Enzyme-mediated cytosine deamination by the bacterial methyltransferase M.*Msp*I

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Most prokaryotic (cytosine-5)-DNA methyltransferases increase the frequency of deamination at the cytosine targeted for methylation *in vitro* in the absence of the cofactor *S*-adenosylmethionine (AdoMet) or the reaction product *S*-adenosylhomocysteine (AdoHcy). We show here that, under the same *in vitro* conditions, the prokaryotic methyltransferase, M.*Msp*I (from *Moraxella* sp.), causes very few cytosine deaminations, suggesting a mechanism in which M.*Msp*I may avoid enzyme-mediated cytosine deamination. Two analogues of AdoMet, sinefungin and 5′-amino-5′-deoxyadenosine, greatly increased the frequency of cytosine deamination mediated by M.*Msp*I presumably by introducing a proton-donating amino group into the catalytic centre, thus facilitating the formation of an unstable enzyme–dihydrocytosine intermediate and hydrolytic deamination. Interestingly, two naturally occurring analogues, adenosine and 5′-methylthio-5′-deoxyadenosine, which do not contain a proton-donating amino group, also weakly increased the deamination frequency by M.*Msp*I, even in the presence of AdoMet or AdoHcy. These analogues may trigger a conformational change in the enzyme without completely inhibiting the access of solvent water to the catalytic centre, thus allowing hydrolytic deamination of the enzyme–dihydrocytosine intermediate. Under normal physiological conditions the enzymes M.*Hpa*II (from *Haemophilus parainfluenzae*), M.*Hha*I (from *Haemophilus hemolytica*) and M.*Msp*I all increased the *in vivo* deamination frequency at the target cytosines with comparable efficiency.

INTRODUCTION

The target cytosines of (cytosine-5)-DNA methyltransferases (Mtases) are frequently mutated to thymine in both pro- and eukaryotes [1–5]. This increased frequency of transition mutations accompanied the evolution of all species harbouring an enzyme with Mtase activity [6] and leads in humans to the observed high frequency of C → T transition mutations in genetic disease and cancer [7–10].

Several mechanisms have been proposed to explain the high frequency of transition mutations at the target cytosines of Mtases. The spontaneous hydrolytic deamination frequency for 5-methylcytosine (5-mC) → T in double-stranded DNA is 2–3-fold higher than that for C → U [11]. The deamination rate at 5-mC is 4–9-fold higher than at C in single-stranded DNA [12,13], and the transient formation of single-stranded DNA during transcription increases the deamination frequency of cytosines on the untranscribed strand [14]. Several prokaryotic Mtases increase the rate of C → U and 5-mC → T deamination at the cytosine targeted for methylation *in vitro* in the absence of the cofactor *S*-adenosylmethionine (AdoMet) and the reaction product *S*-adenosylhomocysteine (AdoHcy) [15–18]. Conformational changes introduced into the AdoMet-binding pocket of Mtase M.*Hpa*II (from *Haemophilus parainfluenzae*) that interfere with efficient AdoMet binding can lead to enzymes with higher C → U deamination frequencies, even in the presence of physiological concentrations of AdoMet and AdoHcy [19]. Two analogues of AdoMet, sinefungin and 5′-amino-5′-deoxyadenosine, can increase the rate of enzyme-mediated deamination, even in the presence of AdoMet and AdoHcy [18].

On the other hand, differences in the G:U and G:T mismatch repair efficiencies can also contribute to the high frequency of transition mutations at methylated cytosines, since they determine the rate of fixation of deaminated C and 5-mC. Specific repair systems such as the very-short-patch (vsp) repair system in bacteria [20] and the G/T glycosylase in mammals [21] have evolved to recognize and repair the G:U and G:T mismatches generated at the target cytosines of Mtases. In non-replicating bacteria, 5-mC is not a mutation hotspot, suggesting that the vsp repair pathways are not fast enough in replicating bacteria so that deaminated C and 5-mC can propagate unrepaired [22]. Since the bacterial uracil DNA glycosylase recognizes uracil in looped and damaged DNA inefficiently, certain G:U mismatches in specific sequences may remain unrepaired [23–25]. The M.*Hha*I (from *Haemophilus hemolytica*) and M.*Hpa*II (from *H. parainfluenzae*) Mtases bind G:U mismatches and abasic sites with high affinity and can interfere with their repair [26,27].

We compare here three bacterial Mtases, M.*Hpa*II, M.*Hha*I and M.*Msp*I, for their ability to induce enzyme-mediated cytosine deamination in the absence of the cofactor AdoMet or the reaction product AdoHcy. Furthermore, the effects of several naturally occurring and artificial analogues of AdoMet and AdoHcy on the DNA binding efficiency and enzyme-mediated cytosine-deamination frequency of M.*Hpa*II, M.*Hha*I and M.*Msp*I were measured. We also determine whether the failure of M.*Msp*I to induce *in vitro* enzyme-mediated C → U transition

Abbreviations used: (dc-)AdoMet, (decarboxylated) *S*-adenosylmethionine; (dc-)AdoHcy, (decarboxylated) *S*-adenosylhomocysteine; 5-mC, 5-methylcytosine; MTA, 5′-methylthio-5′-deoxyadenosine; Mtase, (cytosine-5)-DNA methyltransferase; vsp, very short patch; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.

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mutations results also in a lower *in vivo* mutation frequency when compared with that induced by other enzymes.

