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Evidence of complete cellular repair of 1,N⁶-ethenoadenine, a mutagenic and potential damage for human cancer, revealed by a novel method

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Abstract

1,N⁶-Ethenoadenine (ϵ A) is generated endogenously by lipid peroxidation and exogenously by tumorigenic industrial agents, vinyl chloride, and vinyl carbamate. ϵ A detected in human tissues causes mutation and is implicated in liver, colon and lung cancers. *N*-methyl purine DNA-glycosylase (MPG) is the only enzyme known so far to repair ϵ A. However, the mechanism of in vivo repair of ϵ A and the role of MPG remain enigmatic. Moreover, previous in vivo repair studies for DNA lesions, including ϵ A, focused only on the step of the removal of the base lesion without further insight into the completion of the repair process. This may be in part due to the unavailability of an appropriate in vivo quantitative method to evaluate complete BER process at the basal level. Our newly developed in vivo method is highly sensitive and involves phagemid M13mp18, containing ϵ A at a defined position. The complete repair events have been estimated by plaque assay in *E. coli* with the phagemids recovered from the human cells after cellular processing. We found that the detectable complete (removal and replacement of ϵ A with adenine) repair was observed only 18% in 16 h, but with the repair nearing completion within 24 h in colon cancer, HCT-116, cells. Moreover, MPG is the predominant enzyme for the BER process to remove ϵ A in mammalian cells. Although, the ϵ A is fairly a bulky adduct compared to other small BER substrate lesions, NER pathway is not involved in repair of this adduct. Furthermore, the ϵ A repair in vivo and in vitro is predominant in the G0/G1 phase of the cell cycle.

Keywords

Base excision repair; 1,N⁶-Ethenoadenine; N-Methylpurine-DNA glycosylase; In vivo repair

Introduction

1,N⁶-Ethenoadenine (ϵ A) is generated endogenously by oxidative stress-induced lipid peroxidation and exogenously by exposure of cells to tumorigenic industrial agents such as vinyl chloride, vinyl carbamate and benzene [1–5]. ϵ A was detected in biological samples from animals and also humans, working in the plastic industry and cigarette smokers [6–8]. ϵ A causes predominantly A→T and also A→G and A→C mutations in in vitro translesion DNA synthesis systems with purified enzyme and cell-free extracts as well as in vivo in cell culture systems [9, 10]. This adduct has also been implicated in tumorigenesis of several organs including liver, colon and lung [11, 12]. In a molecular epidemiologic study,

reduction in repair of ϵ A has also been correlated with lung cancer especially of the adenocarcinoma type [11].

MPG is the only enzyme known so far to repair ϵ A via the BER pathway in in vitro reactions [5, 13]. It is a broad substrate enzyme that excises at least 17 structurally different DNA adducts, including, in addition to ϵ A, alkylation and deamination lesions from both the major and minor grooves of duplex DNA [13–20].

The primary steps in the BER pathway are common to all organisms. Until recently they were envisioned simplistically as involving few excision, gap filling, and ligation steps; however, in eukaryotes the repair pathway is quite complex. Mammalian cells appear to have at least two distinct pathways with different repair patch sizes (1 and >1 nucleotides for short and long patches, respectively) for repair of uracil, natural and reduced AP-sites, oxidized bases, and ROS-induced strand-breaks [21–23]. In in vitro reactions, the monofunctional DNA glycosylase, MPG, repairs ϵ A and hypoxanthine (HX) via both short- and long-patch repair pathways (~50% each), while the glycosylase/lyase type enzymes, OGG1 or NTH, repair 8-oxoguanine or thymine glycol, respectively, predominantly via the short-patch (75–80% of total repair events) repair pathway [24–26].

