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QUARTERLY

Mutagenic specificity of imidazole ring-opened 7-methylpurines in M13mp18 phage DNA

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The most abundant lesion formed in DNA upon modification with methylating agents 7-methylguanine, under alkaline conditions is converted into 2,6-diamino-4-hydroxy-5*N*-methyl-formamidopyrimidine (Fapy-7MeGua). We have previously shown that treatment of dimethylsulfate methylated DNA with NaOH creates mutagenic base derivatives leading to a 60-fold increase in the frequency of A→G transitions and a 2–3-fold increase of G→T and G→C transversions. We have analyzed which lesions lead to these mutations. We compared mutagenic spectra in the *lacZ* gene of M13mp18 phage DNA modified with dimethylsulfate and NaOH after selective elimination of damaged bases from molecules used for transfection into SOS-induced *E. coli*.

Partial elimination of Fapy-7MeGua from phage DNA performed by its digestion with formamidopyrimidine-DNA glycosylase resulted in a 2–3-fold decrease of G→T

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Abbreviations: AP site, apurinic/apyrimidinic site; DMS, dimethylsulfate; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; Fapy-7MeAde, 4,6-diamino-5*N*-methyl-formamidopyrimidine; Fapy-7MeGua, 2,6-diamino-4-hydroxy-5*N*-methyl-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine; HPLC, high performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactoside; 1MeAde, 1-methyladenine; 3MeAde, 3-methyladenine; 7MeAde, 7-methyladenine; 7MeGua, 7-methylguanine; MNU, methylnitrosourea; thiotepa, *N,N',N''*-triethylenethiophosphoramide; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

and G→C transversions. Selective depurination of methylated bases (9 h, 37°C, pH 7.0) resulting in almost complete loss of 7MeAde as demonstrated by HPLC analysis of [^{3}H]MNU alkylated phage DNA used as a probe, caused a dramatic, 9-fold decrease of A→G transitions. Alkali-catalysed rearrangement of 7MeAde was followed by HPLC analysis of [^{3}H]MNU alkylated poly(A) and poly(dA). After incubation of these oligonucleotides in NaOH, 7MeAde disappeared from both chromatograms, but only in polyA, 2 new peaks migrating with retention time different from that of 1MeAde, 3MeAde or 7MeAde were detected, suggesting formation of two rotameric forms of Fapy-7MeAde as observed for Fapy-7MeGua. Thus the miscoding lesion, giving rise to A→G transitions derived from 7MeAde was Fapy-7MeAde. Fapy-7MeGua was at least an order of magnitude less mutagenic, but in SOS-induced cells it gave rise to G→T and G→C transversions.

The main product of DNA alkylation by methylating agents, N7-methylguanine (7MeGua) can undergo further processing yielding either apurinic/apyrimidinic (AP) sites or an imidazole ring-opened derivative, 2,6-diamino-4-hydroxy-5*N*-methyl-formamido pyrimidine (Fapy-7MeGua) (Singer & Grunberger, 1983; Boiteux *et al.*, 1984). The rate of imidazole ring-opening of purines is mediated by the structure of substituent in N7 or C8 position of guanine and occurs easily with mustard or ethyleneimine adducts (Hemminki, 1984). Compounds commonly used in cancer chemotherapy, e.g. cyclophosphamide or thiotepa (cf. Abbreviations) produce these kinds of DNA damages (Hemminki & Kallama, 1986). Imidazole ring-opening of purines in DNA substituted in N7 position with methyl or ethyl groups occurs very slowly in physiological conditions, but is accelerated in alkali (Haines *et al.*, 1962). Fapy-7MeGua was, however, found 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 (Beranek *et al.*, 1983; Kadlubar *et al.*, 1984). The imidazole ring of unmodified purines can also be opened during interaction of DNA bases with reactive oxygen species. Reaction of purine bases with hydroxyl radicals leads to the formation of 8-OHGua or 8-OH-Ade free radicals. These unstable intermediates can subsequently undergo oxidation giving respective 8-hydroxy-derivatives, or reduction leading to imidazole ring-opening and formation of unsubstituted Fapy (Fujita & Steenken, 1981; Dizdaroglu, 1992).

DNA glycosylase/AP-lyase specific to unsubstituted and substituted formamidopyrimidines as well as 8-hydroxyguanine – Fpg protein was cloned in *Escherichia coli* (Boiteux *et al.*, 1987). The affinity of the enzyme toward a spectrum of known substrates differs markedly depending on the structure of the excised base. Fapy-7MeGua and 8-OH-Gua are repaired very efficiently, with K_m being as low as 0.6–8 nM (Boiteux *et al.*, 1990; Castaing *et al.*, 1993). The size of the substituent in the N7 or C8 position of imidazole ring-opened guanine seems to be an important determinant of the enzyme activity, since the K_m value for excision of Fapy-7-ethylguanine is 7-fold higher than for excision of Fapy-7MeGua (Tudek *et al.*, 1998) and for guanine imidazole ring-opened adduct of aminofluorene – over 10-fold higher (Boiteux *et al.*, 1989). In eukaryotic cells, repair of 8-OH-Gua, FapyGua and of FapyAde is performed by separate enzymes. In *Saccharomyces cerevisiae* two glycosylases specific for 8-OH-Gua – OGG1 and OGG2 were described (Karahalil *et al.*, 1998; Hazra *et al.*, 1998) and separate proteins, NTG1 and NTG2 excising Fapy as well as oxidized pyrimidines (Senturker *et al.*, 1998).