MATERIALS AND METHODS

Bacteria

The *Escherichia coli* strain ER2357 [*end*A1 *thi*-1 *sup*E44 *mcr*-67 *ung*-1 *dut* Δ(*arg*F-*lac*)U169 Δ (*mcr*C-*mrr*)114::IS10 *rec*A1 F′ *pro*AB *lac*I^q ZΔM15 *zzf*::Tn10(Tet^r)] was kindly provided by Dr. Sha Mi (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.).

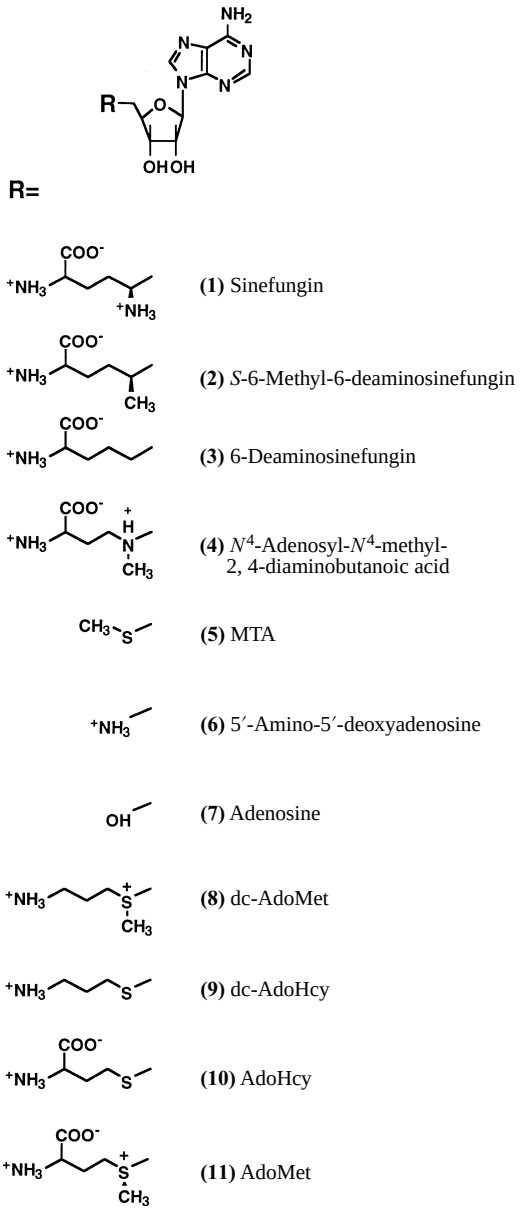

Figure 1 Chemical structure and numbering system of analogues used in this work

During the methylation reaction a methyl group is enzymically transferred from the cofactor AdoMet (**11**) to C-5 of cytosine, thus leading to the reaction product AdoHcy (**10**). Compounds **1–9** are analogues of either the cofactor AdoMet (**11**) or the reaction product AdoHcy (**10**). The chemical structures are drawn under physiological conditions (pH 7.5) and the stereochemistry is indicated if known. Analogues (**5**), (**7**), (**8**), (**9**), AdoHcy (**10**) and AdoMet (**11**) occur naturally in mammalian cells, and sinefungin (**1**) occurs naturally in the soil bacteria *Streptomyces incarnatus* and *Streptomyces griseolus* [55,56].

Materials

The structure and numbering system of the cofactor analogues used in the present study is shown in Figure 1. AdoMet (**11**), M.*Msp*I, M.*Hpa*II and M.*Hha*I Mtases were purchased from New England Biolabs (Beverly, MA, U.S.A.), AdoHcy (**10**), 5′-methylthio-5′-deoxyadenosine (MTA) (**5**), 5′-amino-5′-deoxyadenosine (**6**) and adenosine (**7**) were purchased from Sigma. Sinefungin (**1**), *S*-6-methyl-6-deaminosinefungin (**2**) and 6-deaminosinefungin (**3**) were kindly provided by Dr. H. Rapoport (Department of Chemistry, University of California, Berkeley, CA, U.S.A.) [28]. N^4-Adenosyl-N^4-methyl-2,4-diaminobutanoic acid (**4**) was kindly provided by Dr. A. Gall (Oridigm Corporation, Seattle, WA, U.S.A.) [29]. Decarboxylated AdoMet (dc-AdoMet) (**8**) and decarboxylated AdoHcy (dc-AdoHcy) (**9**) were kindly provided by Dr. J. Wagner (Merrell Dow Research Institute, Strasbourg, France) [30]. All analogues were dissolved in water and concentrations confirmed spectrophotometrically by their A_{260}.

Plasmid construction

The *Hin*dIII/*Hpa*I fragments from the plasmids C**C**GG-pSV2-neo^s and G**C**GC-pSV2-neo^s [15,19,26] containing the mutant neomycin genes were cloned into the *Hin*dIII/*Eco*RV site of pACYC184, leading to C**C**GG-pAC-neo^s and G**C**GC-pAC-neo^s respectively (Figure 2). Plasmid C**C**GG-pAC-neo^s was reverted by incubation with M.*Hpa*II to wild-type C**T**GG-pAC-neo^r and then subjected again to PCR-mediated mutagenesis to generate a new plasmid, **C**CGG-pAC-neo^s, which reverts to wild-type by deamination of the first C (Figure 2). Briefly, two PCR fragments were generated with the primer pair: neoleft (5′-GCAAGAGATTACGCGCAGACC-3′) and neomut1 (5′-GAC-TGGCTGCTACCCGGCGAAGTGC-3′) and the primer pair neomut2 (5′-GCACTTCGCCGGGTAGCAGCCAGTC-3′) and neoright (5′-TCAGGCAGCAGCTGAACCAAC-3′).