The tissue and embryonic fibroblast cell extracts from the MPG null mice were devoid of MPG activity toward ϵ A, Hx, and 3-methyl adenine (m^3 A) as compared to wild type extracts [27–29]. MPG is likely to be the major DNA glycosylase for the repair of all these lesions in mice. On the other hand both MPG null cells and mice, when treated with alkylating agents or urethane, showed surprising in vivo repair kinetics of m^3 A, 7-methyl guanine (m^7 G) and ϵ A; m^7 G was removed equally efficiently in MPG-proficient and -deficient cells, whereas m^3 A and ϵ A were far less efficiently removed in MPG-deficient cells and mice tissues. The removal of both m^3 A and ϵ A adducts was slower than expected [29, 30]. This suggests an alternative repair mechanism or another DNA glycosylase that repairs these lesions (m^3 A, m^7 G and ϵ A) [29, 30]. However, it is noted that the mechanism of in vivo basal repair of ϵ A and other adducts (without challenging cells with DNA damaging agents) that includes the role of MPG is not known yet. Moreover, previous in vivo repair studies for these DNA lesions including ϵ A focused only on the removal of the base lesion without any further insight into subsequent steps of the MPG-BER pathway up to the completion of the repair process [28, 30]. Notably, there is no appropriate and quantitative in vivo method yet available to completely evaluate the total BER process under untreated condition. We have developed a highly sensitive in vivo method. We found that ϵ A is repaired to the end nearly 100% by the BER pathway in 24 h, when a large fraction of human cells are at the G0/G1 phase of the cell cycle. Moreover, MPG-mediated BER, but not NER, is the predominant repair process in mammalian cells.

Materials and methods

Preparation of circular double-stranded DNA constructs containing modified bases

The M13mp18 single-stranded DNA (2 μ g; Bayou Biolabs, LA) was annealed at 80°C for 5 min with 50-fold molar excess of oligonucleotides containing ϵ A or A (control) at *Eco*RI or *Pst*I sites, which had been phosphorylated previously using T4 polynucleotide kinase (New England Biolabs, MA). The oligonucleotides were purchased from Gene Link, NY and the sequences used were 5'-⁶²⁴²CC GAGCTCGAATTCGTAATC⁶²²³-3' [control (*Eco*RI)], 5'-⁶²⁴²CCGAGCTCG ϵ AATTCGTAATC⁶²²³-3' [ϵ A (*Eco*RI)], 5'-⁶²⁸⁶GCATGCCTGCAGGTCGACTCTAG⁶²⁶⁴-3' [control (*Pst*I)], and 5'-⁶²⁸⁶GCATGCCTGC ϵ AGGTCGA CTCTAG⁶²⁶⁴-3' [ϵ A (*Pst*I)]. The numbers in the oligonucleotide sequences correspond to nucleotide positions in the (+) strand of M13mp18 sequences. T7 DNA polymerase (10 units, New England Biolabs, MA) and 1 unit of T4

DNA ligase (USB, OH) were added to the primer template mixture, and the polymerization and ligation reactions were carried out on ice for 5 min, at room temperature for 5 min, at 37°C for 1 h, and at 14°C overnight in the presence of 10-mM ATP, 100-mM DTT, 10-mM dNTPs, and 25-μg BSA/ml. Various forms of circular DNAs in the reaction mixture were clarified from unused primers, dNTPs, and other reactants, using DNA Clean and Concentrator™ 25 cartridges (Zymo Research, CA). An aliquot was checked on 0.8% agarose gel for covalently closed circular (CCC) DNA. Forty-eight replicate reactions were carried out and then pooled for subsequent purification by isopycnic centrifugation on an ethidium bromide/ CsCl gradient.

