Cytosine deaminations catalyzed by DNA cytosine methyltransferases are unlikely to be the major cause of mutational hot spots at sites of cytosine methylation in *Escherichia coli*

(C to T mutations/uracil DNA-glycosylase)

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ABSTRACT Sites of cytosine methylation are hot spots for C to T mutations in *Escherichia coli* DNA. We have developed a genetic reversion assay that allows direct selection of C to T mutations at a site of methylation. Because the mutant gene is on a plasmid, this system can be used to study mutational effects of biochemical agents *in vitro* as well as *in vivo*. Using this system we show that *in vitro* an *E. coli* methyltransferase can cause C to U deaminations at a site of methylation. Reaction conditions that are known to inhibit a side reaction of the methyltransferase also suppress reversion frequency, suggesting that this side reaction is required for deamination. Furthermore, a mutation in the enzyme that eliminates its catalytic activity but not its ability to bind DNA eliminates the ability of the enzyme to cause C to U deaminations. Despite this, *in vivo* experiments strongly suggest that enzyme-catalyzed deaminations of cytosine do not play a major role in making methylation sites in *E. coli* hot spots for mutations. For example, although uracil-DNA glycosylase (Ung) suppresses the occurrence of mutations due to C to U deaminations, the frequency of C to T mutations at a methylation site remains high in *ung*⁺ cells. Furthermore, the reversion frequencies in *ung*⁺ and *ung*⁻ cells are quite similar.

In *Escherichia coli*, the chromosomally coded DNA (cytosine-5-)-methyltransferase [Dcm; S-adenosyl-L-methionine:DNA (cytosine-5-)-methyltransferase, EC 2.1.1.37] methylates position 5 of the second cytosine in the sequence 5'-CCWGG-3' [W is A or T (1, 2)]. Analysis of spontaneous amber mutations in *E. coli* genes has revealed that cytosines methylated by Dcm frequently mutate to thymine (3, 4). Such C to T mutations at sites of methylation are more frequent than any other substitutions at any other base in the gene (3). Because 5-methylcytosine (5MeC) in DNA is known to deaminate to thymine (5, 6), these data have led to the hypothesis that C to T mutations at sites of methylation arise due to the spontaneous hydrolytic deamination of 5MeC in DNA (3).

Recently it was suggested that enzymes that methylate cytosine can cause C to U deaminations (7, 8). This suggestion was based on the similarity of an intermediate in bisulfite-catalyzed deamination of cytosine to a product formed by a side reaction of the cytosine methyltransferases (MTases). Bisulfite increases the rate of deamination of cytosine by adding across the 5,6 double bond and thus reducing conjugation within the pyrimidine ring (9). Cytosine MTases initiate the methyl transfer reaction by attacking C6 of cytosine and creating a negative charge at position 5 (10). If the methyl donor S-adenosylmethionine (SAM) is left out of the reaction, the carbanion can acquire a proton from the surrounding medium to neutralize the charge (11, 12). The

resulting 5-dihydrocytosine derivative is equivalent to the reaction intermediate of bisulfite and hence may be susceptible to deamination. Normally, uracil produced in this manner would be replaced with cytosine by a DNA repair process in which uracil is excised from DNA by a glycosylase (uracil DNA-glycosylase, Ung). If Ung fails to remove this uracil, a C to T mutation would occur.

Recently, experiments were done with *Hpa* II MTase (*M.HpaII*) to test this hypothesis. That study made use of a genetic system in which C to T mutations at a site of methylation by *M.HpaII* could be directly selected for in a reversion test (8). This system was used to show that under certain *in vitro* conditions *M.HpaII* can interact with a site of methylation in free DNA to cause C to U deaminations (8). When this DNA was introduced into *Ung*⁻ *E. coli*, C to T mutations occurred at the site. These results were used to argue that such deaminations may occur in tumor cells and that this side reaction of C5 MTases may play a role in the generation of C to T mutations at sites of methylation.

