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Biological properties of imidazole ring-opened N7-methylguanine in M13mp18 phage DNA

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ABSTRACT

Guanine residues methylated at the N-7 position (7-MeGua) are susceptible to cleavage of the imidazole ring yielding 2,6-diamino-4-hydroxy-5N-methyl-formamidopyrimidine (Fapy-7-MeGua). The presence of Fapy-7-MeGua in DNA template causes stops in DNA synthesis *in vitro* by *E.coli* DNA polymerase I. The biological consequences of Fapy-7-MeGua lesions for survival and mutagenesis were investigated using single-stranded M13mp18 phage DNA. Fapy-7-MeGua lesions were generated *in vitro* in phage DNA by dimethylsulfate (DMS) methylation and subsequent ring opening of 7-MeGua by treatment with NaOH (DMS-base). The presence of Fapy-7-MeGua residues in M13 phage DNA correlated with a significant decrease in transfection efficiency and an increase in mutation frequency in the *lacZ* gene, when transfected into SOS-induced JM105 *E.coli* cells. Sequencing analysis revealed unexpectedly, that mutation rate at guanine sites was only slightly increased, suggesting that Fapy-7-MeGua was not responsible for the overall increase in the mutagenic frequency of DMS-base treated DNA. In contrast, mutation frequency at adenine sites yielding A→G transitions was the most frequent event, 60-fold increased over DMS induced mutations. These results show that treatment with alkali of methylated single-stranded DNA generates a mutagenic adenine derivative, which mispairs with cytosine in SOS induced bacteria. The results also imply that the Fapy-7-MeGua in *E.coli* cells is primarily a lethal lesion.

INTRODUCTION

The main product of DNA alkylation by methylating agents, N^7-methylguanine (7-MeGua), can undergo further processing yielding either apurinic (AP) sites or imidazole ring-opened derivative, 2,6-diamino-4-hydroxy-5N-methyl-formamido-pyrimidine (Fapy-7-MeGua) residue (1–3).

Fapy-7-MeGua has been described *in vivo*, in the liver of rats treated with N,N-dimethylnitrosamine, or 1,2-dimethylhydrazine, and in rat bladder epithelial DNA after treatment with N-methylnitrosourea (4, 5). Furthermore opening of the imidazole ring of guanine yielding a non-methylated Fapy-Gua has been observed in animal (6) and in human tumor tissues (7). *In vitro*, imidazole ring-opened guanine is formed under alkaline conditions in DNA treated with cyclophosphamide metabolites — phosphoramide or nitrogen mustards (8), aflatoxin B1 (9, 10) or N-hydroxy-2-aminofluoren (11). This lesion is also formed upon treatments generating hydroxyl radicals or singlet oxygen (12, 13).

A DNA glycosylase (Formamidopyrimidine-DNA-glycosylase or Fpg protein) that excises both unsubstituted and substituted Fapy lesions as well as 8-hydroxyguanine has been described and cloned from *E.coli* cells (13–19). Fpg protein has also been partially purified from mammalian tissues (20).

Fapy-7-MeGua lesions inhibit *in vitro* DNA synthesis by *E.coli* polymerase I (21) and the stops in DNA synthesis occur one base before potential Fapy-7-MeGua residues in the template (1). These results suggest that Fapy-7-MeGua lesion could be a cell killing lesion after treatment with alkylating agents. To the best of our knowledge, no data are available concerning the potential mutagenic properties of Fapy-7-MeGua lesions.

We report that DMS-base treated M13 phage DNA, containing Fapy-7-MeGua residues shows decreased transfection efficiency and increased mutation frequency when transfected into the SOS-induced *E.coli* cells. Sequencing data of M13 *lacZ* mutants lacking α-complementation revealed that there was no targeting of base substitutions on guanine residues. Unexpectedly, the frequency of A→G transitions increased dramatically in DMS-base mutants, indicating that alkaline treatment of methylated DNA creates a mutagenic adenine derivative.

MATERIALS AND METHODS

Chemicals

Dimethylsulfate (DMS) was from Aldrich; Sequenase version 2.0 sequencing kit from United States Biochemicals; Deoxyadenosine-5′-[alpha-(^{35}S)thio]triphosphate — from Amersham; isopropyl β-D-thiogalactoside (IPTG) — from Boehringer Mannheim; 5-bromo-4-chloro-3-indolyl-β-D-

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galactoside (X-gal) — from Stratagene; [^{3}H]-dimethylsulfate (2.9 Ci/mmol) — from New England Nuclear.

