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Biochemical characterization and DNA repair pathway interactions of Mag1-mediated base excision repair in *Schizosaccharomyces pombe*

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ABSTRACT

The *Schizosaccharomyces pombe mag1* gene encodes a DNA repair enzyme with sequence similarity to the AlkA family of DNA glycosylases, which are essential for the removal of cytotoxic alkylation products, the premutagenic deamination product hypoxanthine and certain cyclic ethenoadducts such as ethenoadenine. In this paper, we have purified the Mag1 protein and characterized its substrate specificity. It appears that the substrate range of Mag1 is limited to the major alkylation products, such as 3-mA, 3-mG and 7-mG, whereas no significant activity was found towards deamination products, ethenoadducts or oxidation products. The efficiency of 3-mA and 3-mG removal was 5–10 times slower for Mag1 than for *Escherichia coli* AlkA whereas the rate of 7-mG removal was similar to the two enzymes. The relatively low efficiency for the removal of cytotoxic 3-methylpurines is consistent with the moderate sensitivity of the *mag1* mutant to methylating agents. Furthermore, we studied the initial steps of Mag1-dependent base excision repair (BER) and genetic interactions with other repair pathways by mutant analysis. The double mutants *mag1 nth1*, *mag1 apn2* and *mag1 rad2* displayed increased resistance to methyl methanesulfonate (MMS) compared with the single mutants *nth1*, *apn2* and *rad2*, respectively, indicating that Mag1 initiates both short-patch (Nth1-dependent) and long-patch (Rad2-dependent) BER of MMS-induced damage. Spontaneous intrachromosomal recombination frequencies increased 3-fold in the *mag1* mutant suggesting that Mag1 and recombinational repair (RR) are both involved in repair of alkylated bases. Finally, we show that the deletion of *mag1* in the background of *rad16*, *nth1* and *rad2* single mutants reduced the total recombination frequencies of all three double mutants, indicating that abasic sites formed as a result of Mag1 removal of spontaneous base lesions are substrates for nucleotide excision repair, long- and short-patch BER and RR.

INTRODUCTION

Genomic integrity depends on the efficient repair of DNA base damage. DNA lesions are induced by endogenous factors, such as reactive oxygen species and alkylating metabolites (*S*-adenosylmethionine), and by exogenous factors, including ultraviolet and ionizing radiation. Of particular importance for the repair of endogenous lesions is the base excision repair (BER) pathway, which is initiated by DNA glycosylases that remove modified bases by cleavage of their *N*-glycosylic bonds. Monofunctional glycosylases remain bound to the abasic site until acted upon by either the hydrolytic activity of an AP (apurininc/apyrimidinic)-endonuclease or by an AP-lyase activity inherent of bifunctional DNA glycosylases. The resulting strand break is further processed to remove the sugar residue at the 5′ or 3′ end by a phosphodiesterase activity (single nucleotide gap; short patch). Alternatively, in cooperation with proliferating cell nuclear antigen the structure-specific nuclease FEN1 removes the 3′ sugar by a strand displacement reaction (2–8 nt; long patch). A DNA

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polymerase fills the nucleotide gap and a DNA ligase sealing the two ends together completes repair (1,2).

Alkylating agents are abundant in the environment and introduce a broad range of lesions in DNA. 3-methylpurines [3-methyladenine (3-mA) and 3-methylguanine (3-mG)] and 1-methyladenine are major cytotoxic lesions repaired by the BER pathway and the oxidative demethylation (3,4), respectively, whereas 6-methylguanine is a mutagenic lesion repaired by alkyltransferases. DNA glycosylases for the removal of alkylated bases were first detected in *Escherichia coli* (5–7) and have subsequently been found in all other organisms investigated, including yeast, mammals and plants. Characterization of mutants deficient in 3-mA DNA glycosylase activity in *E.coli* revealed the presence of two enzymes termed as Tag and AlkA. The extreme alkylation sensitivity of the *E.coli alkA tag* double mutant has been used to identify genes from other organisms by functional complementation of the alkylation sensitivity (8–11). Alkylbase DNA glycosylases can be divided into classes based on sequence homologies. The *E.coli* Tag enzyme is conserved in several bacterial species and plants but is not present in lower eukaryotes and mammalian cells. The *Saccharomyces cerevisiae* Mag enzyme is homologous to *E.coli* AlkA, whereas the mammalian and certain plant enzymes (Aag) are similar to each other and represent a third class. Certain bacterial species such as *Bacillus subtilis* possess homologues to the mammalian Aag family. The DNA glycosylase Mpg II, found in several bacteria and archaea, represents yet another family of enzymes that removes alkylated bases and shares sequence homology with the endonuclease III class of DNA repair enzymes (12). Recently, two new classes of alkylbase DNA glycosylases, designated as AlkC and AlkD, were identified in *Bacillus cereus* (I. Alseth, unpublished data). The structural diversity of alkylbase DNA glycosylases suggest that many different protein scaffolds can be used to recognize and remove alkylated bases. Members of the AlkA and Aag family of 3-mA DNA glycosylases show broad substrate specificity, including activities for the removal of ethenoadducts and deaminated adenine (hypoxanthine) (13–16). In fact, the *B.subtilis* Aag seems to have a more important role in the removal of premutagenic residues induced by deamination and lipid peroxidation than for cytotoxic residues induced by alkylating agents (17).