Cell culture and transfection for in vivo repair study

The HCT-116 cells were cultured in McCoy's medium with 10% fetal calf serum, 2 mM glutamine, and antibiotics (penicillin and streptomycin, 125 units/ml each). The MPG^{+/+} and ^{-/-} SV40 T-antigen-transformed mouse embryonic fibroblast (MEF) cells (gift from Dr. Leona Samson, MIT) were cultured in DMEM complete media supplemented with 2-mercaptoethanol. The NER-deficient (XPA) and NER-proficient (GM00637) cells were cultured in MEM with 10% fetal calf serum and 2-mM glutamine. For transfection experiments, 2×10^6 cells were seeded in a 6-well plate containing 1 ml of complete medium and incubated overnight at 37°C. Four micrograms of closed circular double-stranded damage-containing M13 mp18—εA:T at the *Eco* RI or *Pst* I site or damage-free M13 mp18—A:T (control) were transfected into human cells using Lipofectamine 2000. Following transfection, cells were incubated for 5 h, supplemented with complete media, and harvested at different repair time points (0, 2, 8, 16, 20, 22, and 24 h). The plasmids were then recovered from cells using the Perfect Prep Plasmid Mini Kit (Eppendorf, Germany). Prior to electroporation into XL1-Blue competent *E. coli* cells, the plasmids were individually digested with *Eco* RI and *Pst* I to analyze the presence of unrepaired εA and *Sac* I or *Sal* I for control sites in the same DNA molecules. The efficiency of transfection varied from 10% to 15% in different experiments.

Electro-transformation to *E. coli* and measurement of complete repair of εA

Following recovery, we introduced the post-transfected phagemids with or without restriction enzyme digestion into electrocompetent XL1-Blue cells by the electroporation method. The electroporator was set at 1.8 kV, 200 Ω, and 25 μF. The transformed cells were diluted in SOC media and grown on LB plates containing 0.2-mM IPTG and 40-μg/ml X-gal at 37°C for 16–18 h. The total number of plaques was scored in the restriction enzyme digested versus undigested plates, and the repair efficiency was measured as follows:

$$\text{Total repair} = (1 - \text{No. of plaques from } Eco \text{ RI or } Pst \text{ I digested DNA} / \text{No. of plaques from undigested DNA}) \times 100$$

Cell cycle analysis

At different in vivo repair time points (0–24 h) post-transfected cells were tested for cell cycle analysis by Fluorescence-activated cell sorting (FACS) analysis. In addition enrichment of cells at G1 phase 5×10^5 cells were treated with 0.5 mM L-aminopterin (Sigma-Aldrich, St. Louis, Missouri) for 15 h. S phase synchronization was carried out by double thymidine block. Cells were treated with 3-mM thymidine (Sigma-aldrich, St. Louis, Missouri) for 18 h, followed by washing once with PBS and growing for another 6 h. Thymidine (3 mM) was added again and the cells were incubated for an additional 17 h. The cells from both treatments were then washed twice with PBS and suspended in Citrate-DMSO buffer for DNA staining with Propidium Iodide by Vindeløv staining method [31], followed by DNA analysis by flow cytometry using FACScalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Western Blot analysis

Nuclear extracts from untreated or cell-cycle-arrested human cells (50 μ g soluble protein) were separated by SDS-PAGE (15% polyacrylamide), proteins transferred to a nitrocellulose membrane, and the MPG bands were visualized with the anti-human MPG monoclonal antibodies (1:500 dilution) using enhanced chemi-luminescence protocol (Amersham Life Sciences, Piscataway, NJ) [32].

MPG-mediated excision activity assay

Cell-free nuclear extracts (0.25–1.0 μ g) from untreated HCT-116 and HeLa or Minosine/thymidine-treated HCT-116 (G0/G1 or S phase arrested) cells were individually incubated with 5'-³²P-labeled ϵ A-containing duplex oligonucleotide substrates (4–8 nM) for 10 min at 37°C in an assay buffer (25 mM HEPES-KOH, pH 7.9, 0.5 mM DTT, 10 μ g/ml nuclease-free BSA, 150 mM NaCl and 10% glycerol) in a total volume of 20 μ l. The reaction was stopped by inactivating the enzyme at 75°C for 5 min. The products containing the AP-sites were analyzed as described previously [32, 33].