The two fragments generated were gel-purified, combined and the secondary PCR was performed with the above outside primers neoleft and neoright. The fragment generated was cut with *Xba*I/*Sma*I and cloned into the *Xba*I/*Sma*I sites of the reverted wild-type plasmid C**T**GG-pAC-neo^r. The resulting plasmid **C**CGG-pAC-neo^s was tested by incubation with M.*Msp*I in the presence of sinefungin (**1**) and the reversion to plasmid **T**CGG-pAC-neo^r confirmed by sequencing. The plasmids pUHE-M.*Hpa*II, pUHE-M.*Msp*I and pUHE-M.*Hha*I were kindly provided by Dr. Sha Mi [31].

Gel-mobility-shift assay

The gel-mobility-shift assay has been described previously [18,26]. End labelling was done by filling in the ends of the oligonucleotides with 5′-[α-^{32}P]CTP and sequenase (Amersham). The following oligonucleotides containing a M.*Msp*I target sequence (underlined) were used:

Upper strand:
5′-GCAGTCGCGATGCCGGGTCACCTTGAG-3′

Lower strand:
3′-GTCAGCGCTACGGCCCAGTGGAACTCG-5′

Briefly, the DNA-binding reaction was performed by incubating Mtase (M.*Msp*I, 5 units), 4 pmol of labelled oligonucleotides, 40 pmol of non-specific oligonucleotide [18] in 10 μl of buffered [50 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 mM EDTA/13 %

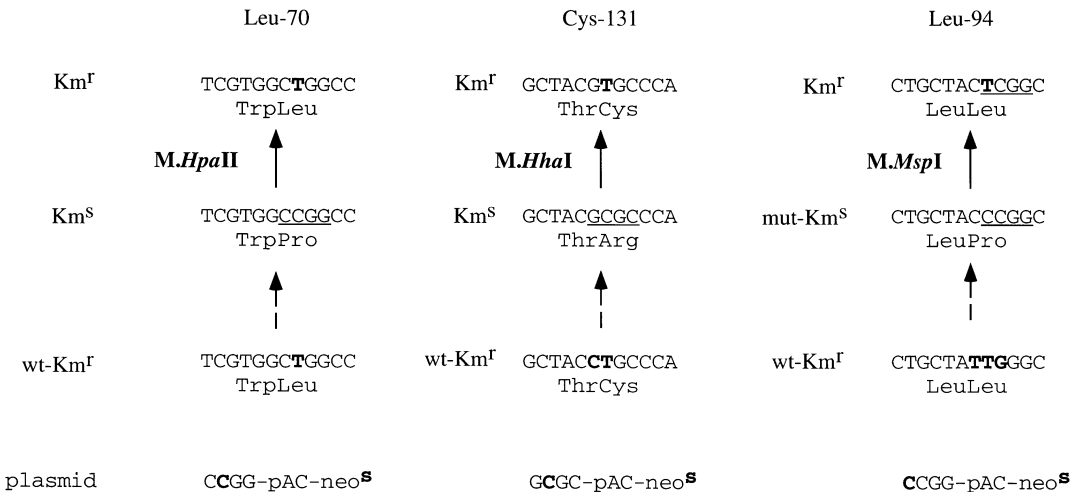

Figure 2 Plasmids constructed containing mutations of the neomycin gene that revert by incubation with the corresponding Mtase

The bases that were mutated by site-directed mutagenesis and by the Mtases are drawn in **bold**, and the newly generated target sequences for the Mtases are underlined.

(v/v) glycerol/0.5 mM dithiothreitol/0.2 μg/μl BSA] reaction mixture and 100 μM each of the AdoMet analogues for 30 min at room temperature. After incubation the samples were electrophoresed on a 6 % non-denaturing polyacrylamide gel for 2 h at 80 V. The bands representing bound and free oligonucleotides were quantified using a phosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the ratio (bound)/(bound + free) was calculated. The results shown represent the mean for two experiments.

Deamination assay

The *in vitro* reversion assay with M.*Hpa*II (C**C**GG-pAC-neo^s) and M.*Hha*I (G**C**GC-pAC-neo^s) was carried out with conditions described previously [11,15,18,26]. For M.*Msp*I, 200 ng of the reporter plasmid **C**CGG-pAC-neo^s was incubated with the DNA Mtase (M.*Msp*I, 5 units) in 20 μl of reaction buffer [for M.*Msp*I: 50 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 mM EDTA/1 mM dithiothreitol/0.2 μg/μl BSA] and the AdoMet analogues for 16 h at 37 °C. The plasmid DNA was extracted with phenol and chloroform and precipitated with ethanol, using glycogen (Boehringer-Mannheim) as a carrier. The DNA was transformed using an electroporator (Bio-Rad) into electro-competent *E. coli* strain ER2357, which is deficient in restriction of 5-mC (*mcr*ABC$^-$) and in uracil glycosylase (*ung*$^-$). An aliquot of bacteria was diluted and plated on chloramphenicol (Cm) plates to score for the **C**CGG-pAC-neo^s transformation efficiency and the remainder plated on kanamycin (Km) plates to score for **T**CGG-pAC-neo^r revertants. The reversion frequency was calculated as (number of Km-resistant bacteria)/(number of Cm-resistant bacteria). Experiments were performed at least twice with similar results.