Schizosaccharomyces pombe encodes one 3-mA DNA glycosylase, Mag1, which belongs to the AlkA/Mag family (11). The *mag1* mutant is less sensitive to methyl methanesulfonate (MMS) exposure than 3-mA DNA glycosylase deficient strains of *E.coli*, *S.cerevisiae* and mammalian cells. Both *rad13* (*S.cerevisiae* Rad2/XPG) and *rhp51* (*S.cerevisiae* Rad51) mutants are hypersensitive to MMS demonstrating that nucleotide excision repair (NER) and recombination repair (RR) are the major pathways for resistance to MMS in *S.pombe*. However, both the BER mutants *nth1* (endonuclease III) and *apn2* (AP-endonuclease 2) are hypersensitive to alkylation damage, suggesting that BER is important for the repair of alkylated DNA (18–20). In this study, we have characterized the enzymatic activities of purified Mag1 and found that the substrate specificity was confined to alkylated bases. Mag1 removed cytotoxic 3-methylpurine lesions 5–10 times less efficiently than *E.coli* AlkA, supporting previous data demonstrating only moderate MMS sensitivity of the *mag1* mutant. Furthermore, we introduced *rad16* (*S.cerevisiae* Rad1/XPF), *rhp55* (*S.cerevisiae* Rad55), *rad2* (*S.cerevisiae* Rad27/Fen1), *nth1* and *apn2* null mutations into *mag1* cells to elucidate pathway interactions in repair of spontaneous and MMS-induced alkylation damage. *mag1 apn2*, *mag1 nth1* and *mag1 rad2* double mutants displayed increased MMS resistance compared with *apn2*, *nth1* and *rad2* single mutants, respectively, indicating that Mag1 initiates both long- and short-patch BER pathway of alkylation damage. Moreover, the *mag1* mutant showed increased spontaneous intrachromosomal recombination frequencies, demonstrating that BER and RR overlap in repair of alkylated base lesions.

MATERIALS AND METHODS

Media, strains and plasmids

Media and general genetic methods for *S.pombe* have been described previously (21). The complete medium was yeast extract medium supplemented with 225 mg l^{-1} adenine, histidine, leucine, lysine and uracil (YES). The minimal medium was Edinburgh minimal medium supplemented with uracil and appropriate amino acids (EMM). MMS was added when necessary. The medium used to select the Ade^+ recombinants in plating assays was YES lacking adenine and supplemented with 200 mg l^{-1} guanine to prevent adenine uptake. *S.pombe* transformations were performed using the lithium acetate method (22). The parental strain for construction of the original *mag1::ura4*$^+$ mutant was sp.011 (*h*$^-$ *ura4-D 18 leu1-32 ade6-704*). The *mag1::ura4*$^+$ mutant was made by targeted gene disruption of the wild-type *mag1*$^+$ allele. The *ura4*$^+$ marker was inserted into the unique BalI site of *mag1*$^+$ cloned in pT7-SCII (Stratagene). The *mag1* gene marker (*mag1::ura4*$^+$) was excised and partially degraded with BAL31, and used for the transformation of sp.011. Stable Ura^+ transformants were screened by Southern-blot analysis (data not shown) to confirm targeted disruption of *mag1*$^+$. The resulting *mag1::ura4*$^+$ strain was designated RHP114. Double mutants were made by random spore analysis, where appropriate, or by tetrad dissection following a sexual crossover. Genotypes of the strains used in this study are shown in Table 1.

Mag1 expression and purification

The *mag1* open reading frame (ORF) was PCR amplified from *S.pombe* genomic DNA by using primers g**gaattcc**atgactttggacattgaa and ccc**aagctt**tcagtgtttcttcggcct (restriction sites in bold). The *mag1*$^+$ fragment was ligated into the EcoRI and HindIII sites of pT7-SCII (Stratagene). *E.coli* strain BL21 (Stratagene) was transformed with pT7-Mag1, grown in 10 litres of Luria–Bertani medium with 100 μg ml^{-1} ampicillin to A_{600} of 1 and induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Extracts were prepared by a combination of plasmolysis and lysozyme treatment as described previously (23). Alkyl base DNA glycosylase activity was measured to monitor Mag1 purification. The cell extract was applied to an Affigel Blue (Bio-Rad) column (2×8 cm^2) equilibrated with buffer A [100 mM Tris–HCl (pH 8.0), 1 mM EDTA, 20% glycerol and 10 mM 2-mercaptoethanol] and step eluted with 1 M KCl in buffer A. Active fractions were pooled, desalted on Sephadex G-25M

Table 1. *S.pombe* strains used in this study

Strain	Genotype
sp.011 wt	*h*$^-$ *ura4-D18 leu1-32 ade6-704*
RHP114	*h*$^-$ *mag1::ura4*$^+$ *ura4-D18 leu1-32 ade6-704*
FO101 wt	*h*$^-$ *ura4-D18 leu1-32 his3-D1*
FO726	*h*$^-$ *mag1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1*
FO260	*h*$^-$ *rad2Δ::LEU2 ura4-D18 leu1-32 his3-D1*
FO732	*h*$^-$ *mag1::ura4*$^+$ *rad2D::LEU2 ura4-D18 leu1-32 his3-D1*
RHP103	*h*$^-$ *apn2::kanMX ura4-D18 leu1-32 his3-D1*
RPH107	*h*$^-$ *apn2::kanMX mag1::ura4 leu1-32 his3-D1*
FO656 wt	*h*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4*
FO841	*h*$^-$ *rad16Δ::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4*
FO661	*h*$^-$ *rhp55Δ::arg3*$^+$ *4ura4-D18 leu1-32 his3-D1 arg3-D4*
FO801	*h*$^+$ *mag1::ura4*$^+$ *rhp55Δ::arg3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*
FO837	*h*$^+$ *mag1::ura4*$^+$ *rad16Δ::ura4*$^+$ *ura4-D18 leu1-32his3-D1 arg3-D1*
FO763	*h*$^+$ *nth1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*
FO831	*h*$^+$ *mag1::ura4*$^+$ *nth1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*
FO665	*h*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO755	*h*$^+$ *mag1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO751	*h*$^+$ *nth1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO830	*h*$^+$ *mag1::ura4*$^+$ *nth1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO181	*h*$^+$ *rad16Δ::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO839	*h*$^+$ *mag1::ura4*$^+$ *rad16Δ::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO487	*h*$^+$ *rad2Δ::LEU2 ura4-D18 leu1-32 his3-D1 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO761	*h*$^+$ *mag1::ura4*$^+$ *rad2Δ::LEU2 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO679	*h*$^+$ *rhp55Δ::arg3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*
FO795	*h*$^+$ *mag1::ura4*$^+$ *rhp55Δ::arg3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3*$^+$*/ade6-L469*

(Column PD-10; Pharmacia) equilibrated with buffer A containing 50 mM KCl and applied to MonoQ column. The column was eluted with a gradient of 0.05–1 M KCl. Active fractions were collected at 150 mM KCl, desalted on Sephadex G-25M and applied to a calf thymus DNA cellulose column (HR 5/5, Pharmacia) equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with a linear gradient of 0.1–1.5 M NaCl and Mag1 protein was eluted between 0.4 and 0.8 M NaCl.