In vitro repair synthesis assay by [α -³²P]dAMP incorporation

The assay was performed essentially as described earlier [34] with slight modification that after repair reactions the clarified ϵ A-modified M13 mp18 close circular DNA was directly analyzed by agarose gel electrophoresis.

Results

Strategy for complete and basal in vivo BER Assay

To evaluate the complete and basal in vivo BER of ϵ A, we engineered in a site-specific manner, closed circular double-stranded M13 mp18 DNA substrates containing a single ϵ A damage. Different constructs were generated: a control without damage and ϵ A. Oligonucleotides containing no damage or ϵ A were annealed to single-stranded M13 mp18 DNAs which were then converted to double-stranded molecules with T7 DNA polymerase and ligated with T4 DNA ligase. The ϵ A was placed in the *Eco* RI or *Pst* I site.

After transfection of these closed circular constructs to the cultured mammalian cells, the phagemid DNAs were recovered and then introduced into XL1-Blue *E. coli* for plaque formation, which was subsequently used for repair assessment (Fig. 1). The principle of this plaque assay-based BER in human or any transfectable mammalian cells is that the repaired DNAs (removal and replacement of ϵ A with adenine reporting complete repair) are linearized by restriction enzymes, *Eco* RI or *Pst* I, and the unrepaired DNAs become resistant to cleavage and remain in covalently closed circular state. The latter has significantly greater (~300 fold) transformation efficiency than its linear counterparts [35]. Thus, only the unrepaired DNA molecules presumably generate plaques. A similar number of cleaved DNA molecules are also transformed into XL1-Blue, and the number of plaques generated is scored for total number of DNA molecules representing both repaired and unrepaired fractions. The unrepaired and repaired fractions are thus quantified for the complete BER capacities of the test mammalian cells.

Construction and confirmation of the constructs for the presence of DNA damage

The control and phagemid DNAs harboring ϵ A at *Eco* RI and *Pst* I sites were prepared in vitro, and the covalently closed circular (CCC) ds DNA were purified by isopycnic centrifugation on an ethidium bromide/CsCl gradient. The CCC DNAs were then tested for the presence of ϵ A by restriction enzyme digestion, assuming that the presence of the DNA damage on the *Eco* RI and *Pst* I cleavage sites will resist the linearization by these enzymes

(Fig. 2). Like standard CCC ds M13 mp18, the control DNA was linearized by *Eco* RI (lane 7) and *Sac* I (lane 8), whereas the DNA carrying *εA* at the *Eco* RI site was linearized by *Sac* I at an unadducted site (lane 11), but resistant to *Eco* RI digestion (lane 10). Likewise, the DNA harboring *εA* at *Pst* I site was resistant to *Pst* I (lane 17), but sensitive to *Sal* I (lane 18), a restriction site un-adducted, but close to the *Pst* I site. The CCC DNA preparation had some linear DNA. However, linear DNA, has very low transformation efficiency, as described earlier, and does not essentially affect the overall repair results.

Thus, the results confirm the preparation of constructs harboring the *εA* modification at the correct positions.

Effect of *εA* on plaque formation

Our in vivo BER assay relies on the plaque-forming ability of the CCC M13 mp18 that are resistant to restriction enzyme digestions. However, the *εA* was then tested for its interference with the plaque-forming ability of the CCC phagemids harboring these modified bases. The results showed that the number of plaques increased with increasing DNA amount (Fig. 3a), but the presence of *εA* in DNA did not have any significant effect on plaque formation (Fig. 3b). Moreover, the linearized form of the control (both *Eco* RI and *Sac* I digested) or the modified base (*Sac* I digested only) containing constructs formed low number of plaques, possibly because some CCC DNA escaped linearization under our assay conditions, whereas the construct resistant to *Eco* RI, because of the presence of *εA*, did form plaques (Fig. 3b). Likewise, the construct containing *εA* at *Pst* I site yielded a comparable number of plaques upon digestion with *Pst* I as the undigested DNA (data not shown).