Independently, we developed a similar genetic system to study the occurrence of C to T mutations at Dcm sites in *E. coli*. Using this system, we have studied the ability of a MTase that methylates Dcm sites to deaminate cytosine in free DNA and the relationship of this enzymatic reaction to mutagenesis at Dcm sites in cells.

MATERIALS AND METHODS

Strains and Plasmids. PUC4-KIXX was from Pharmacia and pUC118 was from our stocks. *E. coli* strains CJ236 (F'Cm^R, *dut-1 ung-1 thi-1 relA1*), GM31 (*dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 galE2 xyl-5 thi-1 mtl-1*), and GM30 (GM31, *dcm*⁺) are from our collection. GM31*lung*⁻ (GM31, *ung-1 tyrA::Tn10*) was obtained from M. Lieb (University of Southern California School of Medicine).

Site-Directed Mutagenesis and Characterization of Mutants. The kanamycin-resistance (Kan^R) cassette from pUC4-KIXX (Pharmacia LKB) was released by *EcoRI* digestion and inserted into pUC118 to create pKanR-WT. Site-directed mutagenesis was performed essentially as described by Kunkel (13). CJ236 (pKanR-WT) cells were infected with the phage R408 and single-stranded DNA was purified (14). Mutagenic primer 5'-GACTGGCTGCTACCAGGCCGAAGT-GCC-3' was used with this DNA to introduce the mutation.

This plasmid was introduced into GM30 and independent Kan^R revertants were isolated. Plasmid from one of the revertants, pKanR-Rev, was subject to sequence analysis.

Abbreviations: 5MeC, 5-methylcytosine; MTase, methyltransferase; SAM, S-adenosylmethionine; *M.HpaII*, *HpaII* MTase; Kan^R, kanamycin resistance; Kan^S, kanamycin sensitive; WT, wild type; *M.EcoRII*, *EcoRII* MTase; SAH, S-adenosylhomocysteine.

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Sequencing was carried out with a thermal cycling kit (GIBCO/BRL). The primer used for sequencing was complementary to bases -48 through -68 relative to codon 94 and was labeled at the 5' end with ^{32}P .

Purification of Wild-Type (WT) and Mutant *EcoRII* MTase (*M.EcoRII*). Purification of the WT *M.EcoRII* using a cellulose phosphate column has been described (15). The enzyme was further purified to apparent homogeneity by successive chromatography on DEAE-Sephacel, FPLC Superdex 75, and heparin Sepharose columns. The Cys-186 to Ala (C186A) mutant of *M.EcoRII* was purified similarly, except that this mutant was coexpressed in the host cell with the *Mva* I MTase. The *Mva* I MTase was expressed from the multicopy plasmid pBC1018 provided by A. Janulaitis (Fermentas, Vilnius, Lithuania) to overcome cell-growth inhibition caused by the mutant (16).

Assay for Enzyme-Catalyzed Deamination of Cytosine. One microgram of pKanS-D94 DNA purified from GM31 was incubated with 0.9 μg (unless specified otherwise) of *M.EcoRII* (specific activity, 5.9 pmol of methyl groups transferred per min per μg of enzyme) in 1 $\times$ MTase buffer (100 mM Tris-HCl, pH 7.8/20 mM EDTA, pH 8.0/0.4 mM dithiothreitol) in a total vol of 80 μl . Incubations were carried out at 37°C and the reaction was stopped at different times by adding SDS to 0.5%. After spin dialysis through a 1-ml G-50 column, the samples were extracted once with phenol/chloroform and once with chloroform and spin dialyzed again. The DNA was dried and dissolved in 20 μl of TE buffer (10 mM Tris-HCl, pH 7.8/0.1 mM EDTA, pH 8.0), and 1.5 μl of this DNA was electroporated into *E. coli* cells by using the Cell-Porator *E. coli* pulser (GIBCO/BRL). Cell cultures were plated onto LB plates with carbenicillin (50 $\mu\text{g}/\text{ml}$) or kanamycin (50 $\mu\text{g}/\text{ml}$). Reversion frequency is defined as the ratio between Kan^R transformants and carbenicillin-resistance transformants.