Media

All media and buffers were prepared according to Sambrook *et al.* (22).

Preparation of phage DNA and its modification

M13mp18 phage was grown in JM105 (supE endA sbcB15 hsdR4 rpsL thi Δ (lac-proAB)/F'lacZΔ M15 in 2YT medium (22) overnight at 37°C. The phage was collected and its DNA was isolated according to Messing (23). Immediately before use, DMS was dissolved in TE buffer (10 mM Tris−HCl pH = 8.0, 1 mM EDTA). M13 phage DNA was incubated with DMS (0.5−2 mM) for 10 min at 37°C. For each DMS concentration, as well as for a control, nonalkylated DNA, 10 μg of phage DNA in a total volume of 100 μl was used. Subsequently, DNA was ethanol precipitated and resuspended in 100 μl of TE buffer.

To obtain Fapy-7-MeGua residues in DNA, immediately after DMS methylation, 50 μl of M13 phage DNA was incubated in 0.2 M NaOH for 20 min at 37°C, then the pH of the solution was adjusted to 8.0 with 1 M Tris-HCl buffer, the DNA was ethanol precipitated and resuspended in TE buffer. Under these conditions, 7-MeGua residues are quantitatively transformed into Fapy-7-MeGua lesions (1−3).

Quantification of modified residues in M13 phage DNA

The number of 7-MeGua residues per M13 DNA molecule generated by increasing concentration of DMS was determined after separation of the various bases by HPLC. Briefly, M13 phage DNA was methylated with [^{3}H] DMS (NEN) and purified by repeated ethanol precipitations. The alkylated bases were released from the DNA by acidic treatment (75% formic acid at 80°C for 1 h) as previously described (3). The formic acid was removed by vacuum and the dried radioactive material was resuspended in 100 μl of water containing marker molecules. The hydrolysate was subjected to HPLC chromatography using C18 μ Bondapak column (Waters). The mobile phase was 25 mM $NH_4H_2PO_4$, pH 4.5, containing 2% methanol (v/v). The column was developed isocratically at 1.5 ml/min. The compounds were detected in eluates of the HPLC by monitoring UV absorption at 254 nm and scintillation counting of fractions. The amount of M13 phage DNA was calculated from UV absorption of total adenine and guanine residues eluted from the column. The amount of 7-MeGua was established on the basis of known specific activity of [^{3}H] DMS and the amount of radioactivity eluting from the column as 7-MeGua. Quantification of Fapy-7-MeGua was based on the determination of 7-MeGua, since under alkaline conditions used in this study 7-MeGua was quantitatively transformed into its imidazole ring-opened derivative (1−3).

Preparation of competent cells and transformation

Bacteria were made competent by the $CaCl_2$ method (22). The SOS system was induced by irradiation of the competent cells with UV-light at 254 nm (70 J/m^2) immediately before transfection. Transfection was performed according to Sambrook *et al.* (22), but under low light conditions. Phage DNA (100 ng) was used to transfect 100 μl of competent cells.

Transfection mixtures were plated on LB solid medium with 2.5 ml of LB soft agar supplemented with 0.4 mM IPTG and 0.5 mg/ml X-gal. The plates were incubated overnight at 37°C and plaques of phage infective centers were scored.

Collecting lacZ mutants and their sequencing

M13 *lacZ* mutants lacking α-complementation (24) and exhibiting low or no β-galactosidase activity were recognized as colorless or light blue plaques on LB plates containing IPTG and X-gal. Mutants for DNA sequencing were collected at 2 mM DMS concentration. Mutant DNA was isolated according to Messing (23) and the M13mp18 *lacZ* gene was sequenced using the dideoxynucleotide chain termination method (25). Sequenase version 2.0 sequencing kit, [^{35}S]dATP and a specific 17 nucleotide primer annealing at base positions for amino acids 65 to 70 of the *lacZ* gene of the M13+ strand sequence (synthesised by Dr E.Lescot, Institut Gustave Roussy, Villejuif) were used for DNA sequencing.