Presence of different Fapy in DNA might have quite divergent consequences, lethal and/or mutagenic. Fapy-7MeGua, when present in DNA, was shown to block DNA synthesis *in vitro* and in *E. coli* (O'Connor *et al.*,

1988; Tudek *et al.*, 1992). On the other hand, carcinogenic potency of aflatoxin B1 was correlated with the presence of its imidazole ring-opened guanine adduct in DNA (Hsieh & Atkinson, 1991). Lethal and mutagenic properties of imidazole ring-opened purines have also been demonstrated in mammalian cells in culture, in which overproduction of *E. coli* Fpg protein diminished lethal and mutagenic effects of thiotepa and aziridine (Cussac & Laval, 1996; Gill *et al.*, 1996). Our previous results also indicate that treatment with NaOH of methylated ssDNA of M13 phage creates mutagenic base derivatives giving rise to A→G transitions (60-fold increase) and G→T and G→C transversions (2–3-fold increase) (Tudek *et al.*, 1992). In this paper we demonstrate that lesions leading to these mutations are Fapy-7MeAde and Fapy-7MeGua.

MATERIALS AND METHODS

Chemicals

Dimethylsulfate (DMS) was from Aldrich; T7 Sequencing Kit from Pharmacia; deoxyadenosine-5′-[α^{35}S]thiotriphosphate – from NEN; isopropyl-β-D-thiogalactoside (IPTG) – from Boehringer Mannheim; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) – from Stratagene; [^{3}H]methylnitrosourea, 18 Ci/mmole – Amersham; polyA – Miles Lab; polydA – 40-mer oligodeoxynucleotides were synthesised by R. Gromadka (IBB PAS).

Modification of phage DNA

M13mp18 phage DNA was grown in JM105 (supE endA sbcB15 hsdR4 rpsL thi Δ (lac-proAB)/F'lacZ Δ M15 in 2YT medium (Sambrook *et al.*, 1989) overnight at 37°C. The phage was collected and its DNA was isolated according to Messing (1983).

DMS, immediately before use, was dissolved in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). Single-stranded M13 phage DNA was incubated with DMS (0.5–1 mM) for 10 min at 37°C. For each DMS concentration, as well as for a control, nonalkylated DNA, 15 μg of phage DNA in a total volume of 150 μl was used. Subsequently, DNA was ethanol precipitated and resuspended in 150 μl of TE buffer, pH 7.00.

Methylated DNA was divided into two parts. One part – 100 μl of DNA, immediately after DMS methylation was incubated with 0.2 M NaOH for 15 min at 37°C for imidazole ring opening of 7-methylpurines. Then, the pH of solution was adjusted to 8.00 with 1 M Tris/HCl buffer, pH 8,00, DNA was ethanol precipitated and resuspended in the Fpg buffer (70 mM Hepes/KOH pH 7.6, 100 mM KCl, 2 mM EDTA).

To obtain M13 phage DNA with a diminished number of Fapy-7MeGua residues, a part of M13 phage DNA modified with DMS+NaOH was digested with the Fpg protein. The enzyme excises Fapy-7MeGua and nicks DNA at AP sites causing linearization of phage DNA which makes it unable to give progeny in *E. coli* and thus eliminates mutations that could be derived from Fapy-7MeGua. Five micrograms of phage DNA was incubated with 0.56 μg of purified homogenous Fpg protein from *E. coli* for 10 min at 37°C. Then the enzymatic protein was removed from the reaction mixture by phenol extraction, the DNA was ethanol precipitated and resuspended in TE buffer.

The second part of M13 phage DNA modified only with DMS was selectively depurinated by 9 h incubation at 37°C, pH 7.00. After depurination DNA was incubated in NaOH to achieve imidazole ring-opening of the remaining 7-methylpurines, adjusted to pH 8.00 with 1 M Tris/HCl pH 8.00, ethanol precipitated and resuspended in TE buffer. Incubation of DNA in NaOH resulting in the cleavage of phosphodiester bonds at AP sites also causes linearization of circular phage DNA molecules. This made phage DNA molecules un-

able to replicate in *E. coli* and eliminated mutations that could be derived from AP sites created by depurination.

Monitoring of nucleic acids and polynucleotide methylation, imidazole ring opening and depurination

M13 phage DNA

In parallel to M13 phage DNA methylation with DMS, radiolabelled probe was prepared and it enabled to monitor DNA methylation, depurination and conversion of bases. The probe – ^{3}H-methylated M13mp18 phage DNA was prepared by reacting [^{3}H]MNU of the specific activity 18 Ci/mmole (Amersham, TRK 785) with DNA in 50 mM cacodylate buffer, pH 7.00, as described (Matijasevic *et al.*, 1996). Although a spectrum of DNA base modifications induced by MNU differs from that induced by DMS we decided to use it since: (i) [^{3}H]MNU was the only available radiolabelled alkylating agent, (ii) [^{3}H]MNU introduces a sufficient amount of methyl groups into N7 position of adenine (Singer & Grunberger, 1983) to monitor base rearrangements under basic and neutral conditions.

Degree of methylation of particular bases was established by HPLC analysis. Prior to HPLC, methylated bases were liberated from DNA by 20 min hydrolysis at 80°C in 0.1 N HCl. After neutralisation the DNA was precipitated. Supernatant was evaporated to dryness (SpeedVac-Savant), the residue was dissolved in 20 mM ammonium formate pH 3.5, supplemented with unlabelled authentic markers as internal standards and separated by HPLC on Nova-Pak C18 column, 60 Å, 4 μm, 4.6 mm × 250 mm (Waters). The column was developed isocratically at a flow rate of 1 ml/min, using 20 mM ammonium formate pH 3.5 as a mobile phase. Retention time of standard methylated bases was measured by UV monitoring at 260 nm. Fractions of 0.5 ml were collected and radioactivity measured by liquid scintillation.

Imidazole ring opening of 7MeGua was monitored in a radiolabelled probe, which was incubated in alkaline conditions in parallel with DMS-methylated DNA. Modified bases were liberated by acidic hydrolysis (0.1 N HCl, 20 min, 80°C) and analysed by HPLC.

Selective depurination of methylated bases was verified by HPLC of the supernatant remaining after precipitation of [^{3}H]MNU-M13 DNA, which had been incubated 9 h at 37°C, pH 7.00. Methylated bases remaining in depurinated DNA were also analysed by HPLC after their liberation by acidic hydrolysis (0.1 N HCl, 20 min, 80°C).