Assay for alkyl base DNA glycosylase activity

N-[H^3]-*N*-methyl-*N*′-nitrosourea (MNU; 1.5 Ci mmol^{-1}) was used to prepare alkylated calf thymus DNA (6000 d.p.m. μg^{-1} DNA) (24). DNA glycosylase activity was assayed in reaction buffer [70 mM MOPS, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA and 5% glycerol] with 7 μg DNA substrate and enzyme as indicated for 30 min at 37°C in a total reaction volume of 50 μl. After DNA precipitation of the reaction mixture, base removal was quantified as radioactivity in the supernatant by using a liquid scintillation counter (Tri-Carb 2900TR; Packard).

Assay for enzyme cleavage of DNA substrates containing base damage

Double-stranded DNA substrates containing single base damages (1,N^6-ethenoadenine:T, hypoxanthine:T, 5-hydroxyuracil:G, 5-hydroxycytosin:G, thymin glycol:A or 5,6-dihydrouracil:G) were generated by labelling the 5′ end of the damage containing oligonucleotide using T4 polynucleotide kinase (MBI Fermentas) and [γ-^{32}P]ATP (3000 Ci mmol^{-1}; Amersham). The labelled oligonucleotides were annealed to complementary strands. Substrate (5 fmol) and Mag1 DNA glycosylase (100 fmol) were incubated in reaction buffer at 37°C for 30 min (total volume of 10 μl). The resulting abasic site after base removal was cleaved by adding 100 mM NaOH and continuing incubation for 20 min at 70°C. The reactions were stopped by adding 100 mM HCl and 10 μl DNA loading buffer (90% formamide and 5 mM EDTA DNA), followed by heat denaturation at 80°C for 3 min and separation of reaction products on 7 M urea–20% polyacrylamide gels (LongRanger) in 1× taurin buffer. The radiolabelled fragments were visualized using a PhosphorImager (445 SI; Molecular Dynamics). 5-Hydroxyuracil, 5-hydroxycytosin, thymin glycol (5*R*, 5*S*) and 5,6-dihydrouracil incubated with *E.coli* endonuclease III (Nth); 1,N^6-ethenoadenine and hypoxanthine incubated with human Aag; and 8-oxoguanine incubated with *E.coli* formamidopyrimidine DNA glycosylase (Fpg) were used as positive controls. The sequence of the DNA substrate used in this study was 5′-GCATGCCTGCACGGXCATGGCCAGATCCCCGGGT-ACCGAG, where X is the damaged base.

DNA-binding assays

DNA binding of Mag1 was determined by electrophoretic mobility shift assay (EMSA) using a 36 bp ^{32}P-5′-end-labeled duplex oligonucleotide containing a single tetrahydrofurane (THF) residue at position 20 (GCTGTTGAGATC-CAGTTCG[THF]AGTAACCCACTCGTGC). DNA (5 fmol) and Mag1 (1 fmol) were incubated in reaction buffer containing 70 mM MOPS, pH 7.5, 1 mM DTT, 1 mM EDTA and 5% glycerol on ice for 15 min in a total volume of 10 μl. The samples were analysed using 10% non-denaturing PAGE at 4°C in 1× taurin buffer. Experiments with competitors were performed as above except that non-damaged DNA (pUC18) was added up to 100 times in excess. The radiolabelled fragments were visualized and quantified using a PhosphorImager.

HPLC analysis of alkylated bases

Reverse phase high-performance liquid chromatography (HPLC) of methylated bases released by purified *S.pombe* Mag1 and *E.coli* AlkA was performed as follows (25). The supernatant was centrifuged through Millipore Ultra-fuge-MC filters (10.000NMWC filter unit) and mixed with non-radioactive methylated bases as markers. Separation of 3-mA, 3-mG and 7-methylguanine (7-mG) were obtained by using HPLC (Spheri-5 RP-18, 220×4.6 mm^2; Brownlee Labs) using a linear gradient of 100–75% (v/v) 0.1 M triethyl ammonium acetate buffer, pH 7.3 or pH 5.4, in methanol for elution (1 ml min^{-1}). Fractions of 0.5 ml were collected and the radioactivity was measured using a liquid scintillation counter. 3-mA, 3-mG and 7-mG reference compounds were obtained from Fluka.

Epistasis analysis for MMS sensitivity

Schizosaccharomyces pombe cells were grown in liquid YES to A_{600} of 0.6–0.8, washed in water, diluted and spread on YES agar plates containing up to 0.01% MMS (two plates per dose) to measure cell survival. Three independent cultures per strain were analysed to allow the determination of standard deviations.

Epistasis analysis for MMS by spot tests

Cells were grown in liquid YES, washed and suspended in water at a density of 3×10^7 to 10^3 cells ml^{-1}. Aliquots (10 µl) of the cell suspensions were spotted onto solid YES media containing various concentrations of MMS.

Spontaneous mitotic recombination assays

Mitotic recombination was assayed by the recovery of Ade$^+$ recombinants from strains containing the intrachromosomal recombination substrate. Frequencies of spontaneous recombinants were determined by fluctuation tests. For each strain, five independent colonies were used and each assay was repeated independently at least three times. Thus, for each strain at least 15 colonies were assayed. Cells from each single colony were resuspended in sterile water and plated at a density of 10^5–10^6 cells per plate (10^4–10^5 cells per plate for very hyper-recombinant strains) onto media selective for Ade$^+$. To determine cell titres, appropriately diluted cells were spread onto several plates containing YES complete media. After grown for four days at 30°C, the number of recombinants and cell titre were determined. The Ade$^+$ recombinant colonies on the selective plates were replicated onto EMM lacking adenine and histidine (and onto media lacking just adenine as a control) to determine the proportion of conversion-type (Ade$^+$ His$^+$) and deletion-type (Ade$^+$ His$^-$) recombinants. For each strain, mean recombinant frequencies were determined for each of the three independent assays. The average recombinant frequencies and standard deviations were determined from these three means, according to Luria and Delbruck (26). The percentage of conversion-types and standard deviations were also determined in the same way. To analyse the statistical significance of differences in recombinant frequencies (and percentage of conversion-types) for a given strain compared with wild-type cells, two sample *t*-tests were used to analyse individual recombinant frequencies and (percentage conversion-types) from all colonies (at least 15 per strain).