Therefore, the modified base (*εA*) does not alter the plaque-forming ability of M13mp18 and thus should be able to report the fraction of unrepaired phagemids, whereas the repaired phagemids when linearized do not form plaques and should report the repaired fractions.

In vivo repair of *εA* in colon cancer HCT-116 cells

The persistence of *εA* adduct has been implicated in colon cancer [36]. We chose HCT-116, which is a colon cancer cell line. *εA* excision activity and the MPG expression level were measured in cell-free extracts from HCT-116 cells. The results show that like HeLa cells, which are well characterized to contain functional MPG [32, 37], these cells are competent in *εA* excision activity (Fig. 4a) and also contain detectable MPG Protein expression (Fig. 4b). Then we tested in vivo repair of *εA* at basal level in HCT-116 cells. We transfected the cells with phagemid containing *εA* and control constructs, and the cells were then harvested at 0- and 22-h repair time points. The plasmids were purified and subjected to repair assay by plaque formation after restriction enzyme digestions. In the control case, there was no damage, so all the plasmids from both time points (both 0 and 22 h) were digested by the restriction enzymes. The control DNA, with no damage after *Eco* RI and *Sac* I digestions, did not show any significant number of plaques (0–4%) as expected at both 0- and 22-h repair time points. *εA* at 0 h showed plaques, when it was digested by *Eco* RI, but showed no plaques after digestion with *Sac* I; however, at 22 h there was a reduced number of plaques after *Eco* RI digestion, which resulted in the repair of *εA*. On the other hand, complete digestion with *Sac* I as usual showed no plaque formation. The *εA* showed plaques at 0 h after digestion with both the restriction enzymes and 45% remaining plaques at 22 h after digestion with the restriction enzymes, suggesting that our new assay system could measure the in vivo repair of *εA* in a specific and reliable manner (Table 1). Further kinetic study showed that the detectable repair of *εA* was only 18% in the first 16 h, but neared completion within 24 h (Fig. 4c). Notably, the remaining 40–60% repair was accomplished in the final 2 h. Then, we placed *εA* at another restriction site, *Pst* I, to test positional effect,

if any, in in vivo repair of ϵ A. The results show that the repair at the *Pst*I site was nearly at a similar level (~50% total repair events at 22 h post-transfection) as at the *Eco*RI site (Table 1).

Next, we tested whether MPG is involved in repair of ϵ A at basal level in untreated mammalian cells.

Role of MPG and nucleotide excision repair in in vivo repair of ϵ A at basal level

The cells or animal treated with DNA damaging agents have shown to primarily involve MPG to repair alkylating drug or urethane-induced base lesions [27–30]. However, it is not known whether MPG is involved in in vivo repair of ϵ A and other adducts when cells are not treated with any DNA damaging agents. Moreover, it is also not known whether the excision of adducts, if by MPG, reaches completion to give rise to normal DNA. To test this hypothesis, we examined the in vivo repair of ϵ A using the phagemid M13mp18 containing ϵ A at the *Eco*RI site in MPG^{-/-} and MPG^{+/+} MEFs. While substantial repair (~80% at 24 h) was found in MPG^{+/+} cells, minimal repair (~10% at 24 h) was detected in MPG^{-/-} cells (Fig. 5a). ϵ A is a heterocyclic adduct and is fairly bulky compared to many substrate lesions (hypoxanthine, 3-methyl adenine, 7-methyl guanine etc.) of MPG or other small adducts (8-Oxoguanine, thymine glycol, uracil etc.) that are repaired by BER pathway. The bulky adducts are generally repaired by Nucleotide Excision Repair (NER) pathway. Therefore, we tested the involvement of NER pathway, in the repair of ϵ A using the same phagemid M13mp18 containing ϵ A at the *Eco*RI site in NER-deficient (XPA) and NER-proficient (GM00637) cells, and found that both XPA and the NER-proficient control cells repaired ϵ A substantially at comparable level (~60–65% at 22 h) (Fig. 5b).