Elimination of Fapy-7MeGua from DMS and NaOH modified M13 DNA by Fpg protein digestion was verified by gas chromatography/isotope dilution mass spectrometry with selected ion monitoring. The DNA samples containing 100 μg of DNA (as determined by spectrophotometry) were supplemented with the following internal standards: 2 nmol of thymine-$\alpha,\alpha,\alpha,6$-$^2H_4$ and 0.5 mmol of Fapy-7MeGua. The samples were lyophilized and hydrolysed with 0.5 ml of 60% formic acid in evacuated and sealed tubes for 30 min at 140°C (Djuric *et al.*, 1991). The hydrolysates were lyophilized and then trimethylsilylated in polytetrafluorethylene-capped hypovials (Pierce Chemical Co.) with 100 μl mixture of BSTFA and acetonitrile (4:1, v/v) by heating for 30 min at 130°C under nitrogen. After hydrolysis and derivatization, the samples were analyzed by gas chromatography/isotope-dilution mass spectrometry with selected ion-monitoring according to the method described by Dizdaroglu (1994).

A Hewlett Packard Model 5890 Series II Model gas chromatograph interfaced to a Hewlett Packard Model 5972 mass selective detector was used. The injection port and GC/MS interface were both maintained at 250°C and the ion source at about 200°C. Separations were carried out using a fused-silica capillary column (Ultra 2, 0.2 mm × 12.5 m, Hewlett Packard) coated with crosslinked 5% phenylmethyl silicone, film thickness 0.33

mm. An aliquot (4 μl) of each derivatized sample was injected without any further treatment into the injection port of the gas chromatograph by means of an autosampler. Thymine-$\alpha,\alpha,\alpha,6$-$^2H_4$ was used as an internal standard for thymine to verify the amount of DNA in chromatin samples (Djuric *et al.*, 1991).

Poly(A) and poly(dA)

Poly(A) and poly(dA) were reacted with [^{3}H]MNU in the same way as M13 phage DNA. The content of methylated bases and conversion of 7MeAde in alkali was monitored by HPLC analysis after liberation of bases by acidic hydrolysis - 0.1 N HCl, 20 min, 80°C.

Preparation of competent cells and transformation

Bacteria were made competent by the $CaCl_2$ method (Sambrook *et al.*, 1989). The SOS system was induced prior to making bacteria competent. At the logarithmic phase of growth in LB medium JM105 was centrifuged, suspended in 0.1 M $MgSO_4$ and irradiated with UV light at 254 nm (40 J/m^2). Subsequently bacteria were centrifuged, resuspended in LB medium and further grown in LB at 37°C for 50 min under low light conditions in order to express SOS functions. Subsequently bacteria were harvested and made competent. 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 and exhibiting low or no β-galactosidase activity were recognized as colorless or light blue plaques on LB plates containing IPTG and X-gal. Mutant DNA was isolated according to Messing (1983) and the M13mp18 *lacZ* gene was sequenced using the dideoxynucleotide chain termination method (Sambrook *et al.* 1989). T7 Sequencing Kit (Pharmacia), [^{35}S]-dATP and a specific 19 nucleotide primer annealing at base positions for amino acids 71-78 of the *lacZ* gene of the M13+ strand were used for DNA sequencing.

RESULTS

Alkylation of DNA bases and their rearrangements

In the radioactive probe [^{3}H]MNU-M13 DNA, processed in parallel with M13 DNA methylated with dimethylsulfate, the predominant modification was 7MeGua (83% of methylated bases), then 1MeAde (8%) and 3MeAde (6%). 7MeAde was a minor lesion and constituted only 2.2% of the alkylated bases (Fig. 1A, Table 1). O^6-methylguanine migrated in HPLC with longer retention time in the conditions used (50 min) and is not shown on chromatograms. In alkali (0.2 M NaOH, 15 min, 37°C) the imidazole ring of 7MeGua was opened leading to formation of 2,6-diamino-4-hydroxy-5*N*-methyl-formamidopyrimidine (Fapy-7MeGua). Fapy-7MeGua occurs in DNA in two rotameric forms, which are separable by chromatographic techniques (Boiteux *et al.*, 1984). Two rotameric forms of Fapy-7MeGua were detected by HPLC of M13 DNA modified with [^{3}H]MNU and NaOH (Fig. 1B). HPLC analysis also showed that conversion of 7MeGua into Fapy-7MeGua was quantitative under the conditions used. After incubation of methylated M13 DNA in NaOH, no 7MeAde could be further detected by HPLC (Fig. 1B), suggesting that this procedure also transformed 7MeAde into Fapy-7MeAde. However, in DNA it was difficult to demonstrate such conversion, since the huge quantity of two

rotameric forms of Fapy-7MeGua, migrating with retention time similar to that of 1MeAde, 3MeAde and expected Fapy-7MeAde was masking transformation of 7MeAde in alkaline conditions in DNA. That is why the fate of 7MeAde was further followed in polydA and polyA. In polyA, about 70% of radioactive material was liberated from the oligonucleotide by mild acidic hydrolysis (0.1 N HCl, 20 min, 80°C) and practically the whole material comigrated with 7MeAde standard in HPLC (Fig. 2A). In poly(dA), at hydrolysis efficiency 80%, only 8% of radioactive material comigrated with 7MeAde and the rest with 1MeAde and 3MeAde (Fig. 3A). After incubation of MNU-modified oligonucleotides in alkali, 7MeAde disappeared from chromatograms of both poly(A) and poly(dA), but only in polyA two new peaks migrating before and between 1MeAde and 3MeAde could be detected (Fig. 2B, 3B). We suggest that Fapy-7MeAde, similarly to Fapy-7MeGua also exists in two rotameric forms, which we were able to separate in HPLC as two peaks migrating before and between 1MeAde and 3MeAde in the conditions used (Fig. 2B).

