Mechanisms for the Involvement of DNA Methylation in Colon Carcinogenesis

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ABSTRACT

C → T transitions at CpG sites are the most prevalent mutations found in the p53 tumor suppressor gene in human colon tumors and in the germline (Li-Fraumeni syndrome). All of the mutational hotspots are methylated at 5-methylcytosine, and it has been hypothesized that the majority of these mutations are caused by spontaneous hydrolytic deamination of this base to thymine. We have previously reported that bacterial methyltransferases induce transition mutations at CpG sites by increasing the deamination rate of C → U when the concentration of the methyl donor S-adenosylmethionine (AdoMet) drops below its K_m, suggesting an alternative mechanism to create these mutations. Unrepaired uracil pairs with adenine during replication, completing the C → T transition mutation. To determine whether this mechanism could contribute to the development of human colon cancer, we examined the level of DNA (cytosine-5)-methyltransferase (MTase) expression, the concentration of AdoMet, and the activity of uracil-DNA glycosylase in human colon tissues, and searched for the presence of mutations in the MTase gene. Using reverse transcription–PCR methods, we found that average MTase mRNA expression levels were only 3.7-fold elevated in tumor tissues compared with surrounding normal mucosa from the same patient. Also, no mutations were found in conserved regions of the gene in 10 tumors sequenced. High-performance liquid chromatographic analysis of extracts from the same tissues showed that AdoMet concentrations were not reduced below the K_m value for the mammalian enzyme, and the concentration ratio of AdoMet:S-adenosylhomocysteine, the breakdown product of AdoMet and the competitive MTase inhibitor, did not differ significantly. Finally, extracts from the tumor tissue efficiently removed uracil from DNA. Therefore, biochemical conditions favoring a mutagenic pathway of C → U → T were not found in a target tissue known to undergo a high rate of C → T transitions at CpG sites.

INTRODUCTION

The dinucleotide CpG has been found to have a special role in the genome and in the development of human cancers. Most of the CpGs in human DNA are methylated at the 5-position of cytosine, which is the only known covalent modification in DNA. Areas of the genome without methylation are clustered as “CpG islands,” which are often associated with promoter regions of genes (1, 2) and are generally methylated on the inactive X chromosome (3) and on imprinted genes (4). Changes in global genomic methylation and methylation patterns are among the most consistent findings in the development of human cancers (5–7). This has led to the hypothesis of an epigenetic mechanism for cancer development, in which changes in methylation interfere with expression of proto-oncogenes and tumor suppressor genes (8). In addition, involvement of methylation is also the most plausible explanation for the high rate of C → T transition mutations at CpG sites, which are frequently found in several human cancers in key genes such as the p53 tumor suppressor gene, especially in colon tumors and in the germline (Li-Fraumeni syndrome; Ref. 9). Five of six mutational hot spots in the p53 gene are CpG sites; 47% of all mutations at these sites in colon tumors are C → T or G → A transitions (10); and all of these hot spots in p53 have been found to be methylated (9, 11, 12), which is consistent with a methylation-induced mutational mechanism. Other genes, such as the factor IX gene, show a similar accumulation of transition mutations at CpG sites (13). In addition, CpG sites are underrepresented in human DNA by a factor of 5 (14), and it is believed that transition mutations from C → T or G → A on the opposite strand, respectively, caused the loss of CpGs during evolution (15, 16). We have argued that these mutations are caused by an endogenous mechanism, because epidemiological studies have not revealed any regional differences in the patterns of germline mutations (17), and no strand bias has been found in the repair process (10).