In vivo reversion assay

Plasmids pUHE-M.*Hpa*II, pUHE-M.*Hha*I and pUHE-M.*Msp*I were transformed into ER2357, and electro-competent bacteria were prepared with standard protocols. These electrocompetent bacteria were freshly transformed before each experiment with C**C**GG-pAC-neo^s, G**C**GC-pAC-neo^s or **C**CGG-pAC-neo^s and plated on ampicillin (Ap) and chloramphenicol (Cm) plates. Single colonies were removed and grown in 20 ml of Luria broth [with Ap (50 μg/ml) and Cm (34 μg/ml)] to an A_{600} of about 0.5–0.8, which corresponds to approx. 10^8–10^9 bacteria/ml. The bacteria were centrifuged and resuspended in 5 ml of Luria broth. A 1 ml aliquot was removed and plasmid DNA isolated and digested with methylation-sensitive restriction enzymes to determine if it was completely methylated by the corresponding Mtases. A sample was serially diluted and plated on Cm plates to score for total number of bacteria. A 1 ml portion was plated on Km plates to score for *in vivo* revertants. The reversion frequency was calculated as (number of colonies on Km plates)/(number of colonies on Cm plates). The experiment was performed in triplicate and the means ± S.D. calculated. The entire experiment was repeated twice.

RESULTS

M.*Msp*I requires the presence of sinefungin or 5′-amino-5′-deoxyadenosine for enzyme-mediated cytosine deamination

Whereas all Mtases use the same overall catalytic methylation mechanism, some differences between the Mtases have been detected, such as the strength of binding to DNA in the presence and absence of the cofactor AdoMet and of the reaction product AdoHcy [32–34]. We were interested in whether Mtases showed also differences with respect to enzyme-mediated cytosine deamination, that could possibly also lead to different rates of mutation. To this end, several new plasmids were constructed containing mutant neomycin genes to measure, by an *in vitro* genetic reversion assay, enzyme-mediated cytosine deamination frequencies by M.*Msp*I, M.*Hpa*II and M.*Hha*I (Figure 2). The main difference between this assay and the one previously described [11,15,18,26] is the use of pACYC184 as the plasmid backbone to quantify reversion of the neomycin gene. Briefly, the plasmids were incubated with the corresponding Mtases, transformed into *ung*$^-$ bacteria, plated on kanamycin (Km) plates to measure the number of revertants and diluted and plated on chloramphenicol (Cm) plates to measure the transformation efficiency. As expected, in the absence of AdoMet and AdoHcy the plasmid C**C**GG-pAC-neo^s reverted to C**T**GG-pAC-neo^r when incubated with M.*Hpa*II, and the plasmid G**C**GC-pAC-neo^s

Table 1 Cytosine deamination frequencies mediated by M.*Hpa*II, M.*Hha*I and M.*Msp*I

The experiment was repeated twice, and the values are means ± S.D.

Mtase	Target plasmid	10^{-7} × *In vitro* reversion frequency	
		With Mtase	Without Mtase
M.*Hpa*II	C**C**GG-pAC-neo^s	1170 ± 71	5 ± 1
M.*Hha*I	G**C**GC-pAC-neo^s	2225 ± 558	7 ± 4
M.*Msp*I	**C**CGG-pAC-neo^s	36 ± 28	3 ± 2

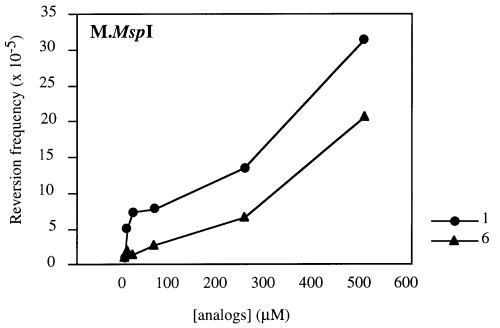

Figure 3 Concentration-dependent increase of enzyme-mediated cytosine deamination frequency by M.*Msp*I with cofactor analogues

The reversion assay was performed as described in the Materials and methods section. Sinefungin (**1**) and 5′-amino-5′-deoxyadenosine (**6**) were at concentrations between 4 and 500 μM.

reverted to G**T**GC-pAC-neo^r when incubated with M.*Hha*I (Table 1).

Interestingly, the plasmid **C**CGG-pAC-neo^s, which reverts by deamination of the first C to **T**CGG-pAC-neo^r, led to very few Km-resistant revertants when incubated with M.*Msp*I in the absence of AdoMet and AdoHcy and when compared with the levels observed with M.*Hpa*II and M.*Hha*I (Table 1). Figure 3 shows that addition of increasing concentrations of the cofactor analogues sinefungin (**1**) and 5′-amino-5′-deoxyadenosine (**6**) dramatically increased the reversion frequency induced by M.*Msp*I. We have previously shown that these two cofactor analogues increase the deamination frequencies of M.*Hpa*II and M.*Sss*I (from *Spiroplasma* sp. strain MQ1) by introducing a proton-donating amino group and possibly by allowing access of solvent water into the catalytic pocket, thus facilitating the formation of an unstable enzyme–dihydrocytosine intermediate and hydrolytic deamination [18]. Our data therefore suggest that M.*Msp*I {which is wild-type (P. Walsh and R. J. Roberts, personal communication; [35])} avoids enzyme-mediated cytosine deamination in the absence of any cofactor analogue by limiting the access of a similar proton donor into the catalytic centre.