Assay for C to T Mutations *in Vivo*. PR215 has been described (17). PDCM72 was constructed by cloning a *Bam*HI/*Sph*I fragment from pDCM35 (18) into pACYC184. To determine reversion frequencies, GM31 or GM31*lung*⁻ carrying pKanS-D94 was transformed with pACYC184, pDCM72, or pR215. Three independent colonies were picked from each transformation and 5-ml cultures were grown to logarithmic phase ($\text{OD}_{550} = 0.3$) in LB medium containing carbenicillin (50 $\mu\text{g}/\text{ml}$) and chloramphenicol (20 $\mu\text{g}/\text{ml}$) (for pACYC184 or pDCM72) or tetracycline (12.5 $\mu\text{g}/\text{ml}$) (for pR215). One milliliter of each culture was added to 3 ml of top agar containing kanamycin (50 $\mu\text{g}/\text{ml}$) and was poured onto plates with kanamycin (50 $\mu\text{g}/\text{ml}$). The total numbers of viable cells in the cultures were determined on LB plates.

RESULTS

A Genetic System to Study 5MeC to T Mutations. To study C to U or 5MeC to T deaminations at sites of Dcm methylation in *E. coli*, we developed a reversion assay that involves the Kan^R gene from transposon Tn5. The principle of the assay is illustrated in Fig. 1. Site-directed mutagenesis was used to introduce a site for Dcm methylation at codon 94 of the gene causing a leucine to proline change in the protein sequence. While cells carrying the WT gene on a multicopy plasmid (pKanR-WT) were resistant to kanamycin (>100 $\mu\text{g}/\text{ml}$), cells carrying the plasmid with the mutant allele (pKanS-D94) were sensitive to as little of the antibiotic as 2 $\mu\text{g}/\text{ml}$. It was expected that substitution of the cytosine that is the substrate for Dcm to thymine would restore the leucine codon and the Kan^R phenotype.

This was confirmed by introducing pKanS-D94 into a Dcm⁺ strain, growing cells carrying this plasmid in the absence of kanamycin, and selecting for revertants on kanamycin-containing plates. Nine Kan^R colonies were picked

FIG. 1. Principle of the genetic assay for C to T mutations at a Dcm site. Codon 94 (TTG) of the Kan^R gene of Tn5 was changed to CCA by site-directed mutagenesis. The newly created Dcm site is underlined. Substitution of the cytosine that is the target for methylation by thymine creates a new codon for leucine at position 94 (CTA) and restores the activity of the enzyme coded by the Kan^R gene.

and their plasmids were analyzed. A C to T change at codon 94 is expected to result in the loss of a *Bst*NI site, and this was confirmed for all the Kan^R revertants (data not shown). Based on this result we conclude that most of the revertants obtained in our reversion assay contain the expected change in codon 94.

The mutation in one of the revertants was identified by DNA sequencing. As expected, the second cytosine within the Dcm site at codon 94 in pKanS-D94 was changed to thymine in pKanR-Rev (Fig. 2). It should be noted that the sequence of the revertant differs from the WT sequence (Fig. 1), and hence the observed revertant could not have arisen as a result of contamination of pKanS-D94 with pKanR-WT.

***M.EcoRII* Can Cause C to U Transitions at a Site of Methylation.** The pathway by which C5 MTases may promote C to U conversion is illustrated in Fig. 3. The conversions can occur if structure I deaminates (Fig. 3). However, when the enzyme is carrying out transfer of methyl groups to C5, structure I should occur infrequently and C to U conversion should be rare. If SAM is omitted from the reaction the formation of structure II is prevented, causing an increase in the occurrence of structure I (Fig. 3). For this reason, the ability of C5 MTases to promote C to U deaminations was tested in the absence of SAM.