Two mechanisms have been proposed to explain why these CpG sites are mutational hot spots in human tumors (9, 18). Amino groups of cytosine and 5mC are less stable in DNA than the amino groups of guanine and adenine and hydrolyze spontaneously, forming uracil and thymine, respectively (19, 20; for review, see Ref. 21). Differences in the repair efficiencies of the U:G versus T:G mismatches created by this process are also likely to contribute substantially to the formation of mutational hot spots at CpG sites (22, 23). On the other hand, the methylation process itself could increase the deamination rate at the target cytosine. During the normal transfer reaction of the methyl group, the MTase flips its target cytosine out of the DNA helix into the catalytic pocket of the enzyme, where a cysteine residue of the enzyme forms a covalent intermediate with the C-6 of the cytosine ring, destroying the aromatic character of the base and activating the C-5 position to accept the methyl group from the cofactor AdoMet (24–26). β elimination of the cysteine moieties of the MTase restores the aromatic character of the base and completes the methylation process (Fig. 1, lower pathway). When AdoMet is present in concentrations below the K_m value for the enzyme, the half-life of the activated intermediate is increased, destabilizing the amino group in the 4-position of the cytosine ring. We have shown previously (27) that the bacterial MTase M.Hpall increases the hydrolytic deamination rate of C → U in vitro by a factor of 10^4 over background under these conditions. Because uracil codes for thymine during replication, unrepaired mismatches would be expected to result in C → T transition mutations (Fig. 1, upper pathway). Subsequently, we (16) and others (28, 29) have demonstrated the same mutational mechanism for the MTases M.HhaI, M.StsI, and M.EcoRII in vitro and in vivo, suggesting that the ability to deaminate the target cytosine is a common feature of all MTases, thus offering an alternative pathway for creating the high rate of C → T transition mutations at methylation sites found in human tumors.

Low AdoMet concentrations might not only lead to an increase in the numbers of mutations via this pathway but also reduce the overall DNA methylation level, because fewer methyl groups would be available for transmethylation reactions. Interestingly, as mentioned before, the content of 5mC in DNA isolated from various colon tumors is significantly reduced compared with that in normal tissue (5, 6, 11, 12, 19).

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3 The abbreviations used are: 5mC, 5-methylcytosine; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; AdoEi, S-adenosylhomocysteine; MTase, cytosine (DNA-5)-methyltransferase; UDG, uracil-DNA glycosylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSCP, single-stranded conformational polymorphism; RT, reverse transcription; HPLC, high-performance liquid chromatography.

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30–32), although regions of hypermethylation of CpG islands have been found (33, 34). In contrast, the overall decrease in 5mC content cannot be explained by reduced MTase activity; in fact, levels of MTase expression are increased in this tissue type (35, 36; see below), and high levels of MTase activity have also been found in various cell lines (37). Further evidence supporting the hypothesis that alterations in DNA methylation are causally related to colon cancer development have been provided by several studies on the MTase and AdoMet for the development of cancer in rodents. Laird et al. (38) found a reduction in tumor incidence in min− mice with reduced activity of MTase, due to heterozygosity of the MTase gene. In addition, treatment of rats with the drug 5-azacytidine, which reduces MTase activity, further reduced the number of polyps detected in these mice. Moreover, overexpression of MTase leads to transformation of NIH/3T3 cells (39). In another approach, feeding rats folate- or methionine-deficient diets, precursors in the biosynthesis of AdoMet, led to lower concentrations of AdoMet in the liver, and the DNA was hypomethylated within 1 week of treatment (40–42). In addition, the rate of liver cancer has been found to be elevated under these conditions. Smith et al. (43) sequenced relevant exons in the p53 gene of hepatomas induced by feeding Fischer 344 rats with a choline-deficient diet and found two C → T transitions at CpG sites of nine mutations detected. It has been shown that diets low in methionine and folate also increase the risk of colon cancer in humans (44), and interestingly, many transformed cells are methionine dependent (45). These findings are consistent with a model that MTase increases the rate of transition mutations at CpG sites in genes such as p53, which are critical for cancer development (Fig. 1, upper pathway), and low AdoMet concentrations could play a role in inducing a mutator phenotype. Interestingly, these findings, except for the accumulation of CpG transition mutations, can also be explained by the epigenetic model of carcinogenesis, and both models are not exclusive.

