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Enzymic removal of 5-methylcytosine from DNA by a human DNA-glycosylase

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ABSTRACT

DNA 5-methylcytosine is a major factor in the silencing of mammalian genes; it is involved in gene expression, differentiation, embryogenesis and neoplastic transformation. A decrease in DNA 5-methylcytosine content is associated with activation of specific genes. There is much evidence indicating this to be an enzymic process, with replacement of 5-methylcytosine by cytosine. We demonstrate here enzymic release of 5-methylcytosines from DNA by a human 5-methylcytosine-DNA glycosylase activity, which affords a possible mechanism for such replacement. This activity generates promutagenic apyrimidinic sites, which can be related to the high frequency of mutations found at DNA 5-methylcytosine loci. The recovery of most released pyrimidines as thymines indicates subsequent deamination of free 5-methylcytosines by a 5-methylcytosine deaminase activity. This prevents possible recycling of 5-methylcytosine into replicative DNA synthesis via a possible 5-methyl-dCTP intermediate synthesized through the pyrimidine salvage pathway. Taken together, these findings indicate mechanisms for removal of 5-methylcytosines from DNA, hypermutability of DNA 5-methylcytosine sites, and exclusion of 5-methylcytosines from DNA during replication.

INTRODUCTION

5-methylcytosine constitutes 0.7–3.0% of vertebrate DNA bases, and is found mostly at CpG dinucleotides; its extent is both tissue-specific and species-specific. Methylation of DNA cytosines is a post-replicative process, catalyzed by DNA methyltransferases (1–3). It is involved in many cellular processes, including gene expression, DNA recombination, DNA replication, DNA repair and chromatin organization (1–5). Many housekeeping genes, inherited in a non-expressed form, are reactivated upon reduction of DNA 5-methylcytosine content (1–7). Altered levels of DNA 5-methylcytosines have been correlated with both neoplastic development and differentiation, in differing cellular systems (1–8). The presence of DNA 5-methylcytosines also constitutes a major factor in the silencing of genes in mammalian cells (1–7). Reduction of DNA 5-methylcytosine content, in the absence of replication, has also been associated with activation of specific genes (9–10). Evidence obtained from differentiating erythroleukemia cells indicates this to be an enzymic process with replacement of 5-methylcytosine by cytosine (10). This process has been termed 'demethylation' of DNA (1–7). One mechanism of DNA demethylation has been demonstrated in developing chicken embryo extracts; it involves the removal of a deoxyribonucleotide containing 5-methylcytosine and subsequent filling of the resultant gap by DNA polymerase 13 (11). This mechanism has not been demonstrated in other systems, and that particular excision nuclease activity is not present in nuclear extracts of human HeLa cells (11).

To determine an enzymic mechanism by which DNA 5-methylcytosine content is reduced *in vitro* by a human cell extract, we synthesized the alternating copolymer poly(dG-5-methyl-dC):polydG-5-methyl-dC), radiolabeled in the methyl group of 5-methylcytosine, for use as a substrate. This polydeoxyribonucleotide was reacted with a nuclear preparation from HeLa cells. Enzymic depyrimidination of 5-methylcytosines from DNA by a human 5-methylcytosine-DNA glycosylase activity was demonstrated. This glycosylase activity generates promutagenic apyrimidinic 4 sites, which can be related to the high frequency of mutations found at DNA 5-methylcytosine loci. Another activity then apparently deaminates free 5-methyl-cytosines to thymines, preventing possible recycling of 5-methylcytosine into replicative DNA synthesis. These findings indicate a possible pathway for the removal of 5-methylcytosines from DNA, a possible basis for the hypermutability of DNA 5-methylcytosine sites, and a means of exclusion of 5-methylcytosines from DNA during replication.