M.EcoRII methylates the same cytosine in DNA as Dcm (1, 2) and is a close homolog of the chromosomally coded MTase (18). It was used in these experiments instead of Dcm because it was available in purified form. Plasmid pKanS-D94 was incubated with excess purified *M.EcoRII* for various lengths of time, and the DNA was introduced into *E. coli* by electroporation. To avoid the removal of uracil by Ung, an *ung*⁻ strain was used as the recipient during electroporation.

FIG. 2. Sequence changes in the Kan^S mutant and its revertant. Portions of a sequencing gel containing the region of the sequence surrounding codon 94 are shown. The relevant sequence from each segment of the gel is indicated to the immediate right of the segments. The cytosine at the third position in codon 95 and the guanine at the first position in codon 96 gave rise to weak bands in the WT sequencing ladder and failed to reproduce in the photograph.

FIG. 3. Relationship between the mechanism of methyl transfer to cytosine and deamination of cytosine. The mechanism of methyl transfer by C5 MTases (10) and the proposed mechanism for enzyme-catalyzed deamination of cytosine (7) are shown. The enzyme is indicated as $E-S^-$. SAH, S-adenosylhomocysteine.

The transformants were scored on plates containing carbenicillin or kanamycin to detect the reversion.

Incubation of pKanS-D94 with *M.EcoRII* prior to transformation led to a time-dependent increase in transformants that were Kan^R (Fig. 4). In as little as 10 min of incubation, a 20-fold increase in reversion frequency was observed and after 5 hr of incubation, a 1000-fold increase in reversion frequency was observed (Fig. 4). In contrast, when the same DNA was transformed into *ung*⁺ cells, a complete suppression of reversion was observed (Fig. 4). These observations are consistent with the hypothesis that *M.EcoRII* can convert the target C to U. Similar results have been reported for *M.HpaII* (8).

Formation of a 5-Dihydrocytosine Intermediate Is Required for Deamination. The proposed mechanism for the C5 MTase-catalyzed deaminations (Fig. 3) predicts that preventing the formation of structure I should suppress the kanamycin-sensitive (Kan^S) to Kan^R reversion frequency. We demonstrated this in three different ways. When SAM was included

FIG. 4. C to U deaminations caused by *M.EcoRII*. pKanS-D94 DNA was treated with *M.EcoRII*, the treated DNA was transformed into *E. coli*, and transformants were scored. *M.EcoRII* was present at 3.4-fold molar excess in the reaction (3.4 enzyme molecules per site of methylation). GM31 (*ung*⁺) and its *ung*⁻ derivative were used as recipients for the transformation.

in the reaction mixture, *M.EcoRII*-induced reversion was abolished at concentrations of the coenzyme above 0.25 μ M (Fig. 5). We interpret this to mean that the formation of structure I is inhibited by SAM and that structure II is too short-lived for creation of thymine through hydrolytic deamination (Fig. 3).

SAH is one of the products of the methyl transfer reaction and it has been found to inhibit the formation of the 5-dihydro intermediate by *HhaI* MTase (11, 12). Although the reasons for this effect remain unclear, we recently found that SAH also inhibits the formation of structure I by *M.EcoRII* (S.G. and A.S.B., unpublished results). When SAH was present during the incubation of *M.EcoRII* with pKanS-D94, significant reduction in reversion frequency was seen at SAH concentrations as low as 0.63 μ M; at SAH concentrations of a few micromolar, enzyme-induced reversion to Kan^R was completely eliminated (Fig. 5).

We also tested a mutant of *M.EcoRII* in which the catalytic cysteine in the enzyme is replaced by alanine. This mutant is capable of sequence-specific binding to substrate DNA, but it is unable to initiate the catalytic reaction (ref. 15; unpub-

FIG. 5. Inhibition of *M.EcoRII*-catalyzed deamination of cytosine by SAM and SAH. pKanS-D94 DNA was incubated with *M.EcoRII* for 5 hr in the presence of various concentrations of SAM or SAH and treated DNA was electroporated into *ung*⁻*E. coli*. The Kan^R reversion frequency at various concentrations of the inhibitor is represented relative to reversion frequency when no inhibitor was present in the reaction (1.04×10^{-4}).