RESULTS

Expression and purification of *S.pombe* Mag1 in *E.coli*

For enzymatic characterization, Mag1 was expressed in the *E.coli* strain BL21 and purified to apparent physical homogeneity. Purification was monitored by standard assays for 3-mA DNA glycosylase activity. The final preparation migrates as a single band with molecular mass of 26 kDa on a SDS polyacrylamide gel, in good agreement with the calculated molecular weight of 26.3 kDa (data not shown). The purified enzyme showed no loss of alkylbase DNA glycosylase activity during storage at 4°C for 2 months or storage at −80°C for 3 years (data not shown).

Slow removal of 3-methylpurines by *S.pombe* Mag1

Escherichia coli AlkA removes a broad range of alkylated base lesions including the major products, such as 3-mA, 3-mG and 7-mG. We compared the ability of Mag1 and AlkA to remove alkylated bases from calf thymus DNA exposed to [^{3}H]methyl-*N*-methyl nitrosourea. As indicated in the linear range of base excision, Mag1 excised 3-mA and 3-mG 5–10 times slower than *E.coli* AlkA whereas the efficiency of 7-mG excision by Mag1 was similar to AlkA (Figure 1); i.e. 1.5 pmol of Mag1 removed 10, 30 and 50 fmol of 3-mG, 3-mA and 7-mG, respectively, whereas 1.5 pmol AlkA removed 80, 250 and 45 fmol of 3-mG, 3-mA and 7-mG, respectively. The difference between AlkA and Mag1 in efficiency of repair of cytotoxic 3-methylpurines indicates that other mechanisms may be more important for the repair of alkylation damage in *S.pombe* than in *E.coli* and may also explain the moderate sensitivity of the *mag1* mutant towards alkylating agents (27).

No removal of oxidized bases, deaminated bases or ethenoadducts by Mag1

Escherichia coli AlkA and *S.cerevisiae* Mag have been reported to remove the premutagenic deamination product hypoxanthine, and the cyclic ethenoproduct 1,N^6-ethenoadenine (15,28). Excision of these lesions was assayed on duplex oligonucleotides containing single lesions incubated with 20-fold excess of enzyme. Mag1 showed no activity towards hypoxanthine or 1,N^6-ethenoadenine (data not shown). Induction of *mag1* transcription by H_2O_2 (data not shown) may indicate a role for Mag1 in the removal of oxidized bases. To test this hypothesis, Mag1 was assayed for activity towards a variety of different oxidized base lesions; however, no activity could be observed against the major oxidation products, such as 8-oxoguanine, thymine glycol, 5,6-dihydrouracil, 5-hydroxyuracil and 5-hydroxycytosine, when incubated with 20-fold excess of enzyme (data not shown). These results show that Mag1 has a narrower substrate range than its counterparts AlkA in *E.coli* and Mag in *S.cerevisiae*.

Disruption of *mag1* partially relieves the MMS sensitivity of *nth1*

As reported previously (27), the *mag1* single mutant shows no hypersensitivity to the alkylating agent MMS under chronic exposure and only a very moderate sensitivity under acute exposure. In contrast, the DNA glycosylase/AP-lyase mutant *nth1* exhibits a strong sensitivity to MMS (Figure 2A), because

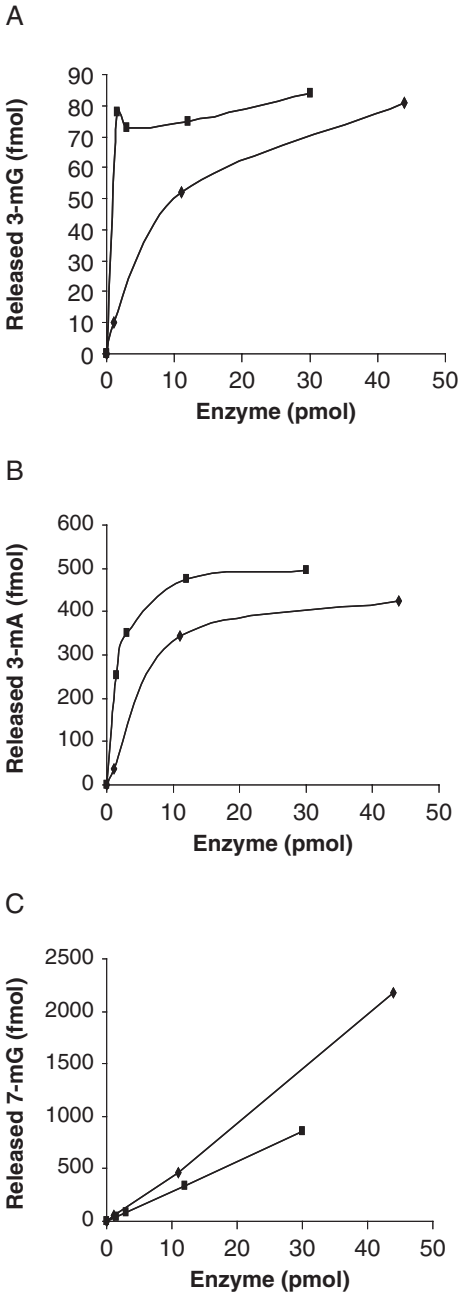

Figure 1. Reverse phase HPLC of methylated bases released from [^{3}H]methyl-*N*-nitrosourea-treated DNA by *S.pombe* Mag1 (diamonds) and *E.coli* AlkA (squares). [^{3}H]methyl-*N*-nitrosourea-treated DNA was incubated with increasing amounts of enzyme at 37°C for 30 min. The DNA was precipitated with ethanol, and the supernatant was analysed using HPLC. Radioactivity in fractions corresponding to 3-mG (**A**), 3-mA (**B**) and 7-mG (**C**) were measured using a liquid scintillation counter.

of a general role of Nth1 in repair of abasic sites in *S.pombe* (18,20). In order to investigate the interaction between Nth1 and Mag1 we tested the double mutant *nth1 mag1* for sensitivity to MMS. The MMS hypersensitivity of the *nth1* mutant was partially relieved by the *mag1* deletion (Figure 2A), indicating that Mag1 and Nth1 operate in the same branch of the BER pathway. Most probably, Nth1 is cleaving the resulting abasic site following base excision by Mag1.

