JOURNAL OF BACTERIOLOGY, Apr. 2000, p. 2104–2112 Vol. 182, No. 8
0021-9193/00/$04.00+0

Contribution of Base Excision Repair, Nucleotide Excision Repair, and DNA Recombination to Alkylation Resistance of the Fission Yeast *Schizosaccharomyces pombe*

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Received 4 August 1999/Accepted 18 January 2000

DNA damage is unavoidable, and organisms across the evolutionary spectrum possess DNA repair pathways that are critical for cell viability and genomic stability. To understand the role of base excision repair (BER) in protecting eukaryotic cells against alkylating agents, we generated *Schizosaccharomyces pombe* strains mutant for the *mag1* 3-methyladenine DNA glycosylase gene. We report that *S. pombe mag1* mutants have only a slightly increased sensitivity to methylation damage, suggesting that Mag1-initiated BER plays a surprisingly minor role in alkylation resistance in this organism. We go on to show that other DNA repair pathways play a larger role than BER in alkylation resistance. Mutations in genes involved in nucleotide excision repair (*rad13*) and recombinational repair (*rhp51*) are much more alkylation sensitive than *mag1* mutants. In addition, *S. pombe* mutant for the flap endonuclease *rad2* gene, whose precise function in DNA repair is unclear, were also more alkylation sensitive than *mag1* mutants. Further, *mag1* and *rad13* interact synergistically for alkylation resistance, and *mag1* and *rhp51* display a surprisingly complex genetic interaction. A model for the role of BER in the generation of alkylation-induced DNA strand breaks in *S. pombe* is discussed.

DNA damage emanates from the inherent chemical instability of nucleic acids, from errors made by DNA polymerase during DNA replication, and from exposure to DNA-damaging agents present in the environment or produced by certain endogenous cellular processes (reviewed in reference 15). All organisms possess a panel of DNA repair mechanisms to repair damaged DNA. DNA excision repair pathways recognize and remove damaged segments from one DNA strand and then resynthesize new DNA, using the opposing undamaged strand as a template. Excision repair includes base excision repair (BER) and nucleotide excision repair (NER). An alternative approach to handling damaged DNA is by recombinational repair. These DNA repair pathways have been best characterized in *Escherichia coli*, but analogous pathways have been found in all organisms examined to date (15).

BER initiation occurs by the action of DNA glycosylases that recognize specific types of damaged or abnormal DNA bases and cleave the glycosylic bond linking the base to the sugar-phosphate backbone. DNA glycosylases recognize bases such as uracil, deaminated adenine (hypoxanthine), and certain alkylated and oxidized purines and pyrimidines (reviewed in references 10, 15, 21, 31, and 47). Releasing a damaged base from the DNA produces an apurinic/apyrimidinic (AP) site, and it is worth noting that AP sites are themselves a form of DNA damage. In addition to being generated by DNA glycosylases, AP sites can be formed spontaneously. AP endonucleases (which cleave 5′ to the AP site) or AP lyases (which cleave 3′ to the AP site) cleave the DNA backbone adjacent to the AP site. AP endonuclease produces a 5′-deoxyribose phosphate moiety, which must be removed to allow subsequent DNA ligation; removal occurs by the action of deoxyribose phosphodiesterase or Flap endonuclease 1 (FEN1). Cleavage by AP lyase produces a 3′ phosphate that cannot prime the new DNA synthesis required for repair. 3′-Phosphodiesterase enzymes remove the 3′-phosphate, generating a 3′-hydroxyl primer. Following modification of the appropriate DNA terminus, DNA polymerase synthesizes a patch of new DNA that can be as small as 1 base or as large as 13 bases; such DNA repair synthesis is followed by DNA ligation (reviewed in references 21, 31, and 47).

Like BER, NER requires several enzymatic steps. Although the general strategy for NER has been highly conserved, the reaction mechanism is more complex in eukaryotes than in prokaryotes (reviewed in references 15, 31, and 47). To initiate NER, the DNA sugar-phosphate backbone is incised both 3′ and 5′ to the damaged base(s). In *E. coli* this reaction requires 3 proteins (UvrA, -B, and -C), whereas in *Saccharomyces cerevisiae* and mammalian cells it requires the concerted action of at least 13 proteins (15, 40). NER was originally thought to repair exclusively bulky DNA lesions and DNA cross-links, known to distort the DNA helix. However, in vivo studies revealed a role for NER in the repair of methylated DNA base lesions that do not cause major helical distortions and in providing cellular resistance to simple methylating agents (38, 48, 49). Indeed, biochemical studies confirmed that subtle types of DNA damage can be substrates for NER, including thymine glycols, 8-oxoguanine, O^4-ethylthymine, O^4-methylthymine, O^6-methylguanine, AP sites, and N^6-methyladenine (17, 22, 26, 36, 38, 46). Thus, NER may play a role in alkylation resistance larger than was originally thought.