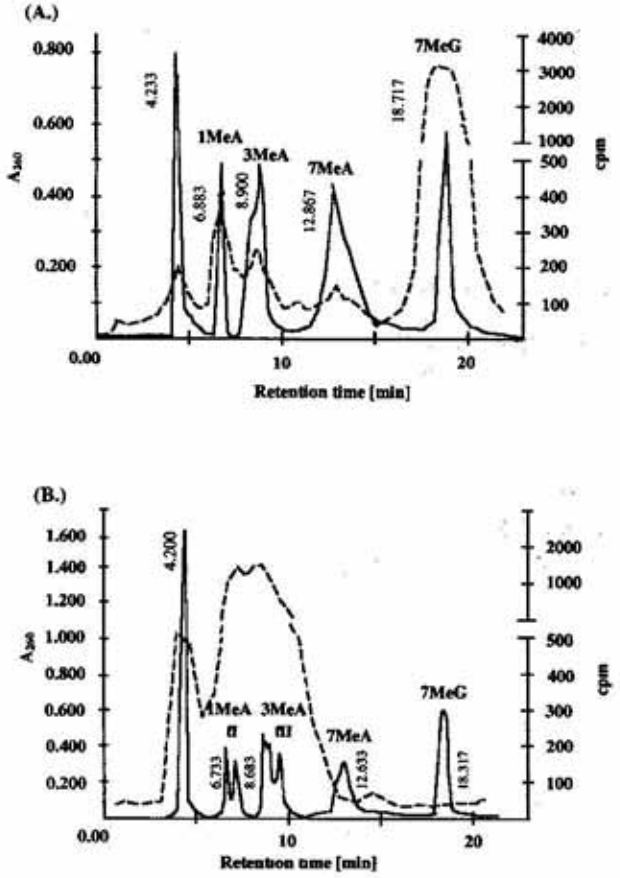

Figure 1. HPLC analysis of methylated bases in: (A) [^{3}H]MNU-methylated M13mp18 phage DNA; (B) [^{3}H]MNU-methylated M13mp18 phage DNA, which after methylation was incubated in alkali (0.2 M NaOH, 15 min 37°C).

Methylated bases were liberated by acidic hydrolysis (0.1 M HCl, 20 min, 80°C), supplemented with authentic markers (solid lines) and developed on Nova-Pak C18 column, 60 Å, 4 μm, 4.6 mm × 250 mm (Waters). The column was developed isocratically at a flow rate of 1 ml/ min, using as a mobile phase 20 mM ammonium formate pH 3.5. Fractions of 0.5 ml were collected and radioactivity measured by liquid scintillation (dotted lines); fI and fII peaks correspond to two rotameric forms of Fapy-7MeGua.

Selective elimination of alkylated bases from M13 DNA

The glycosidic bond of 7MeAde is very unstable in DNA with $t_{1/2}$ for depurination in pH 7.00, 37°C reported to be 3 h (Singer & Grunberger, 1983). We expected that 9 h incubation of alkylated M13 DNA at 37°C, pH 7.00, should eliminate over 80% of 7MeAde residues from methylated DNA. The effectiveness of spontaneous depurination was assessed on radioactive probe ([^{3}H]MNU-M13DNA), which was processed in the same time and exact conditions as M13 DNA methylated with DMS, subsequently used for transfection into *E. coli* and analysis of mutations. Depurination procedure resulted in elimination of at least 73% of 7MeAde residues from M13 DNA, as judged by HPLC (Table 1). 3MeAde and 7MeGua were also partially depurinated (17% and 14% depurination respectively), but the majority of these modifications remained in DNA (Table 1).

We eliminated Fapy-7MeGua residues from M13 DNA used for transfection into *E. coli* by digestion of DMS-NaOH modified DNA with the Fpg protein. The effectiveness of Fapy-7MeGua elimination was verified by the measurement of Fapy-7MeGua content in un-

Table 1. Depurination of methylated bases from [^{3}H]MNU-M13DNA by 9 h incubation at 37°C, pH 7.00, as measured by scintillation counting of fractions separated by HPLC.

Base analog	Radioactivity in the peak (c.p.m.)			Percent of depurination*
	Non-depurinated DNA	Depurinated DNA	Supernatant after depurination	
1MeAde	1643	1407	0	0
3MeAde	1220	494	210	17
7MeAde	450	0	330	73
7MeGua	16452	10164	2365	14
Total radioactivity	19765	12065	2905	

*Percent of depurination was calculated as amount of base analog found in the supernatant compared to its amount in non-depurinated DNA.

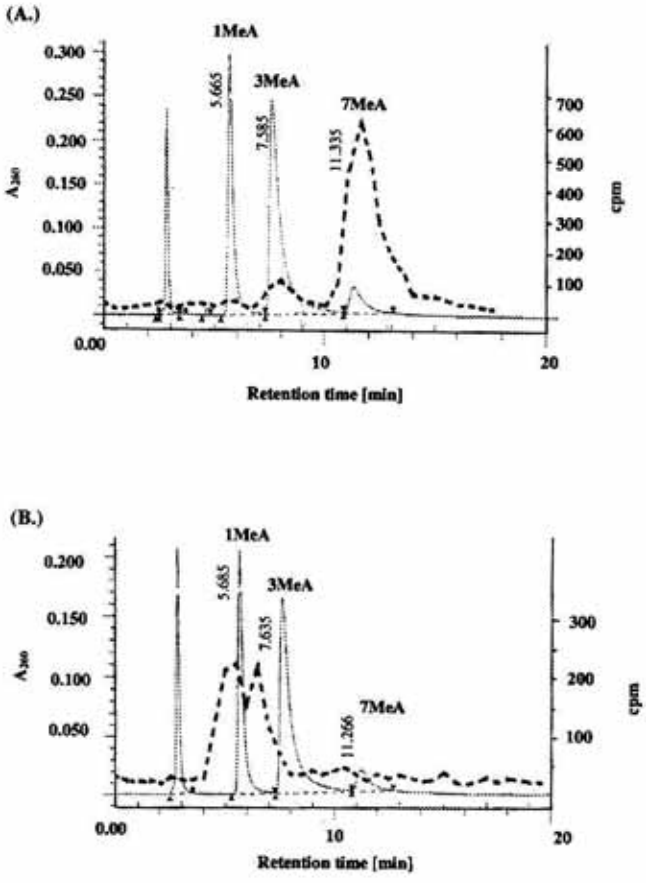

Figure 2. HPLC analysis of methylated bases in: (A) [^{3}H]MNU-methylated poly(A); (B) [^{3}H]MNU-methylated poly(A), which after methylation was incubated in alkali (0.2 M NaOH, 15 min 37°C).