RESULTS

Quantification of imidazole ring-opened 7-methylquanine residues in ss M13mp18 phage DNA

When single-stranded M13 DNA is alkylated with DMS, the spectrum of alkylated bases is the following (1): 1-MeAde (20%), 3-MeAde (7%), 7-MeAde (<1%), 3-MeGua (<1%), 7-MeGua (59%), other methylated bases (14%). To quantitate 7-MeGua residues, M13 phage DNA was modified with [^{3}H] DMS, purified and then acid hydrolyzed in order to release purines from DNA. The alkylated residues were separated by reverse phase HPLC and quantitated by scintillation spectroscopy. As shown in Figure 1, 1.9 residues of 7-MeGua per M13 DNA molecule is generated at 1 mM DMS concentration and this value increases linearly up to 7.5 residue per molecule at 4 mM DMS. Under the alkaline conditions used in this study the quantitative transformation of 7-MeGua into its imidazole ring-opened derivative was observed (1). Thus the number of Fapy-7-MeGua

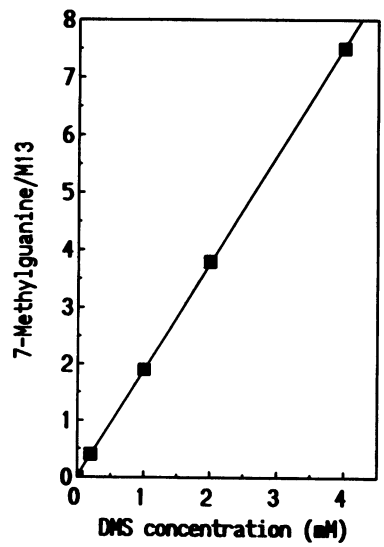

Figure 1. Quantification of 7-methylguanine residues per M13 phage DNA molecule. M13 phage DNA was methylated with increasing concentrations of [^{3}H]-DMS. After purification of DNA and acidic hydrolysis, 7-MeGua residues were quantitated by scintillation spectroscopy following HPLC separation. For the details see Materials and Methods.

lesions per M13 molecule was equal to the number of 7-MeGua residues in the DNA.

Effect of DMS and DMS-base treatments on the survival and mutagenesis of single-stranded M13 phage DNA

After transfection into JM 105 *E.coli* cells, the survival of DMS-base treated M13 DNA, containing Fapy-7-MeGua, was decreased in comparison to DMS treated phage DNA containing 7-MeGua (Figure 2). DMS-base treatment does not induce strand breaks in DNA, as checked by analysis in agarose gel electrophoresis (data not shown). This result suggests that phage DNA inactivation after DMS-base treatment might be due to the conversion of a harmless lesion into another lesion, which blocks DNA synthesis, e.g. the conversion of 7-MeGua into Fapy-7-MeGua (1). Induction of the SOS system by UV irradiation of JM 105 cells increased the M13 phage survival both in DMS and DMS-base treated phage DNAs (Figure 2).

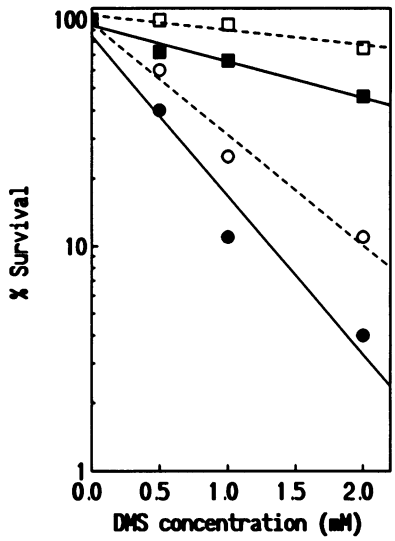

Figure 2. Survival of DMS and DMS-base damaged M13mp18 phage DNA when transfected into *E.coli* cells. M13 DNA was treated *in vitro* with DMS (squares) or DMS-base (circles) and transfected into competent *E.coli* JM105 cells either unirradiated (filled symbols, solid line) or UV irradiated (open symbols, dashed line).