The above studies all point to alterations in DNA methylation patterns and control of the DNA methylation process as having important potential roles in colon cancer development. Alterations in MTase expression levels, mutations in the MTase gene, and variations of AdoMet concentrations may contribute not only to changes in DNA methylation levels and, therefore, to alterations in gene expression, but also to enzymatic deamination of cytosine residues at mutational hot spots (Fig. 1). Therefore, we examined these aspects of DNA methylation in colon cancer specimens and the adjacent normal colonic epithelium.

MATERIALS AND METHODS

Colon Specimen. Human colon cancer tissue and surrounding normal mucosa were obtained from colon cancer patients undergoing colectomy. Normal mucosa was stripped from fat and muscle tissues. Samples were frozen in liquid nitrogen or ethanol and dry ice within 30 min after colectomy and kept at −70°C until use. Colon carcinomas were at least 1 cm in diameter and of high grade and stage.

Analysis of the Methylation Status of p53 Codons 248, 282, and 283. A previously described PCR-based methylation assay was modified to determine the methylation status at mutational hot spots of the p53 gene (11). Genomic DNA (100 ng) was digested for 1 h at 37°C with 10 units of CfoI, HpaII, Rsal, or MspI. HpaII was used as a methylation-sensitive enzyme for the analysis of the methylation status of codons 248 (CGG) and 282 (CGG), and CfoI was used to determine whether codon 283 (CGC) was methylated. The digested DNA (10 ng) was PCR amplified using primers that flanked the codons to be studied. The primers for codon 248 were 5'-GTTGGCCTCTGACTGTACCAC-CATC-3' and 5'-CAAATGGGCTCTAGAAGCTC-3'. The primers for codon 282 and 283 were 5'-ATTATTTCTTAGTTGTTT-3' and 5'-GCTTGTAGTTGCTC-CCTG-3'. The PCR was performed at 94°C for 75 s, 62°C for 75 s, and 72°C for 90 s for 26 cycles, which was in the linear range of the assay. The PCR products were run on 1% agarose gels, transferred to a Zeta probe membrane, and probed with the p53 cDNA (46).

Quantitative RT-PCR Analysis of MTase Expression. A quantitative RT-PCR assay was used to measure the level of MTase mRNA, as described previously (47). Total mRNA was extracted from normal and cancerous colon mucosa. mRNA was reverse transcribed using random hexamers and Molony murine leukemia virus reverse transcriptase (Boehringer Mannheim). The cDNA was then PCR amplified using primers for DNA MTase (5'-GTGGCGAGACACGATGTC-3' and 5'-TTTGTCAGAATGGTGTTG-3') and GAPDH (5'-TGAGGCTTGGTCTACTTCTC-3' and 5'-CAAGTGGCAGC-CAATCG-3'). The PCR conditions were 94°C for 45 s, 55°C for 30 s, and 72°C for 90 s for 22 and 27 cycles for MTase and GAPDH, respectively. The PCR conditions were optimized to assure that the reaction was in the linear phase of amplification. To assure consistency between experiments, a stock of HeLa cell cDNA was serially diluted and run for each experiment.