Adenosine and MTA significantly increase the enzyme-mediated cytosine deamination by M.*Msp*I

Using our newly constructed reversion plasmids we tested several analogues of AdoMet and AdoHcy (Figure 1) for their abilities to increase or decrease enzyme-mediated deamination by M.*Msp*I, M.*Hpa*II and M.*Hha*I, when incubated in the absence

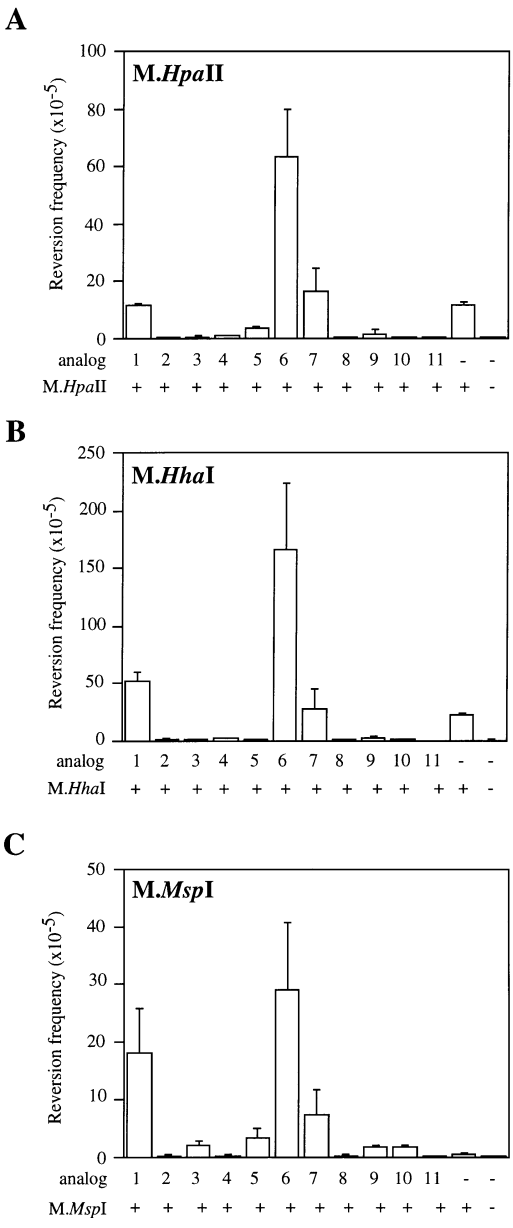

Figure 4 Influence of various cofactor analogues on the enzyme-mediated cytosine deamination frequency *in vitro*

The reversion assay was performed as described in the Materials and methods section. (**A**) The plasmid C**C**GG-pAC-neo^s was incubated with M.*Hpa*II; (**B**) the plasmid G**C**GC-pAC-neo^s was incubated with M.*Hha*I; and (**C**) the plasmid **C**CGG-pAC-neo^s was incubated with M.*Msp*I for 16 h at 37 °C. All analogues (**1–11**) (Figure 1) were at 500 μM.

of AdoMet or AdoHcy (Figures 4A–4C). To take into account the metabolic pathways of AdoMet in bacterial and mammalian cells [10], we included the natural occurring analogues adenosine (**7**), dc-AdoMet (**8**) and dc-AdoHcy (**9**) in addition to the analogues we have previously described [18]. Sinefungin (**1**) and 5′-amino-5′-deoxyadenosine (**6**) both increased deamination mediated by M.*Hpa*II and M.*Hha*I. The remaining analogues [**2**–**4**, **8** and **9**, and AdoHcy (**10**)] all inhibited the deamination reaction mediated by M.*Hpa*II and M.*Hha*I. As expected, analogues with high similarity to AdoMet or AdoHcy, such as analogues (**2**)–(**4**), (**8**) and (**9**), inhibited deamination more

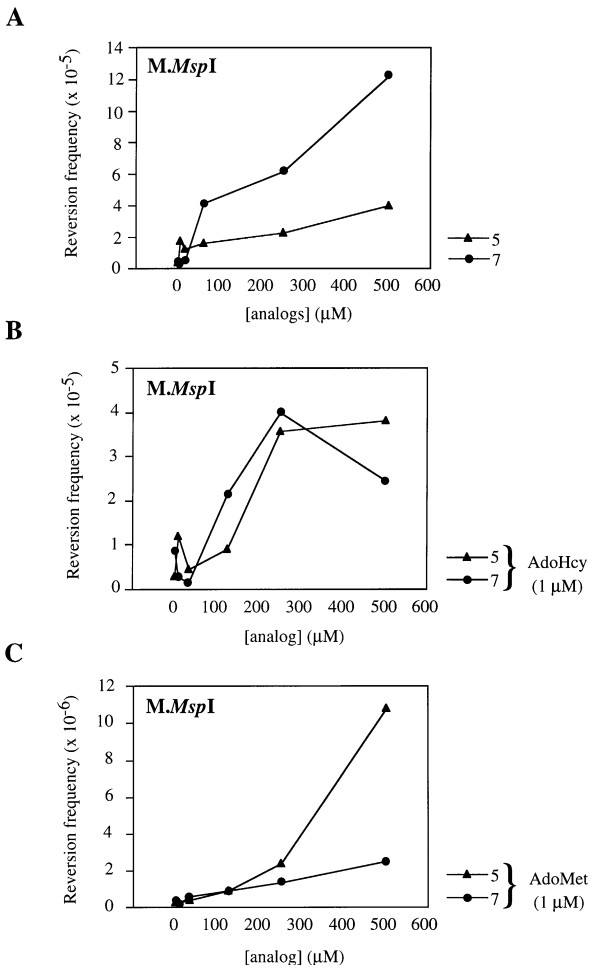

Figure 5 Concentration-dependent increase of enzyme-mediated cytosine deamination by M.*Msp*I with cofactor analogues

The reversion assay was performed as described in the Materials and methods section. Concentrations of MTA (**5**) and adenosine (**7**) were between 4 and 500 μM. (**A**) In the absence of AdoMet or AdoHcy; (**B**) In the presence of 1 μM AdoHcy; (**C**) In the presence of 1 μM AdoMet.