In addition to BER and NER, *Schizosaccharomyces pombe* has an additional DNA excision repair pathway initiated by the enzyme UV damage endonuclease (UVDE) (reviewed in reference 51). UVDE cleaves 5′ to UV photoproducts as well as to other aberrant DNA bases, including cisplatin-cross-linked diadducts, uracil, dihydrouracil, AP sites, and a variety of mismatched normal bases (3, 24, 25). Thus, UVDE-mediated

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excision repair has a wide substrate range and is likely to be important for resistance to a number of DNA-damaging agents. Although the downstream enzymatic components of the *S. pombe* UVDE-mediated excision repair are unidentified, genetic epistasis analysis suggests the involvement of the products of *rad2* (encoding the *S. pombe* FEN1 homologue), *rad18* (an essential gene that plays a role in UV and γ-ray resistance), and *rhp51* (encoding a RecA homologue that plays an essential role in recombination) (reviewed in reference 51).

Strand breaks in DNA are repaired by recombinational repair (RR) mechanisms. Less is known about RR enzymatic mechanisms than about to the excision repair pathways described above, although recent advances have increased our understanding at the molecular genetic level. RR genes have been identified in *S. cerevisiae*, and their homologues have been identified in both *S. pombe* and mammals (reviewed in references 15 and 23). RR repair of DNA strand breaks proceeds by either homologous recombination or illegitimate recombination. In *S. cerevisiae*, homologous recombination predominates for the repair of DNA strand breaks and requires the products of the *RAD52* epistasis group, which includes the *RAD50*, *-51*, *-52*, *-54*, *-55*, and *-57* genes. Mutations in any one of these *S. cerevisiae* genes produces a severe defect in homologous recombination accompanied by sensitivity to the lethal effects of γ rays (an agent which produces both single- and double-strand breaks in DNA), reduced mitotic and meiotic recombination, and defects in mating-type switching (16). In addition to γ rays, *S. cerevisiae* mutant in genes belonging to the *RAD52* group are very sensitive to the killing effects of the simple methylating agent methyl methanesulfonate (MMS). This observation lead to dubbing MMS a radiomimetic, and it has been shown that MMS can induce DNA strand breaks, in addition to alkylated bases (14, 39, 42, 45).

One of the central components of *S. cerevisiae* RR is the Rad51 protein (reviewed in reference 4). *S. cerevisiae* Rad51 homologues are found in *S. pombe*, chickens, and mammals, and these proteins, together with *S. cerevisiae* Rad51, are all homologues of the *E. coli* recombination protein RecA. Biochemical studies show that like RecA, *S. cerevisiae* and human Rad51 form nucleoprotein filaments in the presence of DNA and promote DNA strand transfer and annealing of cDNA. For *S. cerevisiae* and possibly other eukaryotes, the Rad52, Rad55, and Rad57 proteins stimulate such Rad51 activities.

In an effort to develop *S. pombe* as a model organism for the study of cellular responses to alkylating agents, we cloned an *S. pombe* cDNA encoding a 3-methyladenine (3MeA) DNA glycosylase, Mag1 (32). This cDNA was cloned by its ability to suppress the alkylation-sensitive phenotype of 3MeA DNA glycosylase-deficient *E. coli*. The *S. pombe* Mag1 3MeA DNA glycosylase turned out to be homologous to a certain group of 3MeA DNA glycosylases, namely, *E. coli* AlkA, *S. cerevisiae* Mag, and *Bacillus subtilis* AlkA. Structural studies indicate that *E. coli* AlkA (and most likely its homologues) is a member of the helix-hairpin-helix family of DNA glycosylases (28, 50). Members of this family share a common three-dimensional structure and catalytic mechanism. Although *S. pombe mag1* has several features in common with the *E. coli* and *S. cerevisiae* 3MeA DNA glycosylase genes, in contrast to *E. coli alkA* and *S. cerevisiae MAG*, *S. pombe mag1* expression is not inducible by MMS treatment (32).