Quantitative Analysis of AdoMet and AdoHcy Concentrations in Colon Tissue. Colon tissue was homogenized in 2 ml 0.2% perchloric acid, centrifuged for 30 min at full speed in an Eppendorf centrifuge, and subsequently filtered through a 0.2-μm syringe filter. All operations were performed on ice or at 4°C. AdoMet was added before tissue homogenization as an internal standard. The crude extract was analyzed by ion-pair HPLC using a slightly modified method of Wagner et al. (48). A Waters HPLC apparatus equipped with a 440 absorbance detector and a 10-cm Brownlee RP-18 column guarded by a Brownlee Aquaphore ODS precolumn was used. The columns were...
adjusted to 40°C. AdoMet, AdoHcy, and AdoEt were separated by a linear gradient, starting with 100% of eluent A [0.1 M NaH₂PO₄, 2% acetonitrile, and 8 mM octadecanoylsulfonic acid (pH 2.65)] and leading in 40 min to eluent B [0.2 M NaH₂PO₄, 26% acetonitrile, and 8 mM octadecanoylsulfonic acid (pH 3.25)]. The flow rate was set at 1 ml/min. Absorbance was monitored at 254 nm. Under these conditions, the retention times of the standard substances were 18.1 min (AdoMet), 19.6 min (AdoHcy), and 21.5 min (AdoEt). AdoMet and AdoHcy were identified in tissue extracts on the basis of their retention times and cochromatography with reference substances. The purity of the peaks was verified by variation of the mobile phase pH and the concentration of acetonitrile. Standard substances were obtained from Sigma Chemical Co.

Methylation Analysis of the Human Methytransferase cDNA from Human Colon Tissues. cDNA from total RNA was prepared as described above. PCR was performed using primers for regions I–III (m1, 5'-CAAGCCGTTTGAGCAGGAGACCTG-3'; m2, 5'-CCAAGGGATCGCCATCTCGAGGAGATGTG-3'), regions IV–VI (m3, 5'-AAGGGAGACGTGGAGATGCTGTCGGC-3'; m4, 5'-GAAGCCGCTTTAGGAGAAGACAAAGTTCCTGA-3'), and regions IX and X (m5, 5'-CAGCACAACCGTCACCAACCC-3'; m6, 5'-CAACATACAAAGCTTGAATCTCAGGCAAATG-3'; see Fig. 5). PCR products were analyzed for mutations using a SSCP technique and by direct sequencing.

Determination of UDG Activity. We used a reporter plasmid (pSV2neo) containing an ampicillin- and a neomycin-resistance gene. An inactivating point mutation in the neomycin-resistance gene was introduced by site-directed mutagenesis, changing GTGC to GCGC, as described previously (16). A U:G mismatch at this site (GUGC) was created by incubating the plasmid with M.Hhal MTase (a generous gift of Dr. Sha Mi, Cold Spring Harbor Laboratory) for 16 h without AdoMet, which leads to deamination of cytosine to uracil at the recognition site (16). The plasmid was purified by phenol-chloroform extraction and ethanol precipitated, and 100 ng were incubated with various concentrations of extract from human normal and cancerous colon tissue (23) in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2 μg BSA, and 5 μM DTT in a total volume of 10 μl. Purified UDG (Boehringer Mannheim) was also serially diluted and added to the experiment as a control. Subsequently, the plasmid was isolated by phenol-chloroform extraction and ethanol precipitation and electrophoresed into Escherichia coli NR8052 (Δpro-lac, thi, ara, trpE9777, ung1), which are devoid of UDG activity. After incubation on SOB plates containing either ampicillin to score the transformation efficiency and primers specific for GAPDH as a control revealed differences reported by El-Deiry et al. (35) but were similar to the sequence analyzed; HpaII cuts only unmethylated CCGG, andMspI is insensitive for methylation at the CCGG site. B, codons 282 (CGG) and 283 (CGC): Rsal cuts outside of the sequence analyzed; and HpaII and CfoI cut only if their recognition sites are unmethylated.