Table 1. Mutant of *M.EcoRII* defective in deaminating cytosines

Protein	Ratio, protein/sites*	Reversion frequency†	Relative reversion
<i>M.EcoRII</i> (WT)	3.4	$1.5 (\pm 1.4) \times 10^{-3}$	1
<i>M.EcoRII</i> (C186A)	13.6	$1.1 (\pm 0.4) \times 10^{-5}$	0.0073 ± 0.0028
None	0	$6.2 (\pm 5.1) \times 10^{-6}$	0.0041 ± 0.0034

*Ratio of mol of protein to mol of sites for methylation in pKanS-D94 DNA in the reaction.

†Frequency is presented as the arithmetic mean ($\pm$ SD) from three independent trials.

lished results). When pKanS-D94 DNA was incubated with an excess of purified mutant protein and the DNA was electroporated into *ung*⁻ cells, little increase in the Kan^R reversion frequency over background was detected (Table 1). Therefore, the ability of the enzyme to attack C6 of the substrate cytosine is essential for its mutagenic effect.

Frequency of C to T Transitions at a Dcm Site in pKanS-D94 Remains High in an *ung*⁺ Strain. To determine whether methylation at a Dcm site increases the frequency of C to T mutations, the plasmid pKanS-D94 was introduced in a strain deficient in Dcm. In separate experiments, a second plasmid carrying the gene for Dcm or *M.EcoRII* was also introduced in the cells. The presence of Dcm or *M.EcoRII* in the cells caused a 100-fold or a 38-fold increase in the Kan^R reversion frequency, respectively (Table 2). This extends previous observations regarding the existence of high mutation frequencies at Dcm sites in *E. coli* genes and provides a quantitative estimate for the effect. It should be noted that the high mutation rate at the Dcm site is not the result of overproduction of the MTases from multicopy plasmids (19). A high reversion frequency was also seen when pKanS-D94 was introduced in cells that contained the *dcm*⁺ gene in the chromosome. In this case, the reversion frequency in the *dcm*⁺ strain was 17 times higher than in a *dcm*⁻ strain (data not shown). These results clearly show that C5 MTases substantially increase the frequency of C to T mutations at sites of methylation.

These data also show that C to U deaminations may not play a major role in increasing the frequency of transition mutations at Dcm sites *in vivo*. The host used in the *in vivo* experiments was *ung*⁺ and any uracil created by the enzymes should have been removed by Ung (Table 2). Furthermore, when the plasmid carrying the *dcm*⁺ gene was introduced into an *ung*⁻ strain, the Kan^R reversion frequency was comparable to that found in the corresponding *ung*⁺ strain (Table 2, compare lines 2 and 5). This is in contrast to results from experiments in which the pKanS-D94 DNA was incubated with *M.EcoRII* in the absence of coenzyme to cause C to U deaminations and the DNA was electroporated into *ung*⁺ or *ung*⁻ cells (Fig. 3). Therefore, we conclude that although *M.EcoRII* (and presumably Dcm) can catalyze C to U deaminations *in vitro*, such events do not make a major contribution to C to T mutations at sites of methylation *in vivo*.

DISCUSSION

Studies of the distribution of amber mutations in the *lacI* gene of *E. coli* led to the original observation that sites of cytosine

methylation in DNA are hot spots for C to T mutations (3). This observation has been confirmed in a similar study of another *E. coli* gene (4) and in the compilations of mutations in human genes that result in genetic diseases (20). In mammals, C to T mutations are thought to be responsible for the depletion of CG dinucleotides from most vertebrate genomes (20–22). Using a different genetic system we have extended this finding to the gene that codes for Kan^R and have shown that the presence of a MTase in *E. coli* capable of methylating Dcm sites increases the frequency of C to T mutations at Dcm sites by 1–2 orders of magnitude.