The cell cycle status during in vivo repair of ϵ A

Following transfection of the phagemid M13mp18 containing ϵ A the HCT116 cells were analyzed for cell cycle during the entire repair process. The cells at early repair phase ((0–6 h), Fig. 4c) were mostly at S phase (Fig. 6a and b), when there is negligible or undetectable repair. At later repair period ((6–24 h), Fig. 4c) cells were becoming confluent and accumulating increasingly at G0/G1 phase (Fig. 6a and b). The increase in repair of ϵ A was apparently correlated with the increasing G0/G1 and decreasing S phase; all the ϵ A, however, was repaired at 24 h, when 70% or more cells were in G0/G1 phase. Thus, ϵ A repair is predominant apparently at G0/G1 phase compared to the other cell cycle phases. But we reasoned that alternatively, the initial lag in repair may be due to recovery of cells from transfection-related stress and/or time needed for recognition of ϵ A in plasmid DNA by MPG and other BER proteins subsequent to MPG-mediated excision step. To test these possibilities we performed in vitro repair of ϵ A in nuclear extracts from cells arrested in G0/G1 or S phases (Fig. 7a and b). Minosine arrested ~80% cells at G0/G1, whereas double thymidine block could arrest 50–55% of HCT-116 cells at S phase in two independent experiments. Interestingly, the in vitro ϵ A repair synthesis was predominant in G0/G1 compared to S-arrested cell extracts (even the S arrest autoradiograph was overexposed; Fig. 8a) and is reminiscent of in vivo repair pattern (Fig. 4c). Notably, MPG expression was not very different at different in vivo repair time points (Fig. 6c) and also in G0/G1- or S-arrested cell extracts (Fig. 7c). As expected PCNA expression is slightly increased at moderately S-phase-arrested cells (Fig. 7c). Then, the ϵ A excision activity of MPG was tested in the arrested cell extracts. The results show that similar to repair synthesis (Fig. 8a) MPG's cleavage activity was higher in G0/G1 versus S-arrested cell extracts (Fig. 8b). In fact higher protein amount showed some inhibition of MPG activity in extracts from both cell cycle phases (Fig. 8b). This indicates presence of a likely inhibitor of MPG in both G0/G1 and S phases, and possibly more in S phase. Of note, MPG expression was similar in

both phases (Fig. 7c), but both the repair synthesis and cleavage activity were less in S phase. Thus, MPG-mediated BER of ϵ A is regulated by the cell cycle phases in human cells.

Taken altogether, the untreated mammalian cells repair ϵ A in vivo to completion and this predominantly requires MPG. Undoubtedly the repair does not depend on the NER pathway. Moreover, the ϵ A repair in vivo and in vitro is predominant in the G0/G1 phase of the cell cycle.

Discussion

In this study, we investigated the in vivo repair mechanisms of ϵ A by the monofunctional DNA glycosylase, MPG, at basal level. Earlier, we and others have shown that the ϵ A adduct is repaired by mammalian MPGs [13–20]. Although, the mechanism of repair of this type of adduct by MPG has been studied extensively with purified proteins and defined oligonucleotides and a site-specific adduct, its in vivo basal repair mechanisms are yet to be revealed. We found in this study that ϵ A was repaired efficiently in vivo in colon cancer cells (HCT-116), when the latter was not treated with any carcinogen. This elucidates the predetermined BER mode within the cells for ϵ A, which is also generated endogenously. A recent study indicated that 8-oxoG is repaired in vivo preferentially by the long-patch BER [38]. However, that study only followed the extent of long-patch repair without quantifying total repair activity. Here, we have developed a novel method. This DNA modification-restricted and repair-permissible linearization of plasmids and subsequent sensitive plaque assay coupled with human cell transfections provided us with a powerful strategy for extremely sensitive and quantitative assessment of basal repair of DNA damage mimicking the endogenous ones.

