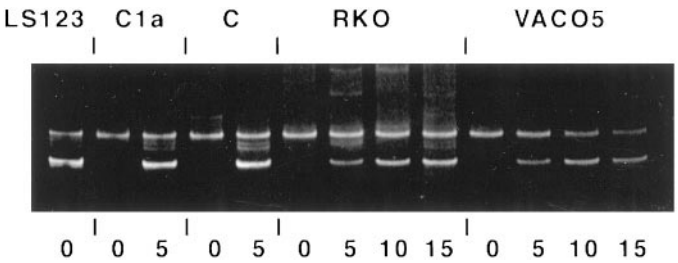

Fig. 2. hMLH1 RNA expression in cell lines treated with 5-aza-2′-deoxycytidine. Cell lines C1a, C, RKO, and VACO5 were treated with 5, 10, or 15 μM 5-aza-2′-deoxycytidine. hMLH1-expressing cell line LS123, without 5-aza-2′-deoxycytidine, is included as a positive control. Total RNA isolated from the cells was reverse-transcribed, and the cDNA was amplified by PCR with two primer sets together, hMLH1 primers and β-actin primers as a control. The relative expression level was calculated by the amount of hMLH1 (lower band) divided by β-actin (upper band). The number below each *lane* indicates the concentration of 5-aza-2′-deoxycytidine.

ated region extended to the CpG sites at −320, −505, −357, and −510, respectively. The six cell lines that did not express hMLH1 (C1a, SW48, VACO5, VACO6, RKO, and C) showed varying degrees of methylation in the entire region from −711 to +15. However, 100% methylation was observed in the region from −248 to −178 in all six cell lines (Fig. 1*B*). To summarize the data, we divided the promoter area into four regions [A (from −711 to −577, containing 23 CpG sites), B (from −552 to −266, 19 CpG sites), C (from −248 to −178, 8 CpG sites), and D (from −109 to +15, 7 CpG sites)] and averaged the percentages of methylation at all CpG sites in each of the four regions (Table 1). We then examined the relationship between the methylation status in regions A, B, C, and D in each of the 24 cell lines and the hMLH1 expression. Methylation in region A seemed not to be critical in silencing the gene expression because all 18 cell lines with normal expression showed methylation in this area (Table 1 and Fig. 1*A*). On the other hand, methylation in region C seemed to play an important role in silencing hMLH1 expression because all 18 cell lines that expressed hMLH1 showed no methylation and the 6 cell lines that did not express hMLH1 showed full methylation in this region (Table 1 and Fig. 1). Methylation in regions B and D may also be important in silencing hMLH1 expression, but methylation in these regions did not provide the best correlation between the methylation status and hMLH1 expression. Four of 18 cell lines with normal hMLH1 expression (HT29, SW620, LS123, and LS174T) showed methylation in region B, and methylation percentages in region D were lower than those in region C in all but one cell line (VACO5) that did not express *hMLH1* genes (Table 1 and Fig. 1). From these results, we conclude that methylation status of CpG sites in region C provides the best correlation and prediction of hMLH1 expression.

The identification of region C is of importance, because the methylation of CpG sites in this region invariably correlates with the loss of expression in all cell lines tested, and methylation outside this region (regions A and B) does not always correlate with inhibition of gene expression. For example, in HT29 cells, the expression of hMLH1 is normal. However, when *Hpa*II digestion was first used for methylation analysis, methylation was observed (data not shown). This inconsistency between the hMLH1 expression and methylation of CpG sites is due to the fact that the four *Hpa*II recognition sites are located in region B (−545, −505, −326, and −320). When the $NaHSO_3$-sequencing method was used to analyze methylation status, partial methylation was detected at CpG sites of these *Hpa*II sites (45%, 45%, 40%, and 20%, respectively; Fig. 1*A*). This suggests that methylation of CpG sites located outside region C does not inhibit hMLH1 expression. Another method, MSP, can also be applied to determine the methylation status. Herman *et al.* (13) selected primers from region A for MSP analysis because of the frequent occurrence of CpG sites in this region. Because all cell lines showed methylation in this region, regardless of whether or not hMLH1 was expressed, the methylation status obtained from MSP of this region may not correlate with the loss of hMLH1 expression. Thus, to analyze the methylation status in hMLH1 promoter by the MSP method, primers located in region C should be used.