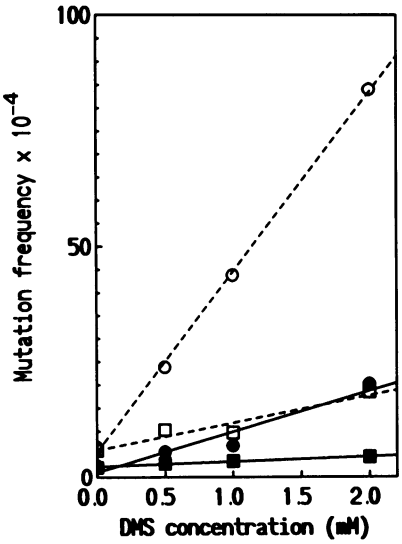

Figure 3. The frequency of *lacZ* gene mutations induced in DMS or DMS-base treated M13mp18 phage DNA after transfection into *E.coli* JM105 cells. Phage DNA treated with DMS (squares) or DMS-base (circles) was transfected into JM105 cells either unirradiated (filled symbols, solid line) or UV irradiated (open symbols, dashed line). Colorless or light blue phage mutants defective in β-galactosidase activity were scored. For the details see Materials and Methods.

Methylation of phage DNA by increasing concentrations of DMS does not modify significantly the mutation frequency observed in the M13 *lacZ* gene (Figure 3), when transfected into SOS noninduced JM105 cells. If the SOS system is induced, there is a 3-fold increase in the mutation frequency compared to the spontaneous rate (Figure 3). In contrast after DMS-base treatment there is an increase in the mutation rate, which has a linear relationship to the DMS concentrations used to alkylate the DNA. The control mutation rate phage DNA, which is not alkylated, but treated with alkali remains on the same level as the spontaneous mutation rate (Figure 3). When DMS-base treated DNA is transfected into SOS induced JM105 cells a dramatic increase in the mutation frequency is observed. These results suggest that in DMS-base treated DNA, there exist lesion(s) which can be bypassed by DNA polymerase under SOS conditions and give rise to mutations. In order to identify this mutagenic DNA modification, the mutagenic spectrum was investigated in *lacZ* gene of DMS-base and DMS induced M13 mutants in UV irradiated *E.coli* cells by means of DNA sequencing.

Analysis of mutations in lacZ gene of DMS-base and DMS treated M13mp18 phage DNA

The *lacZ* system allows the detection of forward mutations and is based on α-complementation between the amino part of β-galactosidase coded by *lacZ* gene of M13 phage (α peptide) and carboxy portion of the enzyme coded by *lacZΔM15* gene of F'episome in bacterial strains with the *lac* operon deleted (26). The production of active β-galactosidase can be visualised as blue

Table 1. Frequency of various classes of mutations in *lacZ* gene of M13mp18 phage.

Class of mutation	DMS number of mutants	DMS %	DMS + base number of mutants	DMS + base %
single base substitution	43	72	43	72
single base substitution + frameshift	0	0	1	2
double base substitution + frameshift	0	0	1	2
frameshift	7	11	6	10
-single base deletion	4	6	5	8
-single base addition	2	3	0	0
-double base deletion	0	0	1	2
-single and double base deletion	1	2	0	0
double deletion of 93 and 54 nucleotides	10	17	6	10
other deletions	0	0	3	5
Total	60	100	60	100

Mutants were collected at 2 mM DMS concentration. Mutation frequency for DMS or DMS-base induced mutants equalled 19×10^{-4} and 84×10^{-4} respectively. For the details see Materials and Methods.