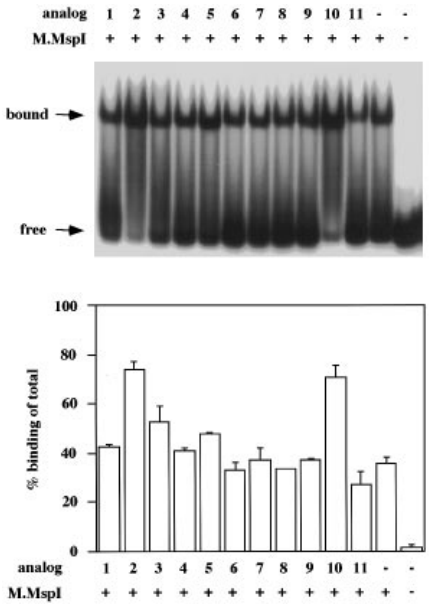

Figure 6 Gel-mobility-shift assays with M.*Msp*I in the presence of cofactor analogues

The gel-mobility-shift assay was performed as described in the Materials and methods section. The analogues were at 100 μM each. The experiments are summarized below the autoradiogram and the S.D. derived from two experiments is indicated by error bars. The numbering **1–11** refers to the cofactor analogues as described in the Materials and methods section and Figure 1.

efficiently than analogues with less similarity, such as MTA (**5**) or adenosine (**7**), which both have a lower affinity for the enzyme (Figures 4A and 4B) [18]. The analogues dc-AdoMet (**8**) and dc-AdoHcy (**9**), which both occur naturally at low concentrations in normal cells but which can accumulate in cells by inhibiting ornithine decarboxylase [36,37], both inhibited the deamination reaction with both enzymes.

As expected from Figure 3, sinefungin (**1**) and 5′-amino-5′-deoxyadenosine (**6**) increased the deamination frequency mediated by M.*Msp*I (Figure 4C). Interestingly, the naturally occurring analogues, MTA (**5**) and adenosine (**7**), neither of which do contain chemical groups that can act as proton donor, led to a significantly higher M.*Msp*I-mediated cytosine deamination frequency than in the absence of any cofactor (Figure 4C). These analogues increased enzyme-mediated deamination by M.*Msp*I in a concentration-dependent manner (Figure 5A), even in the presence of AdoHcy (Figure 5B) and AdoMet ((Figure 5C). We have previously explained the inability of MTA (**5**) to decrease completely enzyme-mediated deamination with M.*Hpa*II and M.*Sss*I by its low affinity for the enzymes, thus leaving enough enzyme unoccupied and accessible to solvent water [18]. However, the increase of deamination seen with M.*Msp*I (Figure 4C) and the incomplete inhibition of enzyme-mediated deamination by M.*Hpa*II and M.*Hha*I (Figures 4A and 4B) is most likely the result of binding of these analogues to the enzymes and the formation of the unstable enzyme–dihydrocytosine intermediate without completely inhibiting the access of solvent water to the catalytic centre. A very small increase was also seen with AdoHcy (**10**) and two other analogues (**3** and **9**), suggesting that these analogues cannot completely prevent the access of solvent water and enzyme-mediated deamination by M.*Msp*I, as was previously shown with other enzymes and AdoHcy [15].

M.*Msp*I binds the target DNA in the absence of cofactors

It seemed possible that the failure of M.*Msp*I to deaminate cytosine in the absence of AdoMet and AdoHcy reflected either the presence of inhibitory cofactors in the enzyme preparation or the requirement of M.*Msp*I to bind certain cofactors for efficient DNA binding. Therefore we used gel-mobility-shift assays to test the efficiency of DNA binding in the absence and presence of various cofactor analogues and the presence of a 10-fold excess of unlabelled competitor oligonucleotide [33] (Figure 6). M.*Msp*I was able to bind DNA in the absence of any cofactor, and since only sinefungin (**1**) and MTA (**5**) increased DNA binding among the analogues that mediated an increase of cytosine deamination (analogues **1**, **5**, **6** and **7**), a cofactor-analogue-mediated change in the strength of DNA binding was not absolutely required for deamination. Addition of AdoHcy (**10**) and analogue (**2**) strongly

Table 2 Expression of the Mtases M.*Hpa*II, M.*Hha*I and M.*Msp*I in *E. coli* increases the *in vivo* frequency of transition mutations at the cytosines targeted for methylation

For reversion frequency the experiment was done in triplicate and the values are means ± S.D. The 'fold increase' is as compared with the result from the control experiment expressing non-complementary Mtase.