Disruption of *mag1* relieves the MMS sensitivity of *apn2*

A 3′ deoxyribophosphate terminus in DNA is highly cytotoxic to the cell due to the inhibition of DNA polymerase activities. Recent results suggest that Apn2 operates downstream of Nth1 in the BER pathway to remove 3′ termini at abasic sites (20). The *apn2* single mutant is very sensitive to killing by MMS whereas the double mutant *nth1 apn2* has reduced MMS sensitivity comparable with that of the *nth1* single mutant (20). Deletion of *mag1* in an *apn2* background relieves the hypersensitivity of the single mutant almost completely (Figure 2B), indicating a major role of Apn2 in repair of Mag1-initiated BER of alkylation damage. Thus, it appears that sequential action of Mag1, Nth1 and Apn2 catalyse the initial steps of short-patch BER of alkylation damage in *S.pombe*.

Disruption of *mag1* relieves the MMS sensitivity of *rad2*

Schizosaccharomyces pombe Rad2 shows sequence similarity to the FEN1 family of structure-specific endonucleases, which are essential for the strand displacement step of the long-patch branch of BER. Previous studies showed that the *rad2* mutant is MMS sensitive indicating a similar role to Rad2 in long-patch repair in *S.pombe* (18). In order to further investigate the importance of long-patch BER of alkylation damage, we analysed the genetic interaction between *rad2* and *mag1*. The *rad2 mag1* double mutant is less sensitive to MMS than the *rad2* single mutant (Figure 2C) suggesting that Rad2 is involved in the processing of a BER reaction intermediate. Therefore, Mag1 may initiate both Rad2-dependent long-patch BER and Nth1-mediated short-patch repair of alkylation damage.

Mag1 binds strongly to abasic sites in the DNA

Mutant analyses suggest that the AP-lyase activity of Nth1 cleaves the abasic site resulting from base excision by Mag1. Several DNA glycosylases show increased turnover in the presence of the enzymes acting in the subsequent step of the BER pathway, e.g. human AP-endonuclease Ape1 stimulates the glycosylase activity of human hNth1 and hOgg1. However, no turnover effect of Mag1 activity was observed by adding increasing amounts of Nth1 (data not shown). In order to analyse the binding affinity to the DNA glycosylase product (abasic site), Mag1 (1 fmol) was incubated with 5-fold excess of AP containing DNA (5 fmol) and analysed by EMSA (Figure 3). The bandshift-complex represented 20% of the substrate (first lane from left), demonstrating that all the Mag1 molecules were bound to the AP-DNA. After addition of 100-fold non-damaged DNA as compared with damaged DNA still 15% of the AP-DNA formed complex with Mag1 (lane 4 from left), showing that 75% of the enzyme was bound to the AP-substrate. These results suggest a strong and specific binding of Mag1 to abasic sites in DNA.

Interactions between Mag1 initiated BER, NER and RR

To investigate the interactions between Mag1 initiated BER and other pathways involved in repair of alkylation damage, including NER and RR, double mutants were constructed and examined for MMS sensitivity by spot tests. The double mutant *mag1 rad16* (*S.cerevisiae* Rad1/ XPF) was much more sensitive to chronic exposure of MMS than the *rad16* and