For the details see Fig. 1.

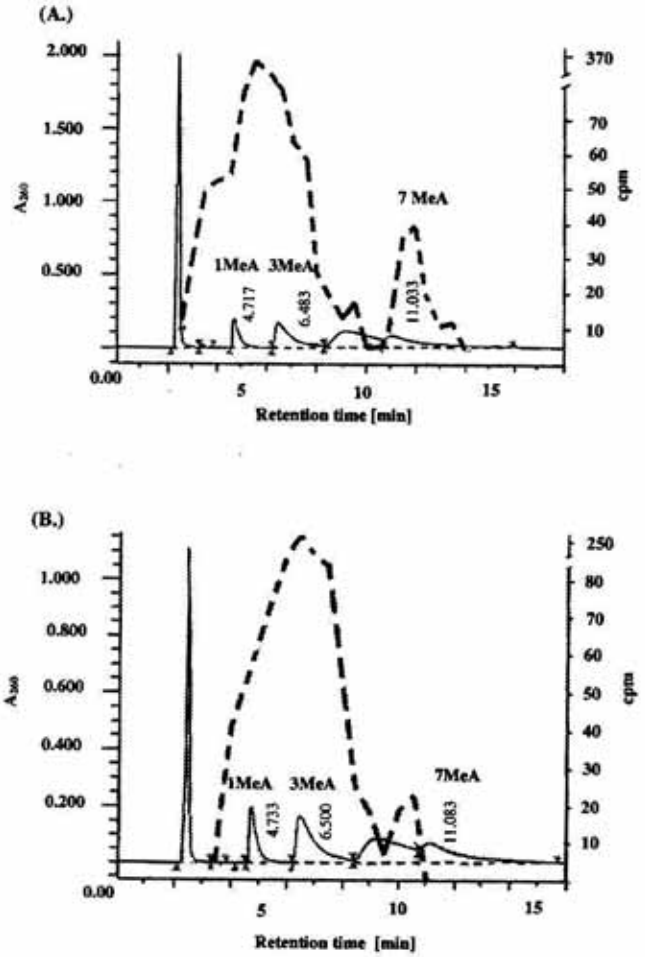

Figure 3. HPLC analysis of methylated bases in: (A) [^{3}H]MNU-methylated poly(dA); (B) [^{3}H]MNU-methylated poly(dA), which after methylation was incubated in alkali (0.2 M NaOH, 15 min, 37°C).

For the details see Fig. 1.

Table 2. Content of Fapy-7MeGua in M13mp18 phage DNA as a function of the Fpg protein treatment

Measured by gas chromatography-mass spectrometry.

DMS (mM)	Fapy-7MeGua (μmoles/mg DNA)	
	-Fpg	+Fpg
0	0	0
0.5	3.107	2.565
2.5	16.726	9.059
5.0	55.22	32.615

digested and Fpg-digested M13 DNA by GC/IDMS-SIM. Table 2 shows that the Fpg protein removed approximately 40% of Fapy-7MeGua residues from M13 DNA molecules used for transfection experiments.

Survival and mutagenesis

Selective depurination of DMS-methylated M13 phage DNA before imidazole ring opening in alkali as well as partial elimination of Fapy-7MeGua residues by digestion with the Fpg protein caused a decrease of phage survival in *E. coli* in comparison with DMS+base treated DNA. Mutation frequency in the *lacZ* gene was increased in the phage progeny obtained after transfection to *E. coli* of M13 DNA digested with the Fpg protein (Fig. 4) and suggested that the Fpg protein did not eliminate the lesion possessing strong mutagenic property. After depurination of 7MeAde from M13 DNA, the frequency of mutations remained on the same level as in the group obtained after transfection of DMS+base modified DNA into *E. coli* (Fig. 4).

Spectrum of mutations

Prevalent mutation type found in M13 *lacZ* gene in 3 different groups of phage progeny were base substitutions (Table 3). Groups of M13 mutants were obtained by transfection to *E. coli*: (i) M13 phage DNA modified only with

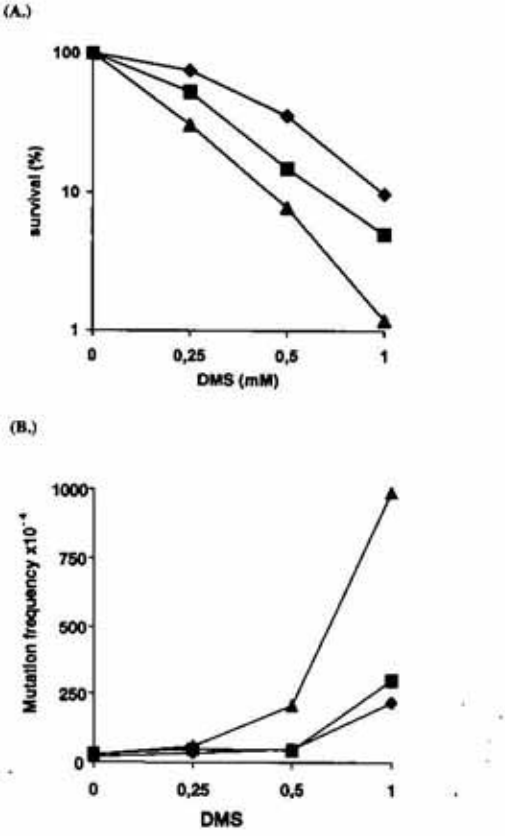

Figure 4. Survival (A) and the frequency of *lacZ* gene mutations (B) in M13mp18 phage DNA modified with: DMS and NaOH (◆); DMS and NaOH, but digested with the Fpg protein (▲); DMS and NaOH, but before ring opening in alkali, depurinated under neutral conditions (9 h, 37°C, pH 7.00 ■).