MATERIALS AND METHODS

The double-stranded polydeoxyribonucleotide poly(dG-5-methyl-dC):poly(dG-5-methyl-dC), [^{3}H]-radiolabeled in the methyl group, was synthesized by methylation of 1 μg of poly(dG-dC):poly(dG-dC) (LKB Pharmacia) by 5 units CpG (SssI) methylase (New England Biolabs) with 1 μCi of S-([^{3}H]-methyl)adenosyl-L-methionine (New England Nuclear;

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specific activity 55–85 Ci/mmol) in a reaction volume of 300 μL for 4 hours at 37 °C. Unlabeled S-adenosyl-L-methionine was added to a final concentration of 30 μM and the reaction continued overnight to achieve methylation of DNA at every cytosine. The DNA was extracted with phenol:chloroform and ethanol-precipitated. The specific activity was 36.5×10^4 cpm/μg. Complete methylation of the DNA segment, 700 base pairs long, was demonstrated by total resistance to digestion by restriction endonuclease HhaI using 2% agarose gel electrophoresis (12). The presence of over 99% of the radiolabel in 5-methylcytosine was demonstrated by denaturation of DNA and digestion of the polymer by Sl nuclease (13). Excess acid phosphatase was present during this reaction. The digestion products were separated by descending chromatography using Whatman No.1 paper in *n*-butanol:water (86:14). The R_f of 5-methyl-2′-deoxycytidine was 0.3, and that of thymidine 0.5, in this system. Radioactive contents of the spots, determined by liquid scintillation counting, demonstrated 99% of the radiolabel to be in 5-methylcytosine.

Radiolabeled substrate poly(dG-5-methyl-dC):polydG-5-methyl-dC), equivalent to 660 pmoles of 5-methylcytosine, was reacted with 13.6 μg of HeLa nuclear extract (HelaScribe, Promega; commonly used in transcription studies) in 100 μL 20 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, at 37 °C for the times indicated. The reaction was stopped by chilling. 50 μL of 1 mg/ml 6 calf thymus DNA was added, with ethanol precipitation, centrifugation, and liquid scintillation counting of the supernatant performed according to Weiss *et al.* (14).

The products released from substrate poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) were determined by two methods: high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). For HPLC analyses, the reaction was stopped by ethanol precipitation and centrifugation was performed (14). The supernatant was lyophilized, dissolved in 200 μL water, filtered, and coinjected with marker compounds into an 8 mm × 10 cm Waters C_{18} Radial-Pak cartridge in a Waters radial compression Z-module with water as the eluant at a flow of 1 mL/min. These fractions were collected at 0.5 min intervals, and their radioactivity determined (14). Thin-layer chromatography of the ethanol-soluble material was performed according to Radany and Friedberg (15), in the presence of marker 5-methylcytosine and thymine. The R_f of 5-methylcytosine was 0.05 and that of thymine was 0.65 in this system.

The formation of DNA abasic sites following enzymic 5-methylcytosine excision was directly demonstrated by reductive labeling of these sites by radiolabeled sodium borhydride. Unlabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) (Pharmacia-LKB) was incubated with increasing concentrations of HeLa nucleoprotein. 300 ng of polydeoxyribonucleotide were in each reaction mixture. After an 18 hour incubation at 37 °C, the tubes were chilled in ice. The DNA was extracted with phenol:chloroform, ethanol-precipitated in 0.3 M sodium acetate, pH 5.2, and washed with 70% ethanol. The precipitate was air-dried, taken up in 50 μL 0.4 M potassium phosphate, pH 6.5, and dissolved after agitation on a vortex mixer. Sodium borhydride (New England Nuclear NET-023; specific activity 359.8 mC/mmol) was thawed in an ice bath after storage at −80°C. 1.6 mL of ice-cold 0.4 M potassium phosphate, pH 6.5, was added to the 5 mCi of $NaBH_4$. 50 μL of the labeled NAB_4 solution was immediately added to each reaction tube, and the reduction proceeded at room temperature for one hour. The reaction mixtures were desalted, and the unreacted $NaBH_4$ removed, by two passages through centricon-10 filters (Amicon). They were spun at 4200 rpm in a Sorvall RC-3 centrifuge equipped with a HG-4L rotor for 30 minutes and 20 minutes, respectively. One mL of dH_2O was added to each filter before every spin. The DNA retained above the filter was collected and counted in Scintiverse (Fisher). A parallel set of experiments using radiolabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) was performed to compare the excision of pyrimdines from DNA with the enzymic generation of abasic sites. 300 ng of DNA was reacted with increasing concentrations of HeLa nuclear extract protein at 37°C for 18 hours. The reaction was stopped by ethanol precipitation, with centrifugation and liquid scintillation counting of the supernatant performed as described (14). The quantity of radiolabeled material released into the ethanol-soluble fraction was the measure of enzymic activity.