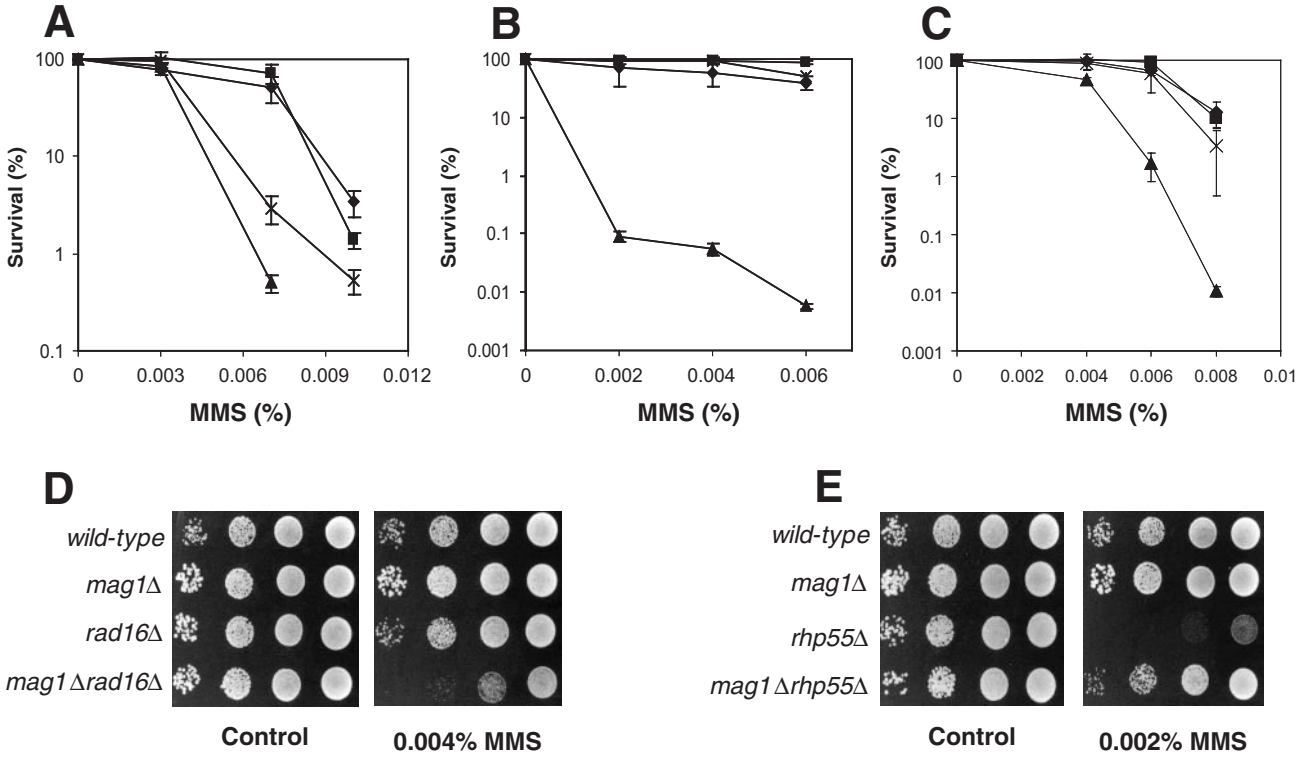

Figure 2. Genetic interactions between *mag1*, *nth1*, *apn2*, *rad2*, *rad16* and *rhp55* analysed for MMS sensitivity. (**A**) *S.pombe* wild type (diamonds), *mag1* (squares), *nth1* (triangles) and *mag1 nth1* (crosses) mutant cells were plated onto solid media containing increasing doses of MMS and assessed for cell survival by colony formation. (**B**) *S.pombe* wild type (diamonds), *mag1* (squares), *apn2* (triangles) and *mag1 apn2* (crosses) mutant cells were plated onto solid media containing increasing doses of MMS and assessed for cell survival by colony formation. (**C**) *S.pombe* wild type (diamonds), *mag1* (squares), *rad2* (triangles) and *mag1 rad2* (crosses) mutant cells were plated onto solid media containing increasing doses of MMS and assessed for cell survival by colony formation. (**D**) An aliquot 10 µl or serially diluted (10^6–10^3 cells ml^{-1}) mid-log phase cultures of wild type, *mag1*, *rad16*, *mag1 rad16* and (**E**) wild type, *mag1*, *rhp55* and *mag1 rhp55* were spotted onto YES plates containing no MMS (control) or MMS doses as indicated.

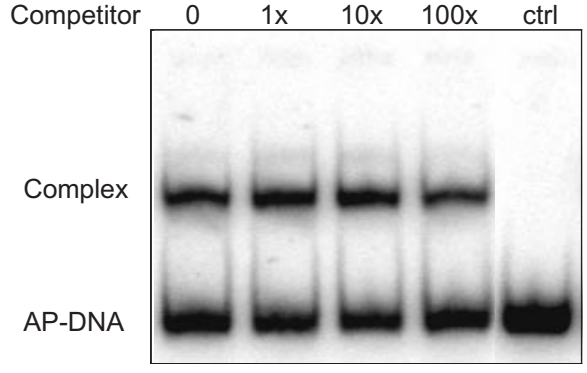

Figure 3. DNA-binding analysis of Mag1. Duplex DNA containing a single THF residue (5 fmol) was incubated with 1 fmol of Mag1and 1–100-fold excess of non-damaged DNA (competitor), or no enzyme (control). The reaction products were separated on 10% polyacrylamide gels and bands visualised using PhosphorImager.

mag1 single mutants (Figure 2D), suggesting an overlap (synergism) between Mag1 initiated BER and NER for the repair of alkylation damage. Analysis of the interaction between Mag1 and Rhp55-dependent recombination showed that the double mutant *mag1 rhp55* was less MMS sensitive compared with the *rhp55* single mutant (Figure 2E). Most probably, Mag1 induces cytotoxic single strand breaks at abasic sites during MMS exposure, which are a major substrate for RR. Alternatively, replication run-off at nicked template DNA during S phase may convert these single strand breaks into double strand breaks, which are also repaired by RR. These data support previous mutant analysis demonstrating a synergistic interaction between Rad13 (NER) and Mag1 for the repair of MMS-induced damage whereas the deletion of *mag1* in an *rhp51* (RR) background partially relieved the MMS sensitivity of the *rhp51* single mutant (27).

Influence of Mag1 function on spontaneous intrachromosomal recombination

In order to study the role of Mag1 in spontaneous mitotic recombination, mutant strains containing non-tandem direct repeats of *ade6*$^-$ heteroalleles with a functional *his3*$^+$ gene between the repeats were constructed (18). Spontaneous Ade$^+$ recombinant frequencies were determined for wild-type and mutant cells. The number of total spontaneous intrachromosomal recombination events was three times higher in the *mag1* mutant than wild type, and the frequency was increased more for deletion-type recombinants (4-fold) than that for conversion-types (2-fold) (Table 2). Thus, both types of mitotic recombination are involved in repair of alkylated bases formed under normal physiology. As shown

Table 2. Spontaneous intrachromosomal recombinant frequencies for wild-type and mutant cells

Strain	Total Ade$^+$			Deletion-types			Conversion-types			
	Frequency	Rel. freq.[a]	P[a]	Frequency	Rel. freq.[a]	P[a]	Frequency	Rel. freq.[a]	P[a]	%
Wild-type (*FO665*)	3.1 ± 0.7	1.0	—	2.2 ± 0.5	1.0	—	1.0 ± 0.2	1.0	—	31 ± 5
mag1 (*FO755*)	10.2 ± 1.8	3.3	***[b]	8.4 ± 1.5	3.9	***[b]	1.8 ± 0.4	1.9	***[b]	18 ± 3
nth1 (*FO751*)	20.5 ± 6.3	6.6	***[b]	9.9 ± 2.7	4.6	***[b]	10.5 ± 4.1	11	***[b]	51 ± 5
mag1 nth1 (*FO830*)	15.7 ± 3.5	5.1	>0.05[c] ***[d]	10.5 ± 2.3	4.9	>0.05[c] ***[d]	5.2 ± 1.4	5.5	***[c] ***[d]	33 ± 4
rad16 (*FO181*)	21.4 ± 3.5	6.9	***[b]	16.0 ± 2.9	7.5	***[b]	5.3 ± 1.0	5.6	***[b]	25 ± 3
mag1 rad16 (*FO839*)	13.0 ± 2.0	4.2	***[e] ***[f]	10.1 ± 1.6	4.7	**[e] ***[f]	2.8 ± 0.6	3.0	***[e] ***[f]	22 ± 3
rad2 (*FO487*)	68 ± 12	22	***[b]	57.8 ± 9.9	27	***[b]	10.1 ± 2.7	11	***[b]	15 ± 3
mag1 rad2 (*FO761*)	43 ± 9	14	***[g] ***[h]	28.9 ± 8.6	13	***[g] ***[h]	14.1 ± 3.0	15	***[g] ***[h]	33 ± 8
rhp55 (*FO679*)	10.2 ± 3.3	3.3	***[b]	10.0 ± 3.2	4.7	***[b]	0.12 ± 0.08	0.1	***[b]	1.1 ± 0.8
mag1 rhp55 (*FO795*)	19.0 ± 3.8	6.1	***[i] ***[j]	18.8 ± 3.8	8.8	***[i] ***[j]	0.17 ± 0.09	0.2	>0.05[i] ***[j]	0.9 ± 0.5