So far, the small base modifications are shown to be repaired by the BER pathway and the bulky adducts by the NER pathway [39]. In vitro reactions showed that BER is the major pathway to repair 8-oxodG, but NER can also act on this oxidized base as a minor pathway [40], but its in vivo repair was shown to be exclusively by the BER pathway [38]. However, the in vitro repair of ϵ A was found to be predominantly by BER, including single and 6–8 nucleotide incorporations, and most likely not by the NER pathway, [26]. Our result shows that ϵ A seems to be repaired in vivo by the BER pathway at basal level, and MPG is the predominant enzyme to remove ϵ A. However, very low repair (~10% at 24 h, Fig 5a) in MPG^{-/-} MEFs may not rule out the possible existence of second MPG-like enzyme. In fact, in *E. coli* there are two functional homologs of MPG: AlkA and Tag; AlkA is induced by DNA damaging agents, while Tag protein is responsible for the basal activity [41]. As with *E. coli* an inducible protein, yet unidentified, may also exist in mammalian cells or animals. A non-MPG-BER pathway may also exist in mammalian cells to repair ϵ A, but our results showed that NER is not involved in repairing this adduct (Fig. 5b).

Although the repair of ϵ A neared completion within 24 h, interestingly, the in vivo BER rate of ϵ A almost doubled at the final 4-h time point. This could be due to the initial lag phase (possibly attributed to the early repair time points) in recruitment of MPG or MPG-BER complex on the damage site, especially, when the cells are not treated with DNA damaging agents and/or due to recovery of cells from transfection-related stress. However, results from this study strongly suggest that during the final 4-h repair period the cells were mostly at G0/G1 phase and in fact, a non-DNA damaging agent, such as minosine-arrested G0/ G1 cell extracts, had significantly higher ϵ A repair capacity. Thus, the repair of endogenously generated ϵ A appears to be assured in the G0/G1 phase prior to the passage of the cells to the S phase for DNA replication. This could possibly be an intrinsic preventive mechanism in human cells to prevent mutations. Furthermore, there might be an inhibitor of MPG present in both G0/G1 and S phases (Compare repair activity in Fig. 8a and b with MPG

expression in Fig. 7c), but possibly more in S phase likely to ensure that MPG does not generate excess AP sites at the S phase. AP sites are potentially more mutagenic and toxic than any of the MPG substrate-damaged bases. In fact deliberate MPG overexpression could not protect cells against alkylating agents; rather, it induced hypersensitivity and hypermutability because of increased production of AP sites and repair imbalances [42–44]. Thus the homeostasis and the changes in the concentration of some inhibitor might act as a regulator for the MPG-mediated BER pathway in various cell cycle phases to ensure efficient and balanced repair of base damage, which are often less toxic and/or mutagenic than their subsequent repair product intermediates, and prevent genomic instability and error-free survival. Identification of the potential inhibitor, which might regulate the MPG-mediated BER in the cells in response to DNA damage remains to be investigated.

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Abbreviations

BER	Base excision repair
MPG	N-Methylpurine DNA glycosylase
Hx	Hypoxanthine
eA	1,N ⁶ -Ethenoadenine
m³A	3-Methyl adenine
m⁷G	7-Methyl guanine
CCC	Covalently closed circular

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Fig. 1.
Strategy for quantitative analysis of in vivo repair of site-specific ϵA

Fig. 2. Sensitivity of M13mp18 constructs containing ϵA to restriction enzyme digestions. Standard M13mp18 RF-DNA was either undigested (ln 3) or digested with *Eco* RI (ln 4) and *Sac* I (ln 5). The CCC M13mp18 carrying no modification (control) at *Eco* RI or *Pst* I site was undigested (ln 6 and ln 13) or digested with *Eco* RI (ln 7), *Sac* I (ln 8), *Pst* I (ln 14), and *Sal* I (ln 15). The CCC M13mp18 carrying ϵA at *Eco* RI or *Pst* I site was undigested (ln 9 and ln 16) and digested with *Eco* RI (ln 10), *Sac* I (ln 11), *Pst* I (ln 17), and *Sal* I (ln 18). “CCC” denotes the covalently closed circular DNA