Modified DNA was transfected into JM 105 strain of *E. coli* induced to express SOS system by UV irradiation (see Materials and Methods).

DMS and NaOH – ("D+b"), (ii) "D+b" DNA digested with the Fpg protein – "FPG", (iii) M13 DNA in which methylated bases were selectively depurinated and subsequently imidazole ring-opened "DEP" (Table 1). Digestion of "D+b" M13 DNA with the Fpg protein resulted in the increase of base substitutions in mutation spectrum from 78% in "D+b" group to 87% in "FPG" group. In "DEP" group, a decrease (to 70%) of base substitutions was observed. Large deletions of 93 and 54 nucleotides being the result of recombination between *lacZ* gene of M13 phage DNA and the F′ factor of *E. coli* (Kunkel, 1984)

Table 3. Types of mutations in the *lacZ* gene of M13 phage.

Type of mutation	"D+b"[a] Number of mutants	%	FPG"[b] Number of mutants	%	"DEP"[c] Number of mutants	%
Base substitution	43	78	48	87	36	70
Frameshift	6	11	5	9	4	8
Del. of 93 and 54 bases	6	11	2	4	11	22
Total	55	100	55	100	51	100

[a]"D+b" mutations were obtained after transfection of DMS and NaOH modified phage DNA into SOS-induced *E. coli*; [b]"FPG" mutants were obtained after transfection to *E. coli* of DMS+NaOH modified phage DNA, but digested with the Fpg protein; [c]"DEP" mutants were obtained after transfection to *E. coli* of methylated M13 DNA, which was selectively depurinated before imidazole ring opening. Del, deletion.

were decreased in the 'FPG' group to only 4%, while they increased among "DEP" mutants, constituting as much as 22% of all mutations identified (Table 3).

Cleavage of DMS and NaOH modified M13 DNA with the Fpg protein eliminated 40% of Fapy-7MeGua residues (Table 2). This was accompanied by the decrease of the frequency of G→T and G→C transversions in comparison with "D+b" group (Table 4). In "FPG" mutants, the most prevalent base substitution found was A→G transition (33%), followed by

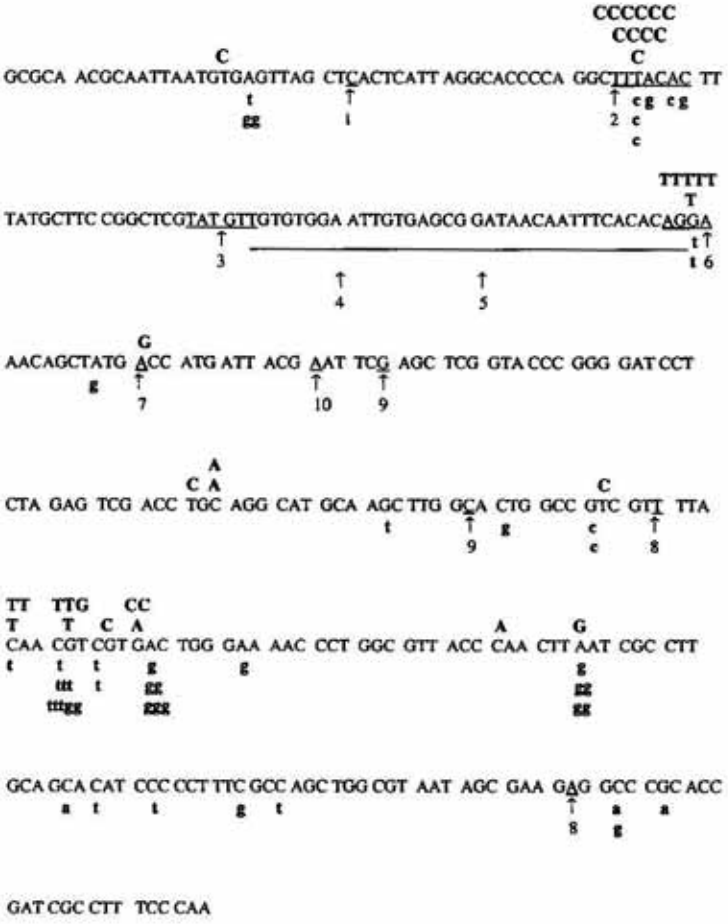

Figure 5. Spectrum of base substitutions in M13mp18 *lacZ* DNA.

The 5'→3' sequence of the *lacZ* fragment of M13mp18 is shown, from the first nucleotide after the *lacI* termination codon through the coding sequence for amino acid 65 of the gene. Base substitution mutations observed after digestion of DMS and NaOH modified M13 DNA with the Fpg protein are shown above the wild type sequence in capital letters; mutations in lower case and below the wild type sequence represent those obtained after limited depurination of methylated M13 DNA, before imidazole ring opening in alkali. (1) CAP site; (2) -35 promoter; (3) -10 promoter; (4) transcription start site; (5) operator site; (6) ribosome binding site; (7) translation start site; (8) first and last nucleotide of 93-nucleotide deletion; (9) first and last nucleotide of 54-nucleotide deletion of the polylinker region; (10) the site of *Eco*RI site and A→G transition in the double 93- and 54-nucleotide deletion mutants.

Table 4. Base substitutions in the *lacZ* gene of M13mp18 phage in the following groups of mutants: "D+b", "FPG" and "DEP"(for explanation see Table 3 and Results).