Recombinant frequencies (per 10^4 viable cells ±SD) were obtained as described in Materials and Methods and are the means from 15 independent colonies (three independent assays, five colonies used per assay). The percentage conversion-types (% ± SD) were determined in the same way. '*' indicates a significant difference at the 95% confidence level ($0.01 < P < 0.05$); '**' indicates a significant difference at the 99% confidence level ($0.001 < P < 0.01$); and '***' indicates a significant difference at the 99.9% confidence level ($P < 0.001$).

[a]Relative frequency (Rel. freq.) is the recombinant frequency of the mutant strain relative to that of the corresponding frequency of the wild-type strain. P-values are from two-sample t-tests in which individual recombinant frequencies and percentage conversion-types from all colonies assayed (15 per strain) were analysed. They were obtained to determine the statistical significance of differences in recombinant frequencies (and percentage of conversion-types) for.

[b]A given single mutant strain compared with the wild-type strain.

[c]The *mag1 nth1* double mutant compared with the *nth1* single mutant.

[d]The *mag1 nth1* double mutant compared with the *mag1* single mutant.

[e]The *mag1 rad16* double mutant compared with the *rad16* single mutant.

[f]The *mag1 rad16* double mutant compared with the *mag1* single mutant.

[g]The *mag1 rad2* double mutant compared with the *rad2* single mutant.

[h]The *mag1 rad2* double mutant compared with the *mag1* single mutant.

[i]The *mag1 rhp55* double mutant compared with the *rhp55* single mutant.

[j]The *mag1 rhp55* double mutant compared with the *mag1* single mutant. P-values > 0.05 indicates no significant difference between the two strains being compared at the 95% confidence level.

previously, *rhp55* cells were significantly hyper-recombinant for deletion-types (4-fold increase) whereas conversion-type recombination was almost absent (18), implying that conversion-type recombination is a Rhp55-dependent pathway. Compared with the single mutants the *mag1 rhp55* double mutant displayed an additive effect for deletion-type hyper-recombination, indicating that there is no overlap in repair of spontaneous DNA damage between Mag1- and Rhp55-dependent deletion-type recombination. As shown previously (18), the total recombination frequency of the single mutants *nth1*, *rad16* and *rad2* also displayed hyper-recombinant phenotypes. *rad2* was the most hyper-recombinant mutant with a 21-fold increase in total recombinants and both *rad16* and *nth1* showed a 7-fold increase. Deletion of Mag1 in the background of *rad16*, *nth1* and *rad2* single mutants reduced the total recombination frequency of all three double mutants; *rad16 mag1* exhibited a reduction in both recombination types whereas the effect on *rad2 mag1* and *nth1 mag1* is accounted for by a reduction of deletion-types and conversion-types, respectively. Since *rad16*, *nth1* and *rad2* represent independent pathways for the repair of abasic sites (18), it thus appears that abasic sites formed as a result of Mag1 removal of spontaneous base lesions are substrates for RR.

DISCUSSION

Schizosaccharomyces pombe possesses one 3-mA DNA glycosylase, Mag1, which is homologous to the *E.coli* AlkA/*S.cerevisiae* Mag family of alkylbase DNA glycosylases. In this paper, we present an extensive *in vitro* and *in vivo* characterization of the Mag1 function. Recombinant Mag1 protein was purified from *E.coli* and tested on various types of base lesions. In contrast to the broad substrate specificity of the *E.coli* and *S.cerevisiae* counterparts, which include alkylated bases, deaminated adenine (hypoxanthine) and cyclic ethenoadducts, such as 1,N^6-ethenoadenine, Mag1 shows a narrow substrate range limited to the major alkylation products 3-mA, 3-mG and 7-mG. HPLC analysis showed that excision of the cytotoxic 3-methylpurine lesions (3-mA and 3-mG) is 5–10-fold less efficient for Mag1 than for AlkA, whereas the affinity for 7-mG was similar. Mutant analysis showed that the *mag1* single mutant exhibits a very moderate sensitivity to MMS-induced damage compared with the *nth1* and *apn2* single mutants. Deletion of *mag1* in an *apn2* background suppressed the MMS hypersensitivity of *apn2* cells completely whereas the *mag1 nth1* and *mag1 rad2* double mutant displayed a partial reduction in MMS sensitivity compared with the *nth1* and *rad2* single mutants, respectively. Thus, it appears that Mag1 initiates both short-patch (Nth1-dependent) and long-patch (Rad2-dependent) BER of MMS-induced damage. Spontaneous mitotic intrachromosomal recombination was increased 3-fold in the *mag1* mutant, demonstrating that Mag1 initiated BER and RR overlap in repair of alkylated bases. Moreover, spontaneous recombination frequencies showed that the NER (Rad16), RR (Rhp55) short-patch (Nth1) and long-patch BER (Rad2) are alternative pathways for the repair of AP-sites generated by Mag1 activity during normal physiological conditions.