Here we set out to determine the biological role of *S. pombe* Mag1 and its contribution to alkylation resistance. This led us to determine the relative roles of BER, NER, and RR in providing *S. pombe* with resistance to alkylating agents. We determined the alkylation sensitivity of *S. pombe mag1* mutants compared to strains deficient in NER, RR, or FEN1. We further determined the interaction between BER, NER, and RR repair pathways for providing alkylation sensitivity.

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

Strain	Genotype	Source
TE236	*leu1-32 ura4-D18*	Derived from 972 h^-
AM006	*leu1-32 ura4-D18 mag1*::*ura4*$^+$	This study
TE793	*leu1-32 ura4-D18 ade6-704 rad2*::*ura4*$^+$	35
TE570	*leu1-32 ura4-D18 ade6-704 rad3*::*ura4*$^+$	1
TE791	*leu1-32 ura4-D18 ade6-704 rad13*::*ura4*$^+$	35
TE792	*leu1-32 ura4-D18 ade6-704 rhp51*::*ura4*$^+$	35
AM008	*leu1-32 ura4-D18 ade6-704 mag1*::*ura4*$^+$ *rad13*::*ura4*$^+$	This study
AM013	*leu1-32 ura4-D18 ade6-704 mag1*::*ura4*$^+$ *rhp51*::*ura4*$^+$	This study
AM019	*leu1-32 ura4-D18 ade6-704 rad13*::*ura4*$^+$ *rhp51*::*ura4*$^+$	This study
AM022	*leu1-32 ura4-D18 ade6-704 mag1*::*ura4*$^+$ *rad13*::*ura4*$^+$ *rhp51*::*ura4*$^+$	This study

MATERIALS AND METHODS

***S. pombe* strains and growth conditions.** The genotypes of strains used in this study are listed in Table 1. *S. pombe* were routinely grown aerobically in rich yeast extract medium supplemented with adenine (2, 33). *S. pombe* strains disrupted at the *rhp51*, *rad13*, or *rad2* locus by *ura4* insertion were generated in the laboratory of A. M. Carr (Sussex University, Sussex, United Kingdom) and kindly given to us by T. Enoch (Harvard Medical School, Boston, Mass.) (1, 35); all three strains were disrupted with the *ura4* gene and able to grow in the absence of uracil. *ura4-D18 leu1-32 S. pombe* (designated strain TE236) obtained from T. Enoch) was used as the wild type for DNA repair. *S. pombe* was cultured using standard techniques (2, 33).

Construction of mutant *S. pombe* strains. A 1.9-kb *Hin*dIII fragment containing the *S. pombe ura4* gene was isolated from the plasmid pUC8-*ura4* (generously provided by C. Hoffman, Boston College, Newton, Mass.), blunt ended with the Klenow fragment of *E. coli* polymerase I and inserted into the unique *Eco*RV site within the *mag1* cDNA (32). The *mag1*::*ura4* DNA fragment was excised from plasmid by *Bcg*I and *Bcl*I digestion, separated from vector by gel electrophoresis, purified using a Qiax DNA purification kit (Qiagen), and used to transform *uraD-18 leu1-32 S. pombe*. Resulting clones were selected for growth in the absence of uracil; the Ura$^+$ phenotype of individual clones was confirmed by serially plating twice onto nonselective rich medium and then plating onto medium lacking uracil and medium containing 5-fluoroorotic acid (5FOA). Clones that were Ura$^+$ and 5FOA sensitive were selected for Southern analysis (Fig. 1). One *mag1*::*ura4* clone (clone 4 in Fig. 1B) was backcrossed three times with *ura4-D18 leu1-32 S. pombe*, and the resulting *mag1 ura4D-18 leu1-32 S. pombe* cells were characterized for sensitivity to the methylating agent MMS. *S. pombe* strains with *ura4* disruptions in multiple DNA repair genes were generated by mating strains of opposite mating type on malt medium agar plates. Resulting progeny were analyzed for growth in the absence of uracil and genotypes were confirmed by Southern analysis.

DNA isolation and Southern hybridization. Genomic DNA isolations were performed as described elsewhere (2). Genomic DNA was digested with the indicated restriction enzymes and size fractionated on 1% agarose gels, transferred to a nylon membrane (Nytran; Schleicher & Schuell), and hybridized to *mag1* cDNA labeled with ^{32}P by the random primer method (NEBlot; New England Biolabs).