This system can also be used to estimate the relative frequencies of C to U and 5MeC to T changes at sites of cytosine methylation. Our data suggest that the latter conversion occurs at 2.3 times the frequency of the former (Table 2, compare lines 4 and 5). Although the *in vitro* frequencies of C to U and 5MeC to T deaminations in double-stranded DNA have not been determined, measurements with nucleotides (5) and single-stranded DNA (6) have suggested that this ratio should be ≈ 4 .

Although the frequencies of Kan^R reversion induced by plasmids carrying genes for Dcm and *M.EcoRII* were significantly different (Table 2), these differences may be the result of the fact that the two plasmids were maintained in cells by using different antibiotics or may be due to different levels of expressions of the two genes rather than any intrinsic differences between the enzymes. Also, results from these experiments cannot be easily extrapolated to effects of chromosomally produced Dcm on rates of mutations at Dcm sites in the *E. coli* chromosome. The presence of the Kan^S gene on a multicopy plasmid is expected to increase the observed reversion frequency.

We have also used this genetic system to show that *M.EcoRII* can convert the target C to U. The inhibition of deamination by SAM and SAH, and the absence of significant deamination by the C186A mutant of *M.EcoRII*, is consistent with the hypothesis that the formation of a 5-dihydrocytosine intermediate (Fig. 3, structure I) is required for this process to take place. It should be noted that the enzyme does form the negatively charged intermediate in the presence of SAH (15). Therefore, formation of this intermediate must not be sufficient to cause C to U deamination. These results are consistent with the results reported for *M.HpaII* (8). The results from the two studies and the generality of the proposal for the mechanism of cytosine methylation (10) suggest that all C5 MTases should be capable of carrying out deamination of the target cytosine.

Our study of *M.EcoRII*-catalyzed deaminations differs from the study of a similar reaction by *M.HpaII* (8) in that we have also explored the effect of *M.EcoRII* on mutagenesis *in vivo*. Using the same plasmid that was used as the test plasmid for the experiments *in vitro*, we have shown that frequency of transition mutations at the Dcm/*EcoRII* site increases when either Dcm or *M.EcoRII* is present in the cells (Table 2). In this way, it was possible to explore the relationship between enzyme-catalyzed cytosine deaminations with the occurrence of mutational hot spots at sites of DNA methylation. When *ung*⁺ cells were used for *in vivo* experiments, any uracil produced by the MTases should have been replaced with

Table 2. Effect of MTases on mutations at a Dcm site *in vivo*

Plasmids*	Host	Reversion frequency†	Relative reversion
pKanS-D94 and pACYC184	<i>ung</i> ⁺	$3.4 (\pm 1.0) \times 10^{-8}$	1
pKanS-D94 and pDCM72	<i>ung</i> ⁺	$3.4 (\pm 0.5) \times 10^{-6}$	100
pKanS-D94 and pR215	<i>ung</i> ⁺	$1.3 (\pm 0.3) \times 10^{-6}$	38
pKanS-D94 and pACYC184	<i>ung</i> ⁻	$1.0 (\pm 0.4) \times 10^{-6}$	1
pKanS-D94 and pDCM72	<i>ung</i> ⁻	$2.3 (\pm 0.3) \times 10^{-6}$	2.3

*pDCM72 and pR215 contain the *dcm*⁺ and *M.EcoRII* genes, respectively.

†Frequency is presented as the arithmetic mean ($\pm$ SD) from three independent trials.

cytosine by the Ung-mediated repair process. Despite this, the frequency of C to T mutations at the Dcm site at codon 94 was increased substantially by the presence of a cognate MTase in the cells. Furthermore, the reversion frequencies in *ung*⁺ and *ung*⁻ cells were quite similar (Table 2).