The effect of DNA hemimethylation on 5-methylcytosine-DNA glycosylase activity was explored. Radiolabeled, fully methylated, poly(dG-5-methyl-dC):poly(dG-5-methyl-dC), equivalent to 725 pmoles of 5-methylcytosine (135,000 cpm), was hybridized with a threefold excess of poly(dG-dC).poly(dG-dC) in 50% formamide, 0.9 M NaCl, 0.09 M sodium citrate, pH 7.4. Initial denaturation was by heating at 82 °C for 5 minutes, followed by reannealing at 61 °C for 30 minutes and 71 °C for 2 hours. The DNA was left at room temperature for 30 minutes; the salt and formamide were removed by two serial passages through centricon-10 filters (Amicon). This rehybridized DNA was used as the hemimethylated substrate. HeLa 5-methylcytosine-DNA glycosylase activity was then assayed as described above.

RESULTS

The possible mechanism of removal of 5-methylcytosine from DNA was investigated by enzymological techniques. The alternating copolymer, poly(dG-5-methyl-dC):polydG-5-methyl-dC) radiolabeled in the methyl group of 5-methylcytosine, was

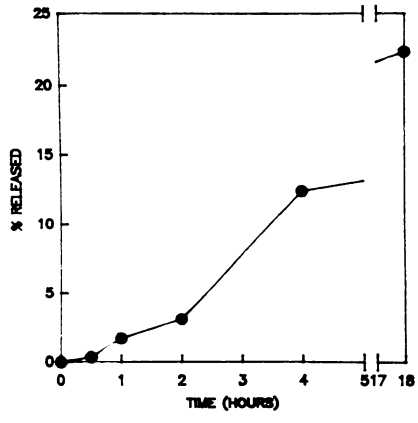

Figure 1. v vs. [T] Plot of release of pyrimidines from poly(dG-5-rnethyl-dC):poly(dG-5-methyl-dC) by a human nuclear preparation. Radiolabeled substrate poly(dG-5-methyl-dC):poly(dG-5-rnethyl-dC), equivalent to 660 pmoles of 5-methylcytosine, was reacted with 13.6 μg of HeLa nuclear extract as described for the times indicated. The reaction was stopped by chilling, and ethanol precipitation, centrifugation, and liquid scintillation counting of the supernatant performed as described. The quantity of radiolabeled material released into the ethanol-soluble fraction was the measure of enzymic activity. Each point represents the average of two independent determinations.

used as substrate with a nuclear preparation from HeLa cells as the enzyme source. The results are shown in figure 1. The quantity of radioactivity released into the ethanol-soluble fraction increased with the time of reaction. The enzymic release of 5-methylcytosine from DNA is dependent on concentration of enzyme protein, as shown in figure 2. This increase in release