Generation of plasmid for expression of *S. cerevisiae APN1* in *S. pombe*. Two primers (5′-CGC GCT CGA GCC TTC GAC ACC TAG CTT T-3′ and 5′-CGC GGG GCC CTA CGT ACG TTG AGA TAA T-3′) were used to amplify the *S. cerevisiae APN1* open reading frame (ORF) and introduce *Apa*I and *Xho*I restriction sites 5′ and 3′, respectively, to the gene. Insertion into the *Apa*I and *Xho*I sites within the *S. pombe* expression vector pREP3-HA (a gift from Dieter Wolf, Harvard School of Public Health, Boston, Mass., and described in reference 13) resulted in a plasmid (pREP-APN1^{sc}) that would express the *S. cerevisiae APN1* gene as a hemagglutinin fusion protein under the control of the thiamine-repressible *nmt1* promoter in *S. pombe*. pREP-APN1^{sc} and or the empty vector were introduced into *S. pombe* strains by using electroporation as described elsewhere (2). Transformants were isolated and maintained in minimal medium containing uracil, adenine, and thiamine (5 μg/ml).

***S. pombe* colony formation in MMS.** Colony-forming ability of *S. pombe* was determined after treatment with MMS, either as a chronic dose in solid medium or as an acute dose in liquid culture. For chronic exposure, serial dilutions of a logarithmically growing *S. pombe* (5×10^6 to 2×10^7 cells/ml) were plated on MMS-containing solid media, and colonies were scored after 3 to 4 days at 30°C. For acute exposure, the indicated doses of MMS were added to logarithmically growing *S. pombe*. Aliquots were removed at the indicated time, serially diluted,

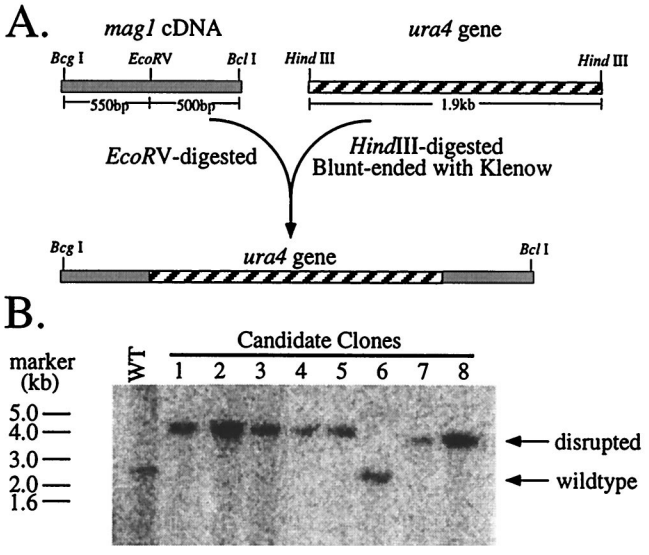

FIG. 1. Disruption of *mag1* in *S. pombe*. (A) The *mag1*::*ura4* disruption construct was made as follows. A blunt-ended DNA fragment containing the *S. pombe ura4* gene (hatched box) was ligated into a unique *Eco*RV site in the *mag1* cDNA (shaded box) as diagrammed. The *Bcg*I/*Bcl*I fragment was isolated, purified, and used to transform *ura4D-18 leu1-32 S. pombe*. (B) Genomic DNA from 5FOA-resistant *S. pombe* clones was isolated, digested with *Hin*dIII, and analyzed by Southern using radiolabeled *mag1* cDNA as a probe. The positions and molecular sizes of marker DNA are indicated.

and spread on solid media. Colony formation was determined after 3 to 5 days of growth at 30°C. In general, MMS doses were selected such that colonies could form in the most sensitive strain and toxicity could be detected in the most resistant strain.

MMS gradient survival assay. Gradient plates, which contain an increasing concentration of MMS across the width of a square petri dish, were made by adding *S. pombe* medium containing agar in two steps in a manner previously described (11). The medium used was either rich yeast extract medium (for *S. pombe* strains not harboring a plasmid) or essential minimal medium supplemented with uracil and adenine as described elsewhere (2) (for strains harboring a plasmid). In the initial step, molten MMS-containing agar was allowed to solidify as a wedge by propping up one edge of the square petri dish approximately 0.5 cm. Following solidification of the first layer, the petri dish was laid flat, and a second layer of molten agar was overlaid and allowed to solidify. Following this second solidification, plates were dried for 5 to 10 min at 55°C with the lids off. The edge of a sterile glass slide was used to transfer stationary-phase *S. pombe* mixed with molten agar from a sterile surface to the MMS gradient plate. In this manner, cells were spread uniformly across the gradient and between samples. MMS sensitivity was determined after allowing 3 to 4 days growth at 30°C.