These results can be interpreted in one of two ways. One possible explanation of these results is that cytosine methylases do cause C to U deaminations at codon 94 *in vivo*, but the resulting uracil is inaccessible to Ung. Alternatively, enzyme-catalyzed (or spontaneous) C to U deaminations may not be the principal cause of the hot spots at sites of cytosine methylation. For example, these mutations may arise due to spontaneous (or enzyme catalyzed) deaminations of 5MeC in DNA to T after completion of the methyl transfer reaction.

We prefer the latter hypothesis for several reasons. First, there is no evidence to support the idea that uracil within codon 94 is protected from the action of Ung. In fact, evidence to the contrary exists. When pKanS-94D DNA that was previously incubated with M.EcoRII was electroporated into the *ung*⁺ strain, the increase in the Kan^R reversion frequency was abolished (Fig. 4). The *ung*⁺ in GM31 was also found to work efficiently in the *in vivo* studies. In the absence of Dcm, the frequency of reversion was 29 times lower in the *ung*⁺ strain than in the *ung*⁻ strain (Table 2, compare lines 1 and 4). There is also direct evidence that the *ung*⁺ strain used in our experiments corrects U-G mismatches to C-G. A U-G mismatch was created at codon 94 by hybridizing a uracil-containing synthetic oligomer to single-stranded DNA from pKanS-D94, and double-stranded DNA was created by enzymatic DNA synthesis followed by ligation. When this DNA was electroporated into *E. coli*, the frequency of Kan^R reversion was substantially lower when *ung*⁺ cells were used as recipients than when *ung*⁻ cells were used (23).

The observed inhibition of MTase-catalyzed C to U deaminations by SAM and SAH also makes it unlikely that this reaction occurs frequently inside cells. Both M.EcoRII and M.HpaII are unable to cause deaminations in the presence of submicromolar concentrations of SAM (Fig. 5 and ref. 8). As the concentration of SAM in *E. coli* is at least 50 μ M (24, 25), intracellular SAM should effectively suppress enzyme-catalyzed deaminations. Furthermore, when the intracellular concentration of SAM drops to $\approx 0.5\%$ of the normal value, growth of *E. coli* is inhibited (25). Therefore, if the *in vivo* levels of SAM in *E. coli* were lowered to the point where enzyme-catalyzed C to U deaminations could occur, cells would cease to divide. In our studies, cells were grown in a rich medium and exponentially growing cells were used for the reversion test.

As noted earlier, Ung-mediated repair of U-G mismatches to C-G is expected to prevent C to U deaminations from contributing to Kan^R reversions *in vivo*. Recently, we found that another mismatch correction system in *E. coli* also corrects U-G mismatches to C-G. Very short patch (VSP) repair is known to correct T-G mismatches that arise when the cytosine that is methylated by Dcm is replaced by thymine (26, 27). We have found that VSP repair can also correct U-G mismatches to C-G (23). Therefore, VSP repair presents an additional barrier to enzyme-catalyzed C to U deaminations from contributing to transition mutations at Dcm sites. It should be noted that the *E. coli* host GM31 used in our experiments contains mutations that result in defective Dcm and an inability to carry out VSP repair (28–31).

The demonstration that C5 MTases catalyze deaminations of substrate C to U represents a strong confirmation of the proposed reaction mechanism of these enzymes (10). It illustrates the predictive capabilities of the mechanism and provides a sensitive genetic test to study an intermediate in

the methyl transfer reaction. But currently there is no evidence to support the idea that such enzyme-catalyzed generation of uracil contributes to transition mutations at sites of cytosine methylation. In fact, the results presented here and in previously published work on M.HpaII (8) strongly suggest that cytosine MTases are unlikely to catalyze C to U deaminations in healthy WT cells. Furthermore, *E. coli* carries multiple repair pathways that can replace any uracil that may arise due to enzyme-catalyzed deaminations of cytosine. Hence 5MeC to T deaminations remain as the most likely cause of mutational hot spots at sites of cytosine methylation in *E. coli*.

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