Mutation	"D+b" Number of mutants	Mutation freq×10^{-5}	%	"FPG" Number of mutants	Mutation freq×10^{-5}	%	"DEP" Number of mutants	Mutation freq×10^{-5}	%
A → G	14	65	30	16	34	33	2	7.2	5.5
A → T	1	5	2	1	2.5	2.5	0	0	0
A → C	1	5	2	1	2.5	2.5	0	0	0
total	16	75	34	18	39	38	2	7.2	5.5
G → A	1	5	2	1	2.5	2	1	3.6	3
G → T	4	18	9	3	5	6	6	18	17
G → C	3	14	7	2	5	4	4	11	11
total	8	37	18	6	12.5	12	11	33	31
C → T	8	37	18	13	29	27	6	18	17
C → A	6	28	12	2	5	4	3	11	8
C → G	0	0	0	6	10	13	1	4	3
total	14	65	30	21	44	44	10	33	28
T → C	2	9	4	3	2.5	6	13	40	36
T → A	3	14	7	0	0	0	0	0	0
T → G	3	14	7	0	0	0	0	0	0
total	8	37	18	3	2.5	6	13	40	36

mutations at cytosine sites in the template (C→T, 27%; C→G, 13%; C→A, 4%) and at thymine sites: the latter constituted only 6% of all base substitutions.

Depurination of the majority of 7MeAde residues from M13 DNA before opening of imidazole rings in alkali (Table 1) changed the frequency of A→G transitions. Only 2 of 36 base substitutions among "DEP" mutants were A→G transitions and they constituted 5% of all mutations found. The frequency of A→G transitions in this group, decreased 9-fold in comparison to "D+b" group and 5-fold in comparison to the "FPG" group (Table 4). In "DEP" mutants the frequency of G→T and G→C transversions was the same as in "D+b" mutants (Table 4). These results suggest that a miscoding adenine lesion pairing with cytosine during replication originates from 7MeAde and most probably is Fapy-7MeAde. Fapy-7MeGua, primarily a lethal damage, under SOS conditions might induce G→T and/or G→C transversions.

An unexpected observation concerned mutations at thymine sites in the template. Action of alkali on M13 DNA methylated with DMS dramatically increased the frequency of all types of thymine substitutions (Tudek *et al.*, 1992) (Table 4). In "DEP" mutants the prevalent mutation observed was T→C transition and it constituted 36% of all mutations found (Table 4). After cleavage of DMS and NaOH modified DNA with the Fpg protein, only 6% of mutations were found at thymine sites in the template and they were exclusively T→C transitions (Table 4). This might suggest that

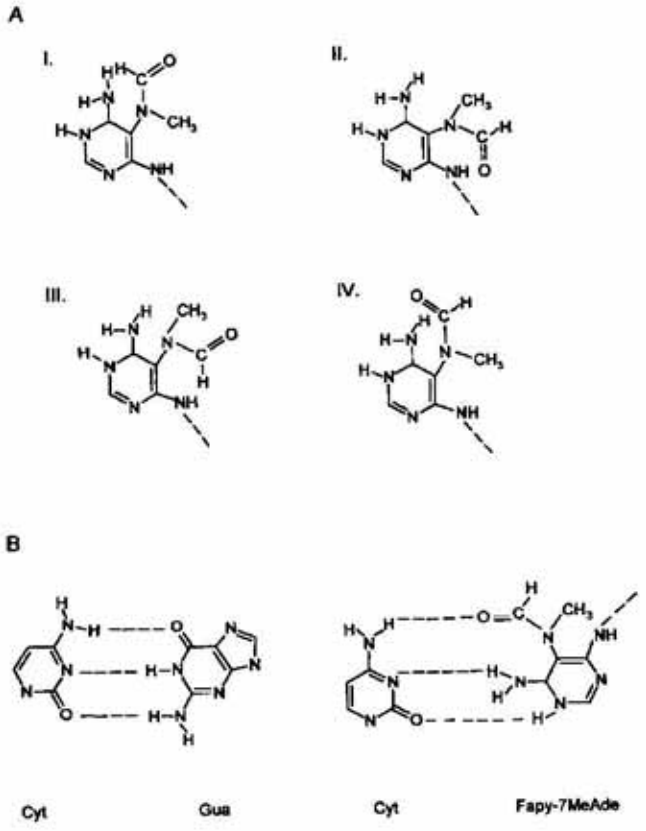

Figure 6. 4,6-Diamino-5*N*-methylformamidopyrimidine (Fapy-7MeAde). (A) Four possible rotameric forms. (B) Hydrogen bonding face of guanine and rotamer IV of Fapy-7MeAde in pair with cytosine – a hypothetical model.

incubation of methylated DNA in alkali creates a mutagenic thymine derivative, which mispairs with guanine during replication and is recognized as a substrate by the Fpg protein. Further studies are, however, necessary for an explanation of this observation.

The pattern of distribution of base substitutions within the *lacZ* sequence in "FPG" mutants did not overlap the one found in "DEP" group (Fig. 5). In the –35 promoter of the *lacZ* gene in "DEP" mutants the "hot spot" at thymine site in the template was observed exclusively with T→C transitions. In "FPG" group T→C substitutions were found in the same site, but they were much less numerous. The same was true for G→T transversions in both groups at ribosome binding site. In the part of the gene coding for protein, several triplets were more frequently mutated than the others. Interestingly, different nucleotides were mutated in the same triplet in "DEP and "FPG" group. For example, in amino-acid position 33 in –GAC– triplet "DEP" mutants had substitutions at guanine site, while in "FPG" group, a hot spot for A→G transitions was localized in the same triplet (Fig. 5).