Mtase	Target plasmid	10^{-10} × Reversion frequency	Fold increase
M.*Hpa*II	C**C**GG-pAC-neo^s	116 ± 16	5.9
M.*Hha*I	G**C**GC-pAC-neo^s	253 ± 120	4.7
M.*Msp*I	**C**CGG-pAC-neo^s	235 ± 38	5.5
M.*Hha*I	C**C**GG-pAC-neo^s	19 ± 25	
M.*Msp*I	G**C**GC-pAC-neo^s	54 ± 26	
M.*Hpa*II	**C**CGG-pAC-neo^s	42 ± 29	

increased the binding that would not occur if saturating concentrations of inhibitory AdoHcy were present in the enzyme preparation. Analogue (**2**) and AdoHcy (**10**) may induce a conformational change of the enzyme that leads to very tight binding [32,33]. Since a significant amount of enzyme is bound to the oligonucleotide in the absence of any cofactor, it is still possible that traces of cofactors are in the enzyme preparation that stimulate such a binding and possibly prevent deamination. However, among the naturally occurring analogues that inhibit deamination, only AdoHcy is abundant and binds strongly. Since we used a highly purified enzyme preparation (New England Biolabs), the presence of such high concentrations of AdoHcy seems unlikely. Other cofactor analogues which prevented deamination (analogues **3**, **4**, **8** and **9**) either increased the binding efficiency slightly or had no significant effect on binding, indicating that these cofactor analogues did not prevent deamination by interfering with DNA binding. AdoMet (**11**) decreases the binding, since the target oligonucleotide becomes methylated during the binding reaction. In summary, the abilities of various cofactor analogues to induce or prevent cytosine deamination did not correlate with their abilities to increase or decrease binding to the DNA target.

Comparison of C → U deamination frequencies *in vivo*

Since M.*Msp*I appeared to possess the unique ability to methylate without the possible side reaction of enzyme-mediated C → U deamination at low AdoMet or AdoHcy concentrations, we were interested in determining whether M.*Msp*I also showed a lower deamination frequency *in vivo*. This would allow us to evaluate whether *in vivo* increases of mutation frequencies seen at the cytosines of various Mtases are mainly the result of the spontaneous 5-mC → T pathway or of the enzyme-mediated 5-mC → T or C → U → T pathways. We took advantage of the fact that the newly constructed neomycin reversion plasmids based on pACYC184 were compatible with plasmids expressing the wild-type Mtases that are based on the ColE1 origin of replication of plasmid pDS1 [31,38]. The C**C**GG-pAC-neo^s, G**C**GC-pAC-neo^s and the **C**CGG-pAC-neo^s plasmids were freshly transformed into *ung*$^-$ bacteria expressing either M.*Hpa*II, M.*Hha*I or M.*Msp*I respectively. As controls, the reversion plasmids were also expressed in bacteria harbouring a non-complementary Mtase, which were not expected to revert the neomycin gene with a high frequency [39]. Single colonies were isolated and grown in 20 ml of Luria broth containing Ap and Cm to an A_{600} of about 0.5–0.8. Plasmid DNA was isolated in all experiments, and the methylation status checked by restriction analysis with methylation-sensitive restriction enzymes and found to be fully methylated in all cases (results not shown). Consistently the expression of the Mtases led to an approx. 5-fold increase in the number of neomycin revertants when compared with the control (Table 2). The exact number of revertants is expected to show some variability, since it depends on factors such as growth conditions, initial number of bacteria, duration of growth, and the different repair rate of the deaminated target cytosines of the various Mtases. However, our results from two experiments suggest that the mechanism which prevents deamination by M.*Msp*I *in vitro* does not lead to a significantly lower *in vivo* deamination frequency than with M.*Hpa*II and M.*Hha*I when measured under normal physiological conditions in *E. coli*. However, since M.*Msp*I does not naturally occur in *E. coli* [40], such a mechanism could play a role under certain physiological conditions in its natural host.

When the physiological conditions in *E. coli* were altered by induction of the Mtases by isopropyl β-D-thiogalactoside (as low as 50 μM), thus increasing the Mtase/cofactor ratio, severe growth retardation with all three Mtase expression plasmids was observed making it impossible to perform the experiment. Since the bacteria (ER2357) were deficient in restriction of 5-mC (mcrABC$^-$) and allowed the transformation of methylated DNA, this growth retardation is most likely due to reasons other than restriction, such as formation of inclusion bodies [35], interference with replication or induction of the SOS response [39].

DISCUSSION

The action of Mtases on DNA have a unidirectional impact on the genomic base composition by increasing the rate of 5-mC → T or C → U → T transition mutations at cytosines that are targeted for methylation, leading to the under-representation of their corresponding recognition sequence in the genome. In prokaryotes, the presence of restriction enzymes with the same recognition sequence probably further increases this unidirectional impact by favouring genomes with fewer recognition sequences that are easier to keep completely methylated [41]. In higher eukaryotes the increased frequency of transition mutations at the CpG dinucleotides targeted by Mtase left traces of this unidirectional mutational drift as evidenced by CpG islands that are usually spared from methylation in the germ line, or by the complementary depletion of CpG dinucleotides in genomic regions that are methylated in the germ line [42]. The rate at which cytosine deaminations occur and the efficiency of their repair may depend on the enzymes involved, thus possibly leading, depending on the organism, to different overall mutation rates at cytosines targeted for methylation. Enzymes with potential mutator activities such as Mtases probably evolved in a way to avoid the generation of high levels of mutations.