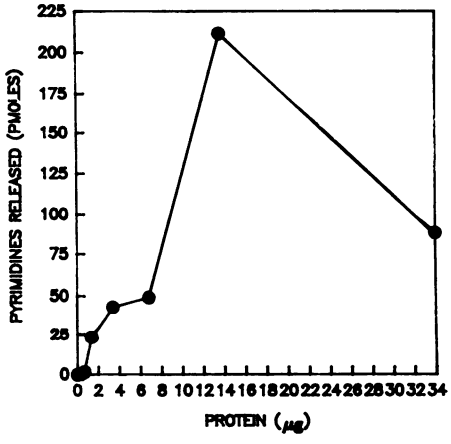

Figure 2. v vs. $[E]_t$ Plot of release of pyrimidines from poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) by a human nuclear preparation. Radiolabeled substrate poly(dG-5-methyl-dC):polydG-5-methyl-dC), equivalent to 530 pmoles of 5-methylcytosine, was reacted with increasing concentrations of HeLa nuclear extract protein at 37 °C for 18 hours. The reaction was stopped, with ethanol precipitation, centrifugation, and liquid scintillation counting of the supernatant performed as described above. The quantity of radiolabeled material released into the ethanol-soluble fraction was the measure of enzymic activity. Each point represents the average of two independent determinations.

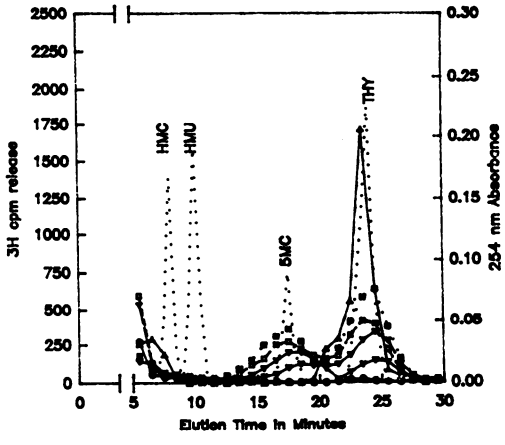

Figure 3. Analysis of DNA pyrimidines released from poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) by a human nuclear preparation Radiolabeled substrate poly(dG-5-methyl-dC):polydG-5-methyl-dC), equivalent to 660 pmoles of 5-methylcytosine, was reacted with 13.6 μg of HeLa nuclear extract as described above for the times indicated. The reaction was stopped, with ethanol precipitation, centrifugation and HPLC analysis performed as described above. The marker compounds, shown by their A_{254} on the right ordinate, are indicated by the dotted line (left to right): (1) 5-hydroxymethylcytosine (8 minutes), (2) 5-hydroxymethyluracil (10 minutes), (3) 5-methylcytosine (17.5 minutes), and, (4) thymine (23.5 minutes). The DNA was reacted with the HeLa nuclear preparation for 0 (●), ½ (▽), 1 (▼), 2 (□), 4 (■) and 18 (△) hours, respectively. The radioactive peaks, shown on the left ordinate, were counted as described above.

of radiolabeled material was linear to 14 pg of nucleoprotein. Enzymic release of radiolobeled pyrimidines was also linear with increasing concentrations of substrate DNA 5-methylcytosines until 3600 pmoles per reaction mixture was reached. The velocity curve was sigmoid (data not shown). Analysis by the Hill equation indicated the enzyme to have about 2.2 binding sites per molecule, with the K' ($[S]_{½}^{2.2}$) = 3.2 pMoles/hour/μg nucleoprotein and the V_{max} = 2.0 pmoles/hour/μg nucleoprotein (data not shown). This indicates the removal of 5-methylcytosine from DNA to be a complex process. Unlike the other glycosylases of base excision repair that remove inappropriate DNA bases, this activity requires the presence of Mg^{+2} in the reaction mixture; it is 90% product-inhibited at 10 mM 5-methylcytosine.