The *E.coli alkA tag* double mutant is deficient in both DNA glycosylases for the removal of the major alkylation products and is extremely sensitive to alkylating agents. Survival experiments with this mutant transformed by a plasmid expressing Mag1 showed that the alkylation sensitivity was

partially suppressed compared with wild type (11), whereas complete restoration was obtained with plasmid expressing AlkA (data not shown). This is consistent with the limited capacity of Mag1 for the removal of the cytotoxic alkylation products, such as 3-mA and 3-mG. The genome complement of *S.pombe* reveals a second ORF (Mag2) with significant similarity to *E.*coli AlkA that may indicate an alternative DNA glycosylase for the removal of alkylated bases. However, biochemical characterization of purified Mag2 showed no activity towards alkylated DNA (unpublished data). Moreover, the *mag2 mag1* double mutant displayed the same resistance to MMS killing as the *mag1* single mutant (unpublished data). Therefore, it appears that other alternative mechanisms, such as NER and RR, play a more prominent role in repair of alkylation damage in *S.pombe*. In comparison, cells deficient in 3-mA DNA glycosylase activity in other organisms, i.e. *E.coli*, *S.cerevisiae* and murine embryonic stem cells, show a much stronger phenotype to MMS-induced damage than *mag1* (27).

Previous studies demonstrate that BER overlaps with NER and RR in repair of MMS-induced damage (18,27). The synergistic effects of combining mutations in *mag1* and NER suggested that alkylated bases are substrates for NER (27). Moreover, the synergistic effects of combining NER mutants and *nth1* in repair of MMS-induced damage indicate that AP-sites are also a common substrate for NER and BER (18). Analysis of spontaneous recombination frequencies in the Mag1 mutant show that RR acts directly on alkylated bases. Furthermore, the deletion of *mag1* in *rhp55* or *rhp51* background partially relieved the MMS sensitivity, suggesting that recombination is important for the repair of cytotoxic intermediates of Mag1-initiated BER, such as abasic sites and single strand breaks. MMS survival data indicate that the combined action of Mag1 and Nth1 on alkylated bases generate single strand breaks, which are major substrates for RR. Thus, it appears that RR acts on three different types of damage induced by MMS, such as alkylated bases, abasic sites and single strand breaks.

The genetic relationship between Mag1 and Nth1 supports previous evidence demonstrating that the AP-lyase activity of Nth1 possesses the major activity for the cleavage of AP-sites in the BER pathway (18,20). In contrast to other organisms, in which AP-endonucleases cleaves the abasic site subsequent to base removal by monofunctional glycosylases, the AP-lyase activity of Nth1 acts downstream of other DNA glycosylases, such as the Mag1, Udg1 (uracil DNA glycoylase) and MutY1 (adenine mismatch DNA glycosylase) (20). In *S.cerevisiae*, the AP-endonuclease Apn1 possesses the major activity for the cleavage of abasic sites, whereas the AP-lyase activity of the endonuclease III homologues Ntg1 and Ntg2 of *S.cerevisiae* only plays a minor role in the incision of abasic sites resulting from the base removal by Mag (29). Furthermore, our mutant studies suggest that the 3′ termini resulting from the combined action of Mag1 and Nth1 is a major substrate for Apn2. Thus, it appears that Apn2 acts primarily as a phosphodiesterase in repair of Mag1 initiated short-patch BER (Figure 4). Alternatively, Apn2 could possibly mediate long-patch BER by AP-endonuclease cleavage of intact abasic sites preceding Rad2-dependent strand displacement. A recent study suggests that Apn2 is the predominant AP-endonuclease activity in *S.pombe* whereas Uve1 and Apn1 initiate an alternative pathway in BER of AP-sites (19). Moreover, Rad2 showed

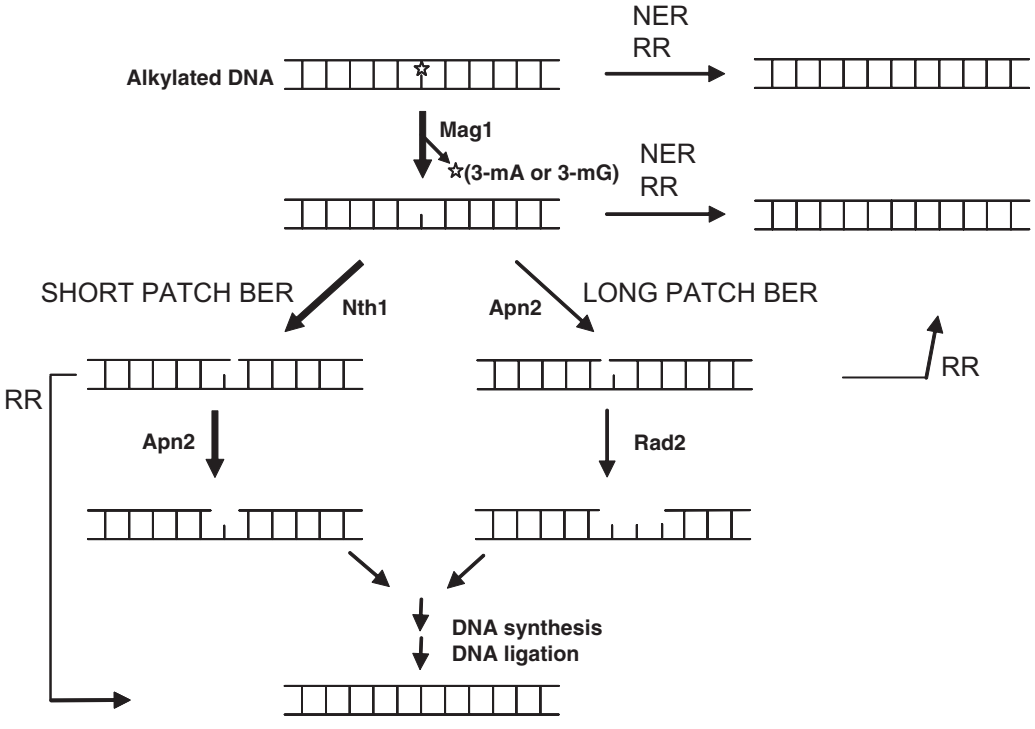

Figure 4. Model for repair of MMS-induced DNA damage in *S.pombe*. Three pathways are involved in repair of alkylated DNA (BER, NER and RR). The major branch of Mag1 initiated BER (thick arrows) is possessed by strand incision at the 3′ side of the abasic site by Nth1 and subsequent removal of the 3′ dRP termini by Apn2.

no genetic interaction with Uve1 or Apn1 in repair of MMS-induced damage (18). However, further analysis of the *rad2 apn2* double mutant is necessary to clarify a potential role of Apn2 in long-patch BER.

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