Several prokaryotic Mtases [M.*Hpa*II, M.*Hha*I, M.*Sss*I, Dcm (from *E. coli*) and M.*Eco*RII (from *E. coli*)] increase *in vitro* the rate of enzyme-mediated C → U → T and 5-mC → T transition mutations at concentrations below the K_m for AdoMet and AdoHcy [15–18,26,39,43]. Some of these enzymes, such as M.*Hpa*II, M.*Eco*RII and Dcm, increased the mutation rate up to 50-fold when expressed *in vivo* [17,19,39]. We describe here one prokaryotic Mtase, M.*Msp*I, which has apparently evolved a safeguard mechanism which prevents enzyme-mediated cytosine deamination under *in vitro* conditions of low AdoMet and AdoHcy concentrations. However, when expressed in *E. coli*, M.*Msp*I did not show a lower mutation rate than other enzymes, suggesting that this mechanism does not play a role in preventing mutations *in vivo*, at least with normal physiological AdoMet

and AdoHcy concentrations. The observed *in vivo* mutation rates may be due to spontaneous 5-mC → T deaminations, to the presence of cofactor analogues such as MTA or adenosine in bacteria that allow enzyme-mediated C → U deaminations, and/or to the unregulated incorporation of uracil in ung⁻ bacteria, followed by strong binding of the Mtases and blockage of repair [26].

Since binding of M.*Msp*I occurs even in the absence of any cofactor the mechanism by which M.*Msp*I prevents enzyme-mediated C → U deamination must occur after DNA binding. In the absence of AdoMet, M.*Msp*I is capable of binding covalently to oligonucleotides containing as target base the mechanism-based inhibitor 2-pyrimidinone 1-β-D-2′-deoxyribofuranoside; however, the binding is increased by addition of the cofactors, AdoMet, AdoHcy and sinefungin, that also increase sequence-specific binding [32,44]. The absence of deamination by M.*Msp*I is therefore not the result of a complete failure to form a covalent bond with C-6 of cytosine, but rather of the absence of conformational changes in the enzyme that potentiate sequence-specific binding and covalent attack and possibly facilitate the access of a proton donor to the catalytic centre. Such a proton donor is positioned into the catalytic centre with sinefungin (**1**) and 5′-amino-5′-deoxyadenosine (**6**), which both lead to a great increase in cytosine deamination mediated by M.*Msp*I. An additional way of increasing the frequency of enzyme-mediated cytosine deamination by M.*Msp*I was observed with the naturally occurring analogues MTA (**5**) and adenosine (**7**). These analogues bind to the enzyme and most likely induce a conformational change in M.*Msp*I without completely preventing the access of solvent water to the catalytic centre that is required to form the unstable enzyme–dihydrocytosine intermediate and for hydrolytic deamination.

Furthermore, it is possible that M.*Msp*I contains two binding sites for the cofactor and requires a fit-induced rearrangement for catalysis triggered by cofactor binding such as has been described for the DNA adenine Mtase (Dam) [44,45]. The crystal structure of the adenine-specific DNA methyltransferase M.*Taq* (from *Thermus aquaticus*) revealed that, owing to its charged amino group, sinefungin is oriented slightly differently to AdoMet in the cofactor-binding site [46,47]. The weak increase of enzyme-mediated cytosine deamination seen with some of the cofactor analogues may be the result of such subtle differences in analogue binding. Our findings suggest that increases of enzyme-mediated cytosine deamination can occur not only by cofactor analogues acting directly as proton donor, but also by analogues such as MTA (**5**) and adenosine (**7**), which mediate a conformational change of the enzyme, potentiate the covalent attack at C-6 of cytosine and/or render the unstable enzyme–dihydrocytosine adduct more accessible to the solvent water. These two analogues have a lower affinity for the enzyme and occur in normal cells at 10–100-fold lower concentrations than AdoMet and AdoHcy [48]. However, it seems possible that chemical modifications introduced to MTA (**5**) and adenosine (**7**) could increase their affinity to the Mtase and their ability to compete with AdoMet and AdoHcy and thus increase also the frequency of enzyme-mediated cytosine deamination. It is also noteworthy that MTA (**5**) accumulates to high concentrations (1–5 μM) in various tumours with homozygous deletion of the methylthioadenosine phosphorylase gene [49–51], and that MTA (**5**) inhibits DNA methylation when added to mammalian cells in culture [52]. Furthermore, the concentration of adenosine (**7**) is 20–30-fold increased in anoxic hepatocytes (to 8–12 μM), and anoxic conditions may occur in solid tumours [53]. Cell lines with a high rate of C → T transition mutations at the CpG dinucleotides have been described which are possibly mediated by the Mtase [54]. Aberrations within the metabolism of AdoMet leading to high level of MTA (**5**), adenosine (**7**) and possibly further cofactor analogues could increase the enzyme-mediated C → U deamination rate in higher eukaryotes by similar mechanisms as described here *in vitro* with the bacterial enzyme M.*Msp*I [10].

Direct proof of the proposed cytosine deamination mechanisms of M.*Msp*I can only be obtained when the crystal structures of M.*Msp*I bound to the target DNA in the absence of AdoMet or AdoHcy and in the presence of sinefungin (**1**), MTA (**5**) or adenosine (**7**) become available. It will be interesting to see whether the cofactor analogues such as MTA (**5**) and adenosine (**7**) induce M.*Msp*I-mediated deamination by potentiating the covalent attack at C-6 of cytosine, by changing the location of key amino acids that can act as proton donors or simply by allowing the access of solvent water into the catalytic pocket.

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