The enzyme-released pyrimidines were characterized both by HPLC and TLC. A profile of the HPLC analysis of these bases is presented in figure 3, while the TLC results are shown in table 1. The findings by both techniques were identical. The pyrimidine products comprised a mixture of 5-methylcytosine and thymine; neither 5-hydroxymethylcytosine nor 5-hydroxymethyluracil were detected. Variable quantities of oligonucleotides were detected during the first five minutes of HPLC column elution ahead of the pyrimidines. These reflect the presence of the HeLa 9 endonucleases that incise DNA at base loss sites (16−17). The ratio of 5-methylcytosine to thymine decreased with the time of reaction. After 18 hours, no 5-methylcytosine was detected. This shows 5-methylcytosine to be rapidly deaminated to thymine following its enzymic release from DNA.

In order to exclude the possibility of non-specific degradation of DNA by HeLa nucleases as being responsible for the apparent glycosylase activities, and to confirm the glycosylic removal of 5-methylcytosine as a free base, generation of apyrimidinic sites in the substrate DNA was directly demonstrated. Unlabeled poly(dG-5-methyl-dC):polydG-5-methyl-dC) was reacted with the HeLa nuclear preparation, the DNA recovered from the reaction mixture, and then reacted with tritiated sodium borhydride. This reagent reduces the aldehydic moiety of DNA abasic sites with an efficiency that exceeds 90% (18). The radioactivity bound to the DNA following reduction by sodium borhydride is therefore a measurement of the number of abasic sites present. These results were compared with those of a simultaneous assay of bases enzymically released from radiolabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC), these are shown in figure 4. The release of pyrimidines from DNA was closely paralleled by induction of abasic sites, which were reductively labeled by the

Table 1. Deamination of enzyme-released 5-methylcytosine

Time (hours)	pmoles recovered 5-methylcytosine	thymine	[5-methylcytosine]/[thymine]
1	0.78	1.45	0.35
2	3.02	7.91	0.28
4	4.23	15.8	0.21
8	6.64	75.8	0.08
18	0	147	0

The DNA was reacted with the HeLa nuclear extract as described in Figure 1. TLC of the ethanol-soluble material was performed in the presence of marker 5-methylcytosine and thymine. The R_f of 5-methylcytosine was 0.05 and that of thymine was 0.65 in this system. Neither TMP nor 5-methyl-dCMP moved beyond the origin. No enzyme-released radioactivity was associated with 5-hydroxymethyl-uracil; definitive separation of 5-methylcytosine from 5-hydroxymethylcytosine could not be achieved in this system as in the HPLC system. Each point represents the average of two independent determinations.

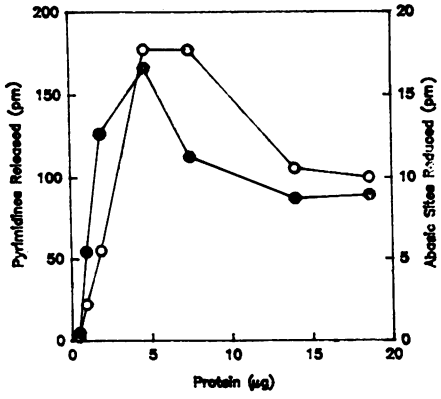

Figure 4. Demonstration of abasic sites in DNA following enzymic 5-methylcytosine excision. 300 ng of unlabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) (Pharmacia-LKB) was incubated with differing concentrations of HeLa nucleoprotein for 18 hours as described in the legend to figure 2. After the reaction, the DNA was recovered and reacted with radiolabeled sodium borhydride as decribed. Reaction mixtures were desalted and unreacted $NaBH_4$ removed, by passages through centricon-10 filters. The DNA retained above the filter after the last spin was collected and counted in Scintiverse (Fisher). Each point represents the average of three independent determinations. A parallel set of experiments using radiolabeled poly(dG-5-methyl-dC):polydG-5-methyl-dC) was performed to compare the excision of pyrimdines from DNA with the enzymic generation of abasic sites. 300 ng of DNA was reacted with increasing concentrations of HeLa nuclear extract protein at 37 °C for 18 hours. Glycosylase activity was assayed as described. The quantity of radiolabeled material released into the ethanol-soluble fraction was the measure of enzymic activity. Each point represents the average of three independent determinations. Left ordinate: [^{3}H]-labeled pyrimidines released from radiolabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) by the HeLa nuclear preparation (open circles); Right ordinate: reduced [^{3}H]-labeled abasic sites detected by the reducing of radiolabeled $NaBH_4$ after enzymic depyrimidination of unlabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) by the HeLa nuclear preparation (closed circles).