RESULTS

Analysis of the Methylation Status of Mutational Hot Spots in the p53 Gene. Previous studies in this (9, 11) and other laboratories (12) have shown that all CpG sites in the p53 gene are methylated in human WBC, kidney, muscle, bladder, and sperm DNA. We first confirmed that the CpG dinucleotides at codons 248, 282, and 283, which are mutational hot spots in colorectal cancer (49), were methylated in normal colon mucosa, adenomas, and carcinomas. Fig. 2 shows a typical analysis with primers flanking codons 248 (Fig. 2A) and 283 (Fig. 2B). Methylation analysis of codon 248 showed strong signals in template DNA cut with CfoI, which cuts outside of the sequence analyzed. No signals were generated when the template DNA was precut with MspI, which is insensitive to methylation of the internal cytosine of the CCGG recognition sequence. On the other hand, the CCGG sequences were methylated in all tissues, because strong signals were generated after digestion with HpaII. Similar results were obtained for codons 282 and 283, which are substrates for the methylation-sensitive enzymes HpaII and CfoI, respectively (Fig. 2B). In this case, Rsal, which cuts outside of the sequence analyzed, was used as a control. Therefore, all of the mutational hot spots examined were methylated in the target colon tissue, showing that these sites are targeted by MTase.

Analysis of Methytransferase Expression Levels. We showed earlier that the levels of active MTase enzyme are often markedly elevated in transformed cell lines relative to their nononcogenic counterparts (37). Also, El-Deiry et al. (35) found considerable over-expression of the MTase mRNA in colorectal tumors, a situation that might lead to an enhanced rate of MTase-induced cytosine deamination. According to our findings in the in vitro studies with the bacterial MTases M.Hhal and M.Hhal (16, 27), deamination of cytosine at the target site is dependent on the concentration of MTase and the cofactor AdoMet. Methylation reaction products, which were generated by incubation of DNA with MTase and [3H]AdoMet, were separated by agarose gel electrophoresis. A, codon 248 (CGG): CfoI cuts outside of the sequence analyzed, HpaII cuts only unmethylated CCGG, and MspI is insensitive for methylation at the CCGG site. B, codons 282 (CGG) and 283 (CCG): Rsal cuts outside of the sequence analyzed; and HpaII and CfoI cut only if their recognition sites are unmethylated.
DNA METHYLATION AND CpG TRANSITION MUTATIONS

HeLa cDNA
Patient

A.

MTase
GAPDH

B.

Fig. 3. Expression of methyltransferase mRNA in human colon tissue. Total RNA was isolated from human colon tissue (N, normal; T, tumor) and HeLa cells. RT-PCR was performed with primers specific for methyltransferase (MTase) and GAPDH, respectively, and products were separated by agarose gel electrophoresis (A). Results were summarized as the ratio of MTase:GAPDH (β).

Fig. 4. Concentration of AdoMet and AdoHcy in human colon tissue samples. Extracts from human colon carcinomas and surrounding mucosa were analyzed for AdoMet and AdoHcy using HPLC. (A-G, tissue from cancer patients; I and 2, tissue from patients without cancer).

as an internal control. The concentrations of both substances were higher in the tumors than in normal tissue, and the concentrations of AdoMet were 8.8 ± 4.4 nmol/g wet tissue in samples from normal mucosa and 30.6 ± 12.3 nmol/g wet tissue in tumor tissue (Fig. 4). Average AdoHcy concentrations were 4.2 ± 2.0 nmol/g wet tissue in normal mucosa and 10.5 ± 7.3 nmol/g wet tissue in tumor tissue. The ratio of AdoMet:AdoHcy was not significantly different comparing normal (2.1 ± 2.2) and tumor (2.9 ± 1.7) tissue. Extracts from mucosal tissue from two patients without cancer showed AdoMet and AdoHcy concentrations in the same range compared with values in normal tissue from cancer patients (Fig. 4, I and 2). We conclude from these data that AdoMet is not limiting for methylating DNA by MTase in this late stage of tumor development.

Mutational Analysis of Human Methyltransferase cDNA. Because we found adequate concentrations of AdoMet for DNA methylation in the tumor tissue, we hypothesized that a mutation in the AdoMet-binding pocket of MTase might create an enzyme that potentially inhibits efficient AdoMet binding. This could prevent efficient cytosine methylation without reducing the DNA-binding ability and thus increasing the frequency of C → T transitions at the target site. Indeed, we have recently shown that point mutations in the highly conserved domain I of the bacterial MTase M.HpaII reduce the ability of the enzymes to methylate DNA and also increase the deamination rate at the target C dramatically in vitro and in vivo (50).