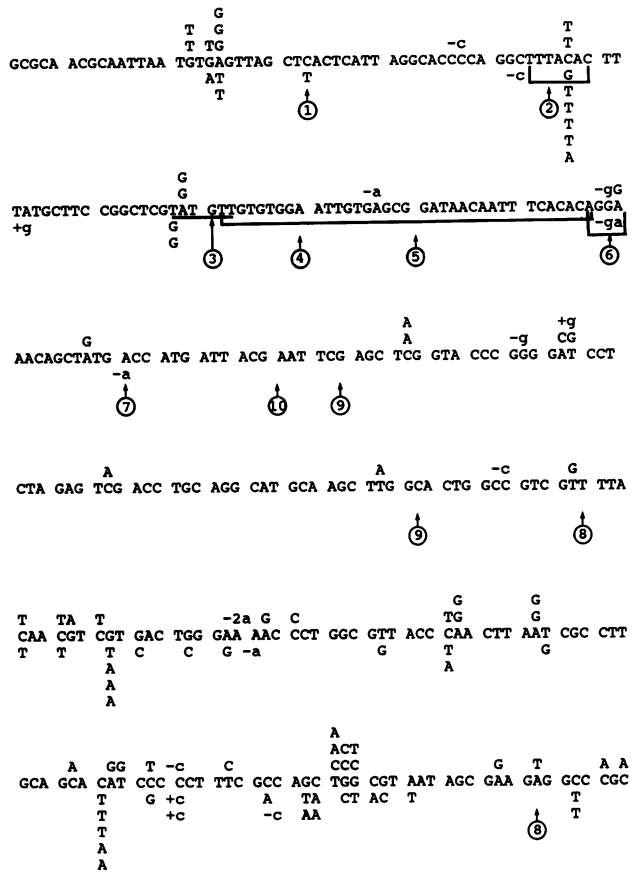

Figure 4. Induced mutational spectrum for base substitutions and frameshifts in M13mp18 *lacZ* DNA. The 5'→3' DNA sequence of the *lacZ* fragment of M13mp18 is shown, from the first nucleotide after the *lacI* termination codon through the coding sequence for aminoacid 61 of the gene. Base substitution and frameshift mutations induced by DMS-base treatment of the phage DNA are shown above the wild-type sequence. Base substitutions and frameshifts, induced by DMS, are shown below the wild type sequence and indicate the nucleotide present in the mutant viral strand. Base substitutions are described as capital letters and frameshifts are described as lower case: a '+' symbol followed by the symbol of the base indicates addition of the given base; a '−' symbol indicates the deletion of the given base. When a frameshift occurs in a run of two or more of the same bases, the exact nucleotide lost is unknown and frameshifts are marked in the center of the run. (1) CAP site; (2) −35 promoter; (3) −10 promoter; (4) transcription start; (5) operator site; (6) ribosome binding site; (7) translation start; (8) first and last nucleotides of 93-nucleotide deletion; (9) first and last nucleotides of 54-nucleotide deletion of the polylinker region; (10) the site of *Eco*RI A→G transition in the double 93- and 54-nucleotide deletion mutants.

plaques on indicator (X-gal) containing plates. Mutation in the *lacZ* gene of the phage would lead to total or partial loss of α-complementation and production of colorless or light blue phage plaques.

More than 200 mutants obtained from DMS and DMS-base treated phage DNA transfected into SOS-induced bacteria were purified and sequenced. Out of them 60 in the DMS group and 60 in DMS-base group show sequence alteration in the 125-base operator/promoter region and 183-base sequence coding for protein. The mutants identified constituted about 50% of all sequenced mutants. Since the *lacZ* gene of M13mp18 phage codes for 168 aminoacids (23) of β-galactosidase protein (α peptide) and we sequenced the DNA fragment covering the regulatory region and 61 amino acids, it is possible that nonidentified mutants carried base alterations in the further, nonsequenced part of the gene.

Table 2. Spectrum of base substitutions in DMS and DMS-base treated ssM13mp18 phage mutants.

Class of base substitution	DMS		DMS-base			
	number of mutants	mutation frequency ($\times 10^{-5}$)	% number of mutants		mutation frequency ($\times 10^{-5}$)	%
G→A	5	8	11	1	5	2
G→T	3	5	7	4	18	9
G→C	4	6	9	3	14	7
Total guanine	12	19	27	8	37	18
A→G	1	1	4	14	65	30
A→T	3	5	7	1	5	2
A→C	0	0	0	1	5	2
Total adenine	4	6	11	16	75	34
C→T	13	20	30	8	37	18
C→A	8	12	18	6	28	12
C→G	2	3	4	0	0	0
Total cytosine	23	35	52	14	65	30
T→C	0	0	0	2	9	4
T→A	0	0	0	3	14	7
T→G	4	6	10	3	14	7
Total thymine	4	6	10	8	37	18
Total base substitutions	43	66	100	46	214	100

Base substitutions from double and triple mutants (base substitutions and frameshifts) are included.