RESULTS

Disruption of the *S. pombe mag1* locus. To study the role of BER in protecting *S. pombe* against the simple methylating agent MMS, we disrupted the *mag1* 3MeA DNA glycosylase gene by insertion of the *ura4* gene. The *ura4* disruption was positioned within the coding region at a predicted cleft-like structure containing the putative Mag1 active site. Amino acids encoding the cleft are highly conserved among *E. coli* AlkA and its homologues; furthermore, in AlkA, structural and biochemical studies have confirmed the existence of the cleft and the importance of this region for catalytic activity (28, 50). Due to Mag1's homology to AlkA, it is very likely that the two enzymes share the same biochemical determinants. In support of this hypothesis, site-directed mutagenesis of a codon encoding an aspartate residue (Asp170) predicted to be essential for catalysis abolished the ability of *S. pombe mag1* to complement the MMS sensitivity of 3MeA DNA glycosylase-deficient *S. cerevisiae* (data not shown); the equivalent aspartate in AlkA (Asp238) is critical for catalytic activity (28, 50). Further, unlike the parental plasmid containing *S. pombe mag1*, a plasmid containing the *ura4*-disrupted *mag1* cDNA did not provide MMS resistance to 3MeA DNA glycosylase-deficient *E. coli* (data not shown). Thus, the biological activity of *mag1* was indeed disrupted by the *ura4* insertion.

Following transformation with the disruption construct, *S. pombe* clones that consistently exhibited the Ura^+ phenotype were screened for the presence of the *mag1*::*ura4* allele by Southern analysis. The *mag1* cDNA hybridized to a *Hin*dIII fragment of approximately 2.5 kb in size in wild-type *S. pombe*. Disruption of *mag1* with *ura4* is predicted to result in a *Hin*dIII fragment approximately 4.5 kb in size. Seven of eight clones tested had the mutated *mag1* allele and had lost the wild-type allele (Fig. 1). In an effort to determine the effect of the *mag1* disruption of 3MeA and 7-methylguanine DNA glycosylase activity in *S. pombe*, repeated attempts were made to measure glycosylase activity in wild-type and *mag1 S. pombe* cell extracts. However, such DNA glycosylase activity was undetectable even in wild-type *S. pombe* extracts, preventing us from determining the effect of *mag1* disruption on activity. We previously showed that the gene transcript is constitutively expressed in *S. pombe*, suggesting that our failure to detect Mag1 activity reflects a problem with in vitro reaction conditions; note that *S. pombe* Mag1 activity can be measured in extracts from *mag1*-expressing *E. coli*, indicating that this gene does indeed encode a 3MeA DNA glycosylase (32).

Sensitivity of *mag1*-deficient *S. pombe* to MMS. In all organisms that we previously tested, inactivation of 3MeA DNA glycosylase genes dramatically increased MMS sensitivity (Fig. 2). However, to our surprise, *mag1 S. pombe* tested for sensitivity to the methylating agent MMS displayed very little MMS sensitivity (Fig. 2). Indeed, when tested under chronic exposure to MMS in gradient plates (11), *mag1 S. pombe* appeared no more MMS sensitive than wild-type cells (Fig. 3B). To reconcile our findings, we reasoned that DNA repair pathways other than BER might play a more prominent role in *S. pombe* alkylation resistance.

MMS sensitivity of *S. pombe* deficient in RR, NER, or FEN1. Since Mag1-initiated BER did not appear to play a major role in alkylation resistance, we set out to determine which DNA repair pathways do contribute to alkylation resistance in *S. pombe*. The MMS sensitivity of various DNA repair-deficient *S. pombe* strains was determined. *rad13*, *rad2*, and *rhp51* strains were chosen based on their known participation in pathways other than BER. The *rad13* gene was originally cloned by virtue of its ability to reverse the UV-sensitive phenotype of *rad13 S. pombe* (7); its product is homologous to mammalian XPG/ERCC5 and to *S. cerevisiae* Rad2 (unrelated to *S. pombe* Rad2), both of which are required for the initiation of NER and have structure-specific 3′-exinuclease activities (18, 19). Similarly *S. pombe rad2* was cloned by its ability to reverse the UV sensitivity of *rad2 S. pombe* and encodes a homologue of *S. cerevisiae* RAD27 and mammalian FEN1 (35). FEN1 is another structure-specific DNA endonuclease, and its substrates include "flaps" of DNA that result from DNA polymerase-mediated displacement of single-stranded DNA 3′ to the newly synthesized DNA. Such structures are thought to occur during lagging-strand DNA synthesis and possibly during BER and UVDE-mediated excision repair (30, 51). In support of a role for *S. pombe* Rad2 in DNA repair, *rad2* strains have a reduced ability to repair cyclobutane pyrimidine dimers and 6-4 photoproducts and presumably accounts for the UV-sensitive phenotype (35). The *S. pombe rhp51* gene was cloned by low-stringency hybridization to *S. cerevisiae RAD51* (34); as