Therefore, we analyzed these highly conserved regions of the MTase cDNA to test whether a similar mutation in domains coding for the AdoMet-binding pocket of the human MTase, which might lead to a mutator phenotype, could be involved in the mutational process in the colon tumors we analyzed (51, 52). RT-PCR analysis was performed using primers flanking the highly conserved regions I–III, IV–VI, and IX and X of the MTase gene (Fig. 5), which are involved in AdoMet binding and catalysis. Portions of the MTase gene were screened for mutations by SSCP. Analysis of 10 tumor samples showed no mutations by SSCP. To confirm these results, PCR products amplified from these conserved regions using cDNA derived from seven colon carcinoma samples as a template were directly sequenced. No mutations or polymorphisms were detected (data not shown). Although the number of samples analyzed was small, the data showed no evidence for a mutated MTase, which might confer a mutator phenotype in human colon cancer.

U:G Mismatch Repair. MTase-induced deamination of cytosine leads to a premutagenic U:G mismatch. Repair of this mismatch is initiated by UDG, which excises uracil, leaving an abasic site. Base
excision is the rate-limiting step in this repair pathway. Subsequently, the plasmid is able to confer kanamycin resistance to E. coli deficient in UDG. Repair activity was scored by comparing the reversion frequency of bacteria transformed with the plasmid, which was incubated with the colon extract, to bacteria transformed with the untreated plasmid (see "Materials and Methods").

In this study, we focused on mechanisms for the generation of C → T mutations and measured parameters that might favor the induction of these mutations during the DNA methylation process by human MTase (Fig. 1). This pathway, which has been validated for several bacterial MTases (16, 27–29), is particularly attractive, because it is specific for methylation sites and may be triggered by a diet low in methyl group-providing substances. The mechanism is also consistent with the observation that inactivation of MTase and reduction of methylation reduces the incidence of polyp formation in a mouse model system (38), and that levels of inactivation of MTase and reduction of methylation are lower in human colon tumours than in normal colon tissue (36). Analysis of normal and transformed colon tissue confirmed that sites that are hot spots for C → T mutations in the p53 tumour suppressor gene are methylated and were, therefore, targeted by MTase. RT-PCR analysis revealed that the average increase of MTase expression was 3.7-fold in colon carcinomas compared with normal adjacent mucosa (Fig. 3). These findings are much lower than what has been reported earlier by El-Diery et al. (35). This group found that values for MTase mRNA expression ranged up to several hundred-fold higher in human colon cancer tissue than in tissue samples from patients with benign disorders. Our data are similar to the 5.4-fold higher MTase enzyme activity detected by Issa et al. (36) in colon cancers compared with normal mucosa. In hepatomas of LEC rats, MTase mRNA expression was also only 2-fold higher compared with normal liver, and MTase activity was increased approximately 3-fold (64).