The distribution of different classes of mutations in both groups of mutants was similar (Table 1). The most frequent type of mutation observed was a single base substitution (72% in both groups). In the DMS-base group, we observed 1 double mutant (single base substitution and frameshift) and 1 triple mutant (double base substitution and frameshift). Frameshift mutations constituted 11% of all changes in DMS group and 10% in DMS-base group (Table 1, Figure 4). Large deletions constituted 17% of all changes in DMS induced mutants and 15% in DMS-base mutants (Table 1). The majority of these large deletions arose by simultaneous elimination of 93 and 54 nucleotides (Table 1, Figure 4) and were presumably the effect of recombination with the F'episome as discussed by Kunkel (27) for M13mp2 phage. In the M13mp18 phage, recombination of M13 and F'episome DNA would result in the loss of M13 *lacZ* sequences corresponding to the 93-nucleotide M15 deletion of F'episome and the 54-nucleotide polylinker sequence (28) cloned to M13mp18 phage, but absent in the F'episome. The fact that the 12-nucleotide sequence separating the 93- and the 54-nucleotide fragments is present in the deletion mutants as well as an adenine−guanine substitution mutation in the *Eco*RI site, representing the original base altered in the construction of M13 hybrid phages, support the contention that simultaneous deletion of 54- and 93-nucleotide fragments is due to recombination of M13 DNA with F'episome.

The distribution of single base substitutions and frameshifts throughout the *lacZ* gene in DMS and DMS-base induced M13 mutants is presented in Figure 4. From 60 mutated sites within the sequenced fragment of the *lacZ* gene, only 13 were shared by both groups of mutants, while the remaining 47 positions were

different. No mutational 'hot-spots' were observed. Classification of base substitutions in DMS and DMS-base induced M13 *lacZ* mutants is presented in Table 2. In DMS induced mutants cytosine was the main target for mutations (52% of all substitutions), then guanine (27%), adenine (11%) and thymine (10%). In DMS-base induced mutants the main target for base substitutions was adenine (34%), then cytosine (30%), guanine (18%) and thymine (18%). These results indicate that after treatment of methylated DNA with alkali there is a change of the mutational spectrum, which might reflect the formation of secondary lesions in the template. The mutation frequency observed at all four base sites in the template was increased in DMS-base mutants as compared with that observed in DMS induced mutants (Table 2). Unexpectedly, the mutation rate at the adenine sites increased over 10 times in DMS-base group (Table 2). In particular the frequency of A→G transitions is 60 times greater than the rate of A→G events observed following DMS treatment of the phage DNA (Table 2). This increase is not apparently the result of deamination of the deoxyadenosine to deoxyinosine under alkaline conditions (29), since base treatment of non-alkylated phage DNA did not increase A→G transition frequency (data not shown). This suggests that the A→G transitions in DMS-base mutants are probably due to secondary lesions generated by alkaline treatment of methylated adenine residues in DNA.

The frequency of mutations at guanine sites in the template following treatment with DMS-base is twice that observed with DMS (Table 2). Most notably G→T and G→C transversions increased 2−3 times, while the frequency of G→A transition reflecting O^6-MeGua mispairing properties (30) remained on the same level.

DISCUSSION

When dimethylsulfate is used to alkylate single stranded M13 DNA, 59% of all methylations occur at N-7 position of guanine and this lesion is quantitatively transformed into Fapy-7-MeGua under the alkaline conditions used (1, 3). This predominance of Fapy-7-MeGua formation, in DMS-base treated single-stranded DNA prompted us to evaluate the lethal and mutagenic potential of Fapy-7-MeGua residues in hybrid M13 phage DNA.

The results reported in this work show that the conversion of 7-MeGua into Fapy-7-MeGua in ss M13mp18 DNA decreased the survival and increased the mutation frequency in SOS induced *E.coli* cells. We propose that the inactivation of phage DNA by DMS-base treatment is due to Fapy-7-MeGua for the following reasons. The measurement of base modifications in M13 DNA shows that the number of Fapy-7-MeGua residues per M13 DNA molecule increased linearly with DMS concentration (Figure 1). Furthermore, the inactivation of the biological activity as measured in transfection assays show that approximately one Fapy-7-MeGua residue was required per lethal hit (Fig.2), which suggests that Fapy-7-MeGua *in vivo* in *E.coli* cells is a cell killing lesion. This is in agreement with our earlier *in vitro* experiments showing that Fapy-7-MeGua was a block to DNA synthesis by *E.coli* polymerase I, which stopped one base before the lesion (1). Other modified bases like 1-MeAde, 3-MeAde or 3-MeCyt might contribute to inactivation of the phage both after DMS and DMS-base treatment. Although these modifications inhibit DNA synthesis (31), they can be bypassed if the fidelity of DNA polymerase is decreased (32, 33).