Table 2. Effect of DNA hemimethylation on excision of 5-methylcytosine from DNA

DNA	radioactivity released (%)	% recovered as 5-methylcytosine	% recovered as thymine
Totally Methylated	32.6	0.8	31.7
Hemimethylated	4.8	1.7	3.1

Radiolabeled, fully methylated, poly(dG-5-methyl-dC):poly(dG-5-methyl-dC), equivalent to 725 pmoles of 5-methylcytosine cpm), was hybridized to the unlabeled poly(dG-dC):poly(dG-dC) as described. Salts and formamide were removed by two serial passages through centricon-10 filters. HeLa 5-methylcytosine-DNA glycosylase activity was then assayed as described. The control reaction, with a fully methylated substrate, was performed by hybridizing radiolabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) with unlabeled poly(dG-5-methyl-dC):poly(dG-5-methyl-dC) in the identical manner. Each point represents the average of two independent determinations.

borhydride. It is of interest that about five to ten times as many bases were released from DNA as apyrimidinic sites were labeled. This indicates the removal of apyrimidinic sites from DNA by the HeLa base excision repair system to be substantially accomplished during the eighteen hour reaction time.

The effects of substrate hemimethylation on the enzymic depyrimidination of 5-methylcytosine from DNA was examined, and the results are shown in table 2. 5-methylcytosine was excised from both fully methylated and hemimethylated DNAs. However, glycosylic activity against the fully methylated polymer was seven-fold greater than that against hemimethylated DNA. This indicates an *in vitro* preference of the enzyme for a fully methylated substrate. The data in table 2 also indicate that the extent of deamination of released 5-methylcytosine to thymine was reduced following its release from the hemimethylated DNA.

DISCUSSION

Activation of repressed genes is potentiated by loss of DNA 5-methylcytosines (1−7). It was demonstrated that demethylation of both DNA strands at critical CpG loci is essential for transcription and that this was a two-stage process (19). During differentiation, there is evidence for enzymic replacement of 5-methylcytosine by cytosine (10−11). Our demonstration of enzymic depyrimidination of 5-methylcytosine indicates a possible role for the pathway of DNA base excision repair preceeding gene expression. The apyrimidinic site, which is stoichiometrically generated upon release of 5-methylcytosine, would then be repaired by excision of the 5′-deoxyribosephosphate residue. Possible excising activities include endonucleases and lyases acting at abasic sites and 5′-deoxyribosephosphatase (20). Subsequent repair replication of the missing DCMP would then be completed by DNA polymerases and DNA ligases (20). Gene expression could then proceed by the established mechanisms following such replacement of DNA 5-methylcytosine by cytosine. The pathway of DNA nucleotide excision repair has been shown to be involved in DNA demethylation (11). Our results indicate the involvement of DNA base excision repair to be of possible consequence in the removal of DNA 5-methylctyosines as well.

The glycosylic activity generates a DNA apyrimidinic site at the position of the released 5-methylcytosine (figure 4). This formation of abasic sites can be related to the mutations found at high frequencies at DNA 5-methylcytosine loci; these have resulted in marked depletion of CpG loci in vertebrate DNAs and in the point mutations related to many human diseases (1−7, 21−22). These account for 30% to 40% of human germ line mutations (21−22). Particular notice has been taken of such mutations in the human tumor supressor gene p53 (21−24). It has been proposed that such mutations are due to deamination of 5-methylcytosine to thymine 12 (25−26). This reaction proceeds at 3.5 times the rate of cytosine deamination to uracil (27−29). However, this rate seems insufficient to account for the established high mutation incidence, considering the paucity of *in vitro* deamination and the documented high efficiency of the repair of G:T mismatches by human cells (21−22, 30−33). Enzymic generation of DNA apyrimidinic sites (figure 4), possibly during the routine process of demethylation prior to gene expression, suggests another pathway for the introduction of mutations at 5-methylcytosine sites.