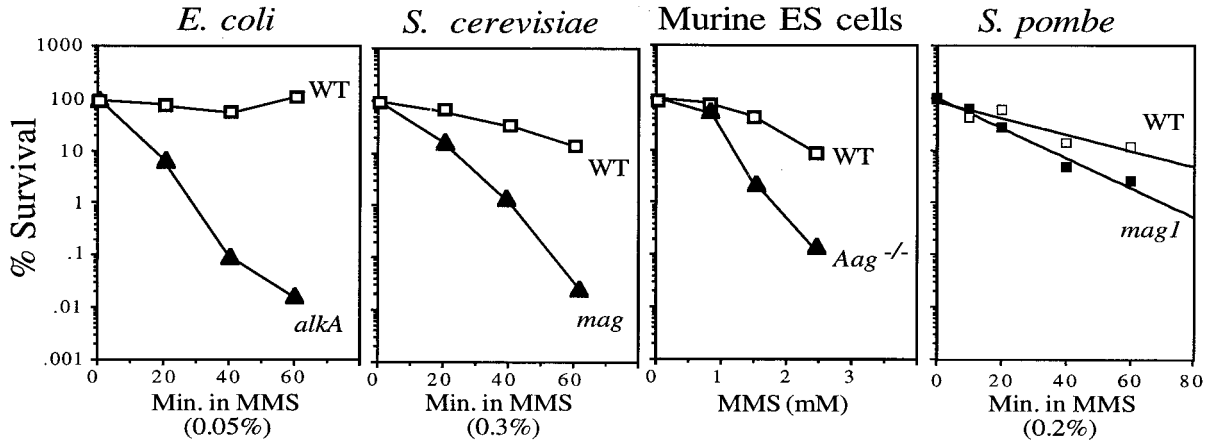

FIG. 2. MMS sensitivity of 3MeA DNA glycosylase-deficient cells. The colony-forming ability of cells from various organisms following MMS treatment was determined. For *E. coli*, *S. cerevisiae*, and murine embryonic stem cells, doses and times are as indicated and data shown are adapted from references 8, 12, and 32. For *S. pombe*, 0.2% MMS was added to logarithmically growing wild-type (WT; TE236) or *mag1* (AM006) *S. pombe*. Aliquots were collected at the indicated times and scored for colony-forming ability as described in Materials and Methods. The data shown for *S. pombe* are the mean from five independent experiments.

mentioned, Rad51 from both yeasts are RecA homologues and are thus involved in DNA strand exchange during RR (4).

The MMS sensitivity of wild-type, *mag1*, *rhp51* (RR-deficient), *rad13* (NER-deficient), and *rad2* (FEN1-deficient) *S. pombe* single mutants was determined by two different methods. The first method measured colony-forming ability following MMS exposure for up to 1 h (acute exposure) (Fig. 3A), and the second method measured growth of *S. pombe* along an MMS gradient plate (chronic exposure) (Fig. 3B). As previously indicated, 3MeA DNA glycosylase deficiency alone (*mag1*) had a small but measurable effect on the colony-forming ability of *S. pombe* given an acute dose of MMS. In contrast, the survival of *mag1 S. pombe* given a chronic dose of MMS from an MMS gradient plate was the same as for the wild type (Fig. 3). In contrast, FEN1-deficient (*rad2*) *S. pombe* was sensitive to MMS in both assays, although sensitivity was greater in the MMS gradient plates (Fig. 3). NER-deficient (*rad13*) *S. pombe* was considerably more sensitive to MMS in both assays and, surprisingly, much more sensitive to MMS than *mag1 S. pombe*. NER and FEN1 deficiencies had quantitatively similar effects on sensitivity to chronic MMS exposure, although they are most likely involved in different DNA repair pathways. RR-deficient (*rhp51*) *S. pombe* was profoundly sensitive to both acute and chronic MMS exposures (Fig. 3).