The decisive parameter that determines whether the MTase methylates its target cytosine or induces deamination (Fig. 1, upper and lower pathways) is the concentration of AdoMet available to the enzyme during the methylation process. In our analysis, AdoMet concentrations were increased in some tumours or not significantly changed in carcinoma tissue compared with normal mucosa from the same patient or from patients without cancer. Assuming that the intracellular water content is in the range of 70–85% (65, 66), the average intracellular concentration of AdoMet in normal mucosa can be calculated to be approximately 11 µM, which is similar to that which has been found in rat colon (17 nmol/mg protein; Ref. 67), and average concentrations in the carcinoma tissue were approximately 38 µM (Fig. 4). The Km of human MTase from HeLa cells for AdoMet has been determined to be 3.25 µM, and Km values of other eukaryotic MTases are in the same range (rat, 0.5–6.6 µM; Refs. 68 and 69; rice, 2.6–6 µM; Ref. 70; and wheat, 5–6 µM; Ref. 71). Although this is approximately 10–100-fold higher than the Km of bacterial MTases for AdoMet (24, 72), it is unlikely that extensive induction of cytosine deamination by MTase is possible under these high concentrations of AdoMet. In addition, we found that the concentration of AdoHcy that has been shown to inhibit the deamination reaction (27) was higher in the tumor tissue as well. It should be noted that a direct comparison of concentration data obtained from tumor and mucosal tissue is difficult, because mucosal tissue is heterogeneous, and rapidly proliferating cells are located only in the crypt base (73). Therefore, caution should be used in using MTase expression levels measured for the entire mucosa as indicators for MTase levels in colon cells at the bases of the crypts. Thus, we cannot exclude that cytosine deamination occurs as a side reaction at very low efficiency and might contribute to the burden of DNA repair. Moreover, it has been shown that a diet low in methionine, which is a direct precursor in the biosynthesis of AdoMet, and low in other methyl group-providing factors can reduce intracellular AdoMet levels and also reduce DNA methylation (40–42). Therefore, it is possible that special conditions in human tissue might result in intracellular concentrations of AdoMet below the Km of MTase, creating conditions favorable for the induction of cytosine deamination (Fig. 1, upper pathway). Because it is not possible to
follow fluctuations of AdoMet or AdoHcy concentrations over time in tissue during the initial or later stages of tumor development, a transient shortage of AdoMet might enable the MTase to induce deaminations at its target cytosine in colon tissue. However, the concentrations of AdoMet we measured in the normal colon or tumor tissue exclude the possibility that a continuous shortage of AdoMet caused by a defect in AdoMet synthesis exists in these cells. In addition, a drop of AdoMet concentration by a factor of approximately 4 to concentrations below the Km for MTase would be expected to affect all enzymes using this cofactor for transmethylation reactions and, thus, would be more cytotoxic than mutagenic for a cell.

Recently, we have shown (50) that bacterial MTases carrying mutations in the AdoMet-binding pocket, which presumably prevents efficient binding of the cofactor, can induce C → U deaminations in vitro and in vivo (Fig. 1, upper pathway) at normal concentrations of AdoMet, even in the presence of a functional UDG repair system. Therefore, we screened tumors for the presence of similar mutations in the AdoMet-binding pocket of the human MTase gene. The fact that such mutations were not observed in the colon tumors argues against this mechanism being of importance in colon carcinogenesis.

An additional factor determining whether a MTase-induced deamination reaction contributes to the generation of C → T mutations is the ability of cells to repair U/G mismatches generated by this process. Therefore, we measured the ability of colon extracts to repair these mismatches. Excision of uracil was efficient in all samples tested (Fig. 6; Ref. 23). Moreover, UDG expression has been shown to be maximal in S-phase (74, 75), when MTase activity peaks. Although it is unlikely that MTase forms a tight complex with the U/G mismatch formed by the deamination reaction, or that a small fraction of uracil gets methylated by MTase, thus escaping repair by UDG (16, 76), the high efficiency of UDG repair found in the human colon extracts makes it unlikely that a U/G mismatch persists in DNA until replication completes the C → T mutation.

None of the parameters tested suggest that biochemical conditions favor a MTase-induced pathway for the formation of C → U → T transitions in human tissue (Fig. 1, upper pathway). Spontaneous deamination of 5mC, therefore, is the most likely mechanism by which the majority of these mutations are formed (Fig. 1, lower pathway). Nevertheless, it is difficult to exclude the possibility that low-efficiency side reactions of enzymatic methylation do not contribute to the generation of these mutations. The mechanisms by which methyl-deficient diets contribute to carcinogenesis and the reason for the decreased number of polyps formed in transgenic mice with lower MTase levels (38) may, therefore, be best explained by an epigenetic pathway.

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