Fig. 3. Effect of (a) DNA amount and ϵA , and (b) restriction enzyme digestion on plaque formation. Data represent the mean values with standard error derived from three independent experiments

Fig. 4. MPG-mediated in vitro excision activity (**a**), immunodetection of MPG (**b**), and in vivo repair kinetics of ϵ A (**c**) in colon cancer HCT-116 cells. Four micrograms of CCC M13mp18 DNA carrying no modification or ϵ A at *Eco* RI site was transfected for repair analysis in HCT-116 cells. Data represent the mean values with standard error derived from three independent experiments. See “Materials and methods” for further details

Fig. 5.

In vivo repair of ϵ A in MPG (WT and knock-out) MEFs and NER-deficient (XPA) and -proficient (GM00637) fibroblasts. Transfection was similarly performed as described in Fig. 4. Data represent the mean values with standard error derived from three independent experiments

Fig. 6. Cell cycle progression of HCT-116 cells after transfection of M13mp18 DNA constructs for in vivo repair at different time points. **(a)** Cells were transfected for repair analysis as described in Fig. 4c, then fixed, stained with Propidium Iodide, and examined for DNA content by flow cytometer. The peaks represent the cells in G0/G1, S and G2/M phases of cell cycle. **(b)** The cell fractions present in different cell cycle phases were derived from panel A and plotted against repair time points. **(c)** Immunodetection of MPG and PCNA at different repair time points

Fig. 7.

Cell cycle profiles of HCT-116 cells synchronized to G₀/G₁ and S phases. **(a)** Cells were arrested at G₀/G₁ and S phases by treatment with L-aminosine and Thymidine, respectively. See “Materials and methods” for treatment details. Exp-1 and 2 represent two independent experiments. **(b)** The cell fractions present in different cell cycle phases after G₀/G₁ and S phase arrests were derived from panel A. **(c)** Immunodetection of MPG and PCNA in G₀/G₁ and S-arrested cells

A *In vitro* repair synthesis on ϵ A

B ϵ A excision activity

Fig. 8.

In vitro DNA repair synthesis and excision activity in extracts from G0/G1- and S-phase-arrested HCT-116 cells. **(a)** DNA repair synthesis on ϵ A-containing M13mp18 DNA. The cell-free nuclear extracts (0–60 μ g per reaction) were incubated for 3 h at 30°C with ϵ A-containing M13mp18 DNA (300 ng) in repair synthesis reaction (details described in the Experimental procedures). **(a)** Photograph of ethidium bromide-stained gel (upper) and autoradiogram of the dried gel (lower). **(b)** MPG-mediated ϵ A excision. Cell-free nuclear extracts (0.5 and 1.0 μ g) were reacted with a 50-mer 5'- 32 P-labeled ϵ A-containing duplex oligonucleotide (8 nM) for 10 min at 37°C. The details of the reaction conditions are described under "Materials and methods." Data represent the mean values with standard error derived from three independent experiments

Table 1

In vivo repair of *eA* in colon cancer HCT-116 cells

Remaining restriction enzyme resistant plaques (%)										
Damage type	<i>Eco</i> RI		<i>Sac</i> I		<i>Pst</i> I		<i>Sal</i> I		Repair (%) at 22 h	
	0 h	22 h	0 h	22 h	0 h	22 h	0 h	22 h	0 h	22 h
Control (<i>Eco</i> RI)	0	1	0	6	3	1	0	1		
<i>eA</i> (<i>Eco</i> RI)	100	45 ± 2	0	2						55 ± 2
<i>eA</i> (<i>dPst</i> I)					100	51 ± 5	0	1		49 ± 5

^a Control (*Pst* I) showed similar result as the control (*Eco* RI) sequence