MMS sensitivity of *S. pombe* deficient in both Mag1 and NER. To determine whether Mag1-initiated BER and Rad13-initiated NER play redundant roles in DNA alkylation repair, *mag1 rad13 S. pombe* double mutants were made. Candidate double-mutant clones were genotyped by Southern analysis (data not shown). Using *ura4* as a probe, a characteristic *ura4*-containing restriction fragment corresponding to each mutant allele was detected; in the double mutant, both *ura4* insertion alleles were present. In the absence of NER, the *mag1* mutation had a much more profound effect on MMS sensitivity than in the presence of NER (i.e., in wild-type *S. pombe*) (Fig. 4). Thus, *rad13 mag1 S. pombe* were much more sensitive to MMS compared to *S. pombe* mutated in *rad13* alone (Fig. 4). The synergistic interaction of *rad13* and *mag1* for the MMS-sensitive phenotype was observed in several independent clones (data not shown), and such interaction indicates that the two repair pathways do indeed play redundant roles.

MMS sensitivity of *S. pombe* deficient in both Mag1 and RR. To determine whether Mag1-initiated BER and Rhp51-initiated RR play redundant roles in DNA alkylation repair, we generated a *mag1 rhp51 S. pombe* double mutant that turned out to display a quite unexpected phenotype. *mag1 rhp51* cells were actually less MMS sensitive than the *rhp51* single mutant (Fig. 5A), and this phenotype was consistent for several clones obtained from independent crosses (data not shown). However, it is worth noting that this unexpected genetic interaction between *mag1* and *rhp51* was observed upon chronic exposure of *S. pombe* to MMS (Fig. 5A) and was not observed upon acute MMS exposure; i.e., *mag1 rhp51* and *rhp51 S. pombe* strains were similarly MMS sensitive when treated with various doses of MMS for 1 h (Fig. 5B).

During BER, a single-stranded DNA break is formed by AP endonuclease at the abasic site produced by DNA glycosylase. Classic studies have shown that the number of MMS-induced DNA strand breaks increases for some time after MMS removal (37, 45); it has been suggested that glycosylases and AP endonucleases are responsible for some fraction of these MMS-induced DNA strand breaks. If the BER enzymes required for the repair of such single-strand DNA breaks were limiting, an accumulation of DNA strand breaks could result. Alternatively, AP sites could give rise to DNA strand breaks due to their inherent chemical instability or their ability to block DNA replication and thus indirectly result in strand breaks. Since, the major role of RR is to repair DNA strand breaks, we hypothesized that DNA strand breaks generated during BER could be substrates for RR, and that in the absence of RR these BER-induced DNA strand breaks could contribute to MMS-induced cytotoxicity. Thus, without Mag1-initiated BER, fewer MMS-induced DNA strand breaks may accumulate, rendering RR-deficient cells more MMS resistant as in Fig. 5A. However, why such MMS resistance is apparent only during chronic exposure remains unclear.

To determine whether Mag1-induced cytotoxicity (in RR-deficient cells) is due to excessive AP sites or other downstream intermediates of BER, we expressed the *S. cerevisiae* AP endonuclease gene, *APN1* in *S. pombe*. Expression of *APN1* in *rhp51 S. pombe* cells partially reversed their MMS sensitivity (Fig. 6). It is worth noting that the level of MMS resistance observed in *rhp51 S. pombe* expressing *APN1* is similar to that observed in *mag1 rhp51 S. pombe* with vector. Thus, expression of *APN1* appears to reverse the negative contribution of Mag1 to MMS sensitivity, suggesting that Mag1-induced cytotoxicity is due to excessive AP sites. Note that expression of *APN1* in