This alternative mechanism of mutagenesis at 5-methylcytosine loci involves intermediate DNA apyrimidinic sites. Base loss sites are known to be promutagenic lesions (34). Abasic sites, whether apurinic or apyrimidinic, are stable in DNA (35−36). DNA polymerases often preferentially insert a dAMP moiety into newly synthesized DNA opposite a base loss site (37−38). Were the abasic site to remain unrepaired, the result would be the insertion of an adenine on the opposite strand after one round of replication instead of a guanine. This would lead to a subsequent a C → T transition mutation on the first strand following another DNA

replication. Therefore, should the apyrimidinic sites not be completely repaired, their persistence following 5-methylcytosine removal before gene activation could account for the substantial number of transition mutations detected at CpG loci. These would result from enzymic depyrimidinations by 5-methylcytosine-DNA glycosylase, rather than from *in situ* deaminations of DNA 5-methylcytosines to thymines. Such thymines are removed by the mismatch repair system (30–33). Because enzymic depyrimidination of 5-methylcytosine involves no formation of G:T base pairs at any point, no protection can be conferred by the DNA mismatch repair system against such mutagenesis.

Chromatographic analyses of the released pyrimidines demonstrated the presence of thymines, possibly by deamination of the released 5-methylcytosines β(figure 3 and table 1). This would effectively remove free 5-methylcytosine from cellular metabolism. The effects of this base on cellular functions are unknown. Deamination would also prevent the recycling of free 5-methylcytosine into DNA during replication via a 5-methyl-dC-TP formed through the pyrimidine salvage pathway. This involves conversion of bases, nucleosides or deoxyribonucleosides into rNTPs or dNTPs by a diverse variety of enzymes (39). It could theoretically lead to incorporation of 5-methyl-dCTP into DNA at random loci opposite guanines during replicative synthesis, with resultant alterations of gene expression. Electroporation of 5-methyl-dCTP into mammalian cells demonstrated such aberrant incorporation of 5-methylcytosine into DNA, with resultant silencing of a number of genes (40–41). A number of seemingly redundant human enzyme activities have been detected which deaminate the putative 5-methyl-dCTP precursors at various stages. 5-methyl-2′-deoxycytidine is deaminated to thymidine by cytidine deaminase (42). 5-methyl-dCMP is deaminated to TMP by a 5-methyl-dCMP deaminase (43). Purification of the human 5-methylcytosine-deaminating activity is necessary to establish whether this is a separate enzyme or if it resides on either the cytosine deaminase molecule or the 5-methylcytosine-DNA glycosylase molecule. However, both the necessity of exclusion of inappropriate incorporation of 5-methylcytosine into DNA, and the requirement of confining methylation of DNA cytosines to postreplicative activities, are evident from the multiple activities that deaminate 5-methylcytosine-containing moieties at various points in the pyrimidine salvage pathway.

We have shown that 5-methylcytosine is enzymically depyrimidinated from DNA by a human enzyme. It is possible that this activity is involved in activation of genes during gene expression, differentiation, embryogenesis and neoplastic transformation. The resultant DNA apyrimidinic site may be etiogenic in genesis of the many mutations found at DNA 5-methylcytosine loci. Subsequent deamination of free 5-methylcytosine may therefore not only prevent inappropriate silencing of cellular genes, but protect the genome from mutations resulting from possible inappropriate 5-methylcytosine incorporation into DNA. Purification and characterization of these activities are necessary for elucidation of these functions.

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