DMS treatment of phage DNA results in increased mutation frequency when transfected into SOS induced bacteria. The spectrum of base substitutions induced by DMS treatment of the *lacZ* gene in single-stranded DNA differs from the spectrum published for the *lacI* gene in double-stranded DNA by the higher incidence of G:C→T:A transversions and a lower incidence of G:C→A:T transitions (Table 2; 34). The G→A transitions are limited to 11% of DMS induced base substitutions (Table 2) and might reflect the G:T mispairing potential of O^6-MeGua lesions during DNA replication (30). The C→T transitions (30% of all base changes) may originate from spontaneous cytosine deamination to uracil (35). The presence of uracil in DNA templates leads to C→T transitions either by the direct incorporation of adenine opposite uracil (36) or the preferential insertion of adenine opposite abasic sites (27, 37, 38) created by uracil-DNA-glycosylase (39). Both C→T transitions and the second most abundant class C→A transversions (18% of all base substitutions) could also derive from 3-MeCyt. This base damage represents 9.5% of total methylation in single-stranded DNA (40) and exhibits miscoding properties with dAMP and dTMP during *in vitro* DNA synthesis with *E.coli* polymerase I (33, 41).

After treatment under alkaline conditions of DMS modified M13 phage DNA, the spectrum of mutations changed, with the A→G transition being the main event observed (Table 2). The A→G transitions are known to be induced by adenosine deamination to inosine or as a result of 1-MeAde mispairing with cytosine during *in vitro* DNA synthesis in the presence of Mn^{+2} ions (32). The A→G transitions have also been described as the main event in MNU treated, SOS induced *E.coli* cells and have been correlated with the mutagenic effect of 3-MeAde presence in the DNA (42). However in the experiments reported above, the increase in the mutation frequency of DMS-base treated DNA was the function of both DNA methylation and its subsequent treatment with the base. Under such conditions, the following transformations of adenine derivatives are known: (i) 1-MeAde could be transformed into 6-MeAde, a naturally occuring methylated base pairing with thymine (40, 43, 44). However, under conditions used in this study 1-MeAde undergoes very limited, if any, transformation into 6-MeAde (3). (ii) 7-MeAde undergoes imidazole ring opening forming Fapy-7-MeAde (44). The potential mutagenic properties of Fapy-7-MeAde are not known.

The quantitatively predominant secondary DNA modification after alkylation with DMS and treatment with the base is Fapy-7-MeGua (1). If this lesion has mutagenic potential, an increase in mutations at guanine sites in the template would have been expected. The frequency of G→C and G→T transversions increased 2−3-fold over the rate induced by DMS (Table 2). These results suggest that some Fapy-7-MeGua residues may be mutagenic, but their participation in the increase of mutation frequency of DMS-base treated phage is rather marginal.

After alkaline treatment, the frequency of mutations at thymine sites in the template increased 6-fold, suggesting formation of secondary, mutagenic thymine derivative. However the observed effect could be independent on alkylation, since in nonalkylated, but treated with NaOH phage DNA the frequency of mutations at thymine sites in the template was also high (data not shown).

We also observed that alkaline treatment induced a 2-fold increase in mutation frequency at cytosine sites in the template in DMS-base induced mutants (Table 2). This might be caused by cytosine deamination to uracil under alkaline conditions (29) or creation of mutagenic derivatives from alkylated cytosine moieties.

Results presented in this paper show that alkali treatment of

single-stranded DNA previously methylated with dimethylsulfate creates at least two secondary lesions with different biological significance: Fapy-7-MeGua , whose presence in the DNA correlates with observed lethal effects but does not play a major role in mutagenesis, and a mutagenic adenine derivative.

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