DISCUSSION

We used dimethylsulfate and single-stranded M13 phage DNA to investigate mutagenic adenine derivative created in alkali from methylated base, since this SN_2 alkylating agent introduces less then 0.2% of methyl groups into O^6 position of guanine in ssDNA and quite important amount of methyl groups into N7 and N3 adenine positions (Singer & Grunberger, 1983), so that mutations at adenine sites in the template could be easily detected and quantified (Tudek *et al.*, 1992). For chemical analysis of rearrangements of methylated adenine derivatives, we, however, used [^{3}H]methylnitrosourea, since this was the only available radioactive methylating agent introducing enough amount of methyl groups into adenine N7 position (Singer & Grunberger, 1983) for moni-

toring base rearrangements in alkaline and neutral conditions. MNU could not, however be used for genetic experiments, since as much as 3% of methyl groups are introduced by this compound into O^6 guanine position in ssDNA and presence of this highly miscoding derivative in DNA results in formation of almost exclusively G:C→A:T transitions (Horsfall *et al.*, 1990).

The profile of adenine methylation in different positions was different for polynucleotides containing ribose and deoxyribose (Figs. 1–3). While in M13 DNA and in poly(dA), 7MeAde was a minor lesion, in poly(A), 7MeAde was the main product of adenine alkylation, since the efficiency of poly(A) hydrolysis for HPLC was 70% (not shown) and practically all radioactivity comigrated with 7MeAde standard (Figs. 1A, 2A, 3A). Similarly, after reaction of methylating (MMS) and ethylating (EMS) agents with free adenosine and oligoribonucleotides, methyl groups were more frequently detected in adenine N7 position, although 1MeAde and 3MeAde were also abundant (Singer *et al.*, 1974). Since the glycosidic bond of 7MeAde is less stable than glycosidic bonds of 3MeAde and 1MeAde, it is possible that the used mild hydrolysis liberated only 7MeAde from poly(A) and this permitted to follow rearrangements of this base in alkaline conditions. Others have also found adenine N7 position as a minor alkylation site in polydeoxynucleotides (Bodell & Singer, 1979; Lawley & Brookes, 1964). This could partially be due to the fact that the glycosidic bond of 7MedAde is extremely labile in DNA, about three orders of magnitude more labile than in ribonucleotides. $t_{1/2}$ for 7MeAde depurination from DNA equals only 3 h in 37°C, pH 7.00, while for 3MeAde – 26 h and for 7MeGua – 155 h (Singer & Grunberger, 1983), so during the alkylation procedure an important fraction of 7MeAde could be lost due to depurination.

Recently it was shown that elimination of 7MeAde from DNA can also occur by enzymatic excision by 3-methyladenine-DNA-glycosylase II from *E. coli* (AlkA protein) (Bjelland & Seeberg, 1996). In alkali, 7-methylpurines were shown to convert into ring-opened Fapy. Two rotameric forms of Fapy-7MeGua (Fig. 1B) (Boiteux *et al.*, 1984) and Fapy-7EtGua were separated by HPLC (Tudek *et al.*, 1998). We have found for the first time that 7MeAde when present in poly(A), in NaOH was also converted into derivative migrating in HPLC as 2 peaks similarly to Fapy-7MeGua, however with different time of retention. We suggest that this derivative is Fapy-7MeAde. We wanted to demonstrate the possible mutagenic properties of imidazole ring-opened purines in nucleic acids. Fapy-7MeGua in DNA blocks replication one base before the lesion both *in vitro* (O'Connor *et al.*, 1988) and in *E. coli* (Tudek *et al.*, 1992). Partial elimination of Fapy-7MeGua by digestion of M13 DNA with the Fpg protein diminished the frequency of G→T and G→C transversions, which suggested that these mutations were derived from Fapy-7MeGua (Table 4). Depurination of 7MeAde from M13 DNA before imidazole ring-opening in alkali resulted in dramatic 9-fold decrease of the frequency of A→G transitions. This suggests that mutagenic adenine modification specifically triggering A→G transitions originates from 7MeAde and most probably is Fapy-7MeAde. Fapy-7MeAde would be approximately two orders of magnitude more mutagenic than Fapy-7MeGua, since as a minor lesion (40-fold less abundant in DNA than Fapy-7MeGua, Table 1) was giving rise to 7-fold more mutations than Fapy-7MeGua (Table 4).

Disruption of imidazole ring of purine changes the hydrogen bonding face of the base and increases the number of possible conformations in the double helix. Four rotameric forms of Fapy-7MeGua and Fapy-7MeAde are possible and the existence of two of them was confirmed by NMR studies for Fapy-7MeGua (Boiteux *et al.*, 1984) and by HPLC (Fig. 1B, 2B). We have recently sug-

gested that oxidatively formed Fapy-adenine triggers SOS-dependent A→G transitions, since one of its possible rotameric forms resembles guanine very much and can pair with cytosine during replication (Grąziewicz *et al.*, 1999). We have also observed that unsubstituted FapyAde is a moderate chain terminator, when present in DNA and its inhibitory effect depends on DNA polymerase and sequence context (our unpublished results). Fapy-7MeAde might have similar properties. Due to the structural flexibility gained by imidazole ring opening, Fapy-7MeAde could probably both stop DNA synthesis and induce A→G transitions, since one of its possible conformations resembles hydrogen bonding face of guanine (Fig. 6). A similar increase of toxicity accompanied by 20-fold increase of mutagenicity was observed when pyrimidine ring of $1,N^6$-ethenoadenine was opened in alkali (Basu *et al.*, 1993).

Spontaneous formation of Fapy-7MeAde is very likely to occur in ribonucleic acids. Studies on alkylation of free adenosine revealed that 7-methyladenosine also converts spontaneously to Fapy-7-methyladenosine in neutral conditions (Singer *et al.*, 1974). In our study 7MeAde in poly(A) also spontaneously converted into product(s) migrating in HPLC similarly to those observed after action of NaOH on alkylated poly(A) (not shown). More than 50% of 7MeAde converted into Fapy-7MeAde in 24 h (not shown). Spontaneous imidazole ring opening of 7MeAde in DNA cannot be excluded. Although in DNA the base undergoes depurination very quickly, a portion of it might spontaneously convert into Fapy-7MeAde and this might probably explain a small portion of A:T→G:C transitions observed in bacterial mutagenesis with SN_2 alkylating agents (Horsfall *et al.*, 1990).

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