5-Aza-2′-Deoxycytidine Treatment Leads to Reexpression of hMLH1 and Demethylation of Its Promoter. To confirm the critical role of methylation of CpG sites in region C (−248 to −178), four cell lines that did not express hMLH1 (C1a, VACO5, RKO, and C) were treated with 5, 10, or 15 μM 5-aza-2′-deoxycytidine. hMLH1 expression was detected in all four cell lines after the treatment. The expression levels were dependent on the cell type and dosage of 5-aza-2′-deoxycytidine. In cell lines C1a and C, 5 μM 5-aza-2′-deoxycytidine induced the expression of hMLH1 to the level of normal-expressing cell line LS123. In cell lines RKO and VACO5, the expression levels of hMLH1 induced by 5 μM 5-aza-2′-deoxycytidine was low, but levels increased when the dosage was raised to 10 or 15 μM (Fig. 2). In SW48, the expression level of hMLH1 induced by 10 μM was also higher than 5 μM (data not shown).

The methylation status of CpG sites in the whole promoter region from −711 to +15 was determined in the four 5-aza-2′-deoxycytidine-treated cell lines. The extent of demethylation in regions A, B, C, and D of each four cell lines was obtained (Table 2). In two of the four cell lines (VACO5 and C), the extent of demethylation in regions A, B, C, and D was similar (around 20% and 30%, respectively), whereas in two other cell lines (C1a and RKO), the extent of demethylation in regions C and D was significantly higher than in regions A and B. These observations are consistent with the notion that methylation of the more proximal regions, especially region C, plays an important role in the regulation of hMLH1 expression.

The methylation status in 6 cell lines without hMLH1 expression and 18 cell lines with normal expression showed that methylation in a proximal region (−248 to −178) can silence expression, whereas methylation in two upstream regions (−711 to −577 and −552 to −266) may be less efficient for the silencing effect. 5-Aza-2′-deoxycytidine is an agent which inhibits DNA methyltransferase, resulting in the demethylation of DNA (13, 14). In this study, we showed that different regions of the hMLH1 promoter responded to 5-aza-2′-deoxycytidine differently (*i.e.*, the extent of demethylation varied in different regions). In regions C and D, demethylation was usually more significant than in regions A and B. This observation suggests the presence of a methylation-response element in or near region C (18), which plays an important role in regulating the expression of the gene through methylation and demethylation.

Sp1 element is a DNA sequence to which the transcription factor Sp1 binds. The 10-bp consensus sequence of the Sp1 element is (G/T)(G/A)GG(C/A)G(G/T)(G/A)(G/A)(C/T), and its core sequences

Table 2 *Demethylation in different regions of the hMLH1 promoter after 5-aza-2′-deoxycytidine treatment*

Cell line	Demethylation extent[a] in different regions			
	A[b]	B	C	D
C1a	4%	23%	40%	40%
VACO5	22%	17%	19%	24%
RKO	0%	1%	29%	29%
C	29%	32%	33%	29%

[a] The extent of demethylation was calculated by averaging the percentage of methylation of all CpG sites in the region before 5-aza-2′-deoxycytidine treatment subtracted by the average percentage of methylation after treatment. The concentrations of 5-aza-2′-deoxycytidine used to treat cell lines C1a, VACO5, RKO, and C were 5, 15, 10, and 5 μM, respectively.

[b] Regions A , B, C, and D are the same as in Table 1.

are GGGCGG or GGCGGG. Recent studies have shown that CpG sites in promoter regions are protected from methylation by a cluster of Sp1 elements in a variety of genes (19, 20). By comparing the consensus and the core sequences of Sp1 element with the 5′ flanking region of hMLH1 (from −711 to +15), we identified nine Sp1 elements that were located at −658, −580, −547, −461, −441, −393, −366, −182, and −85, respectively. Seven of the nine Sp1 elements are located in regions A and B. This is consistent with the recent discovery that the methylated flanks of promoters are segregated from the unmethylated CpG sites by an Sp1-rich boundary region (20). The cluster of Sp1 elements in the hMLH1 promoter region suggests that Sp1 may be involved in regulation of *hMLH1* gene expression in a mechanism involving methylation. Additional experiments are necessary to determine the differences in Sp1 binding between the cells that do and do not express hMLH1.

Acknowledgments

We thank Drs. James Gum and Suzanne Crawley for helpful discussions and suggestions during the course of this work.

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Methylation of CpG in a Small Region of the hMLH1 Promoter Invariably Correlates with the Absence of Gene Expression

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Cancer Res 1999;59:2029-2023.