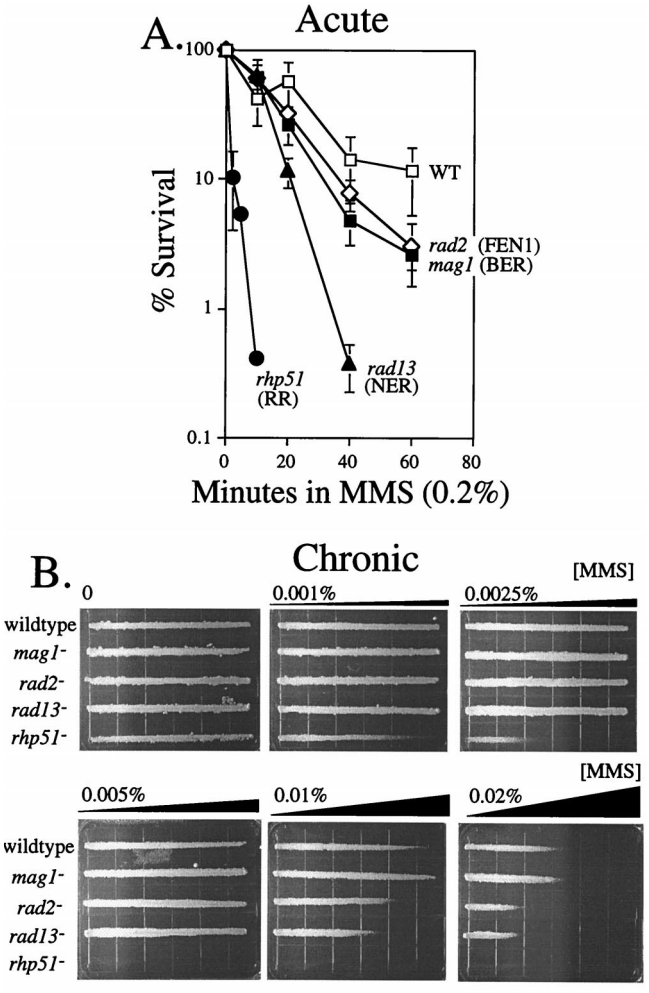

FIG. 3. MMS sensitivity of *S. pombe* deficient in DNA repair. (A) MMS (0.2%) was added to logarithmically growing *S. pombe*. Aliquots were collected at the indicated times and scored for colony-forming ability as described in the Materials and Methods. The strains analyzed were wild-type (TE236), *mag1* (AM006), *rad2* (TE793), *rad13* (TE791), and *rhp51* (TE792) *S. pombe*. The data shown are the mean and standard error from three experiments for *rad2*, *rad13*, and *rhp51* and five experiments for wild type and *mag1*. (B) MMS gradient plate analysis was performed on the indicated strains as described in Materials and Methods. The concentration of MMS indicated reflects the concentration of the bottom layer of agar and corresponds to the highest concentration of the gradient.

the absence of Mag1 activity had no effect on MMS sensitivity (Fig. 6).

Since NER also plays a role in the repair of MMS-induced DNA damage, we postulated that MMS-induced NER intermediates such as single-strand DNA gaps might also generate RR substrates. To test this, we determined the MMS sensitivity of *rad13 rhp51* double-mutant (NER and RR-deficient) and *mag1 rad13 rhp51* triple-mutant (Mag1-, NER-, and RR-deficient) *S. pombe*. All genotypes were confirmed by Southern analysis (data not shown). It turned out that *rad13 rhp51 S. pombe* was in fact more MMS sensitive than the *rhp51* single mutant, suggesting that RR substrates do not accumulate during NER and that the interaction between BER and RR is quite different from that between NER and RR (Fig. 7A). The additive nature of the *rhp51* and *rad13* MMS sensitivity confirms that *rhp51* and *rad13* are involved in different DNA repair pathways and suggests that NER intermediates do not normally become RR substrates. Finally, the *mag1* mutation

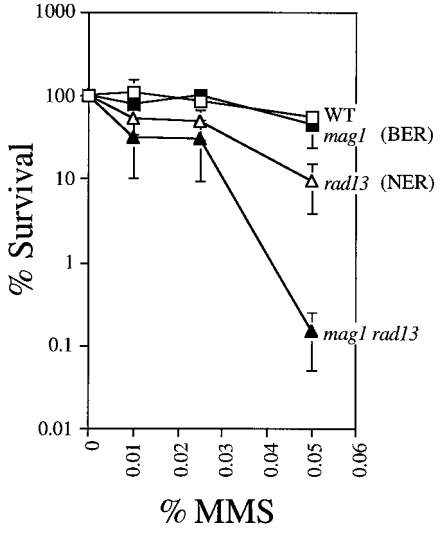

FIG. 4. MMS sensitivity of *S. pombe* mutant for *mag1* and NER. Logarithmically growing wild-type (WT; TE236), *mag1* (AM006), *rad13* (TE791), and *mag1 rad13* (AM008) *S. pombe* strains were treated with the indicated dose of MMS for 1 h as described in Materials and Methods. Aliquots were collected and analyzed for colony-forming ability. The data shown are the mean and standard error of three independent experiments.

still conferred MMS resistance upon *rad13 rhp51* double mutants, just at it had upon the *rhp51* single mutant; thus, even in NER-deficient cells *mag1* and *rhp51* display their unexpected genetic interaction (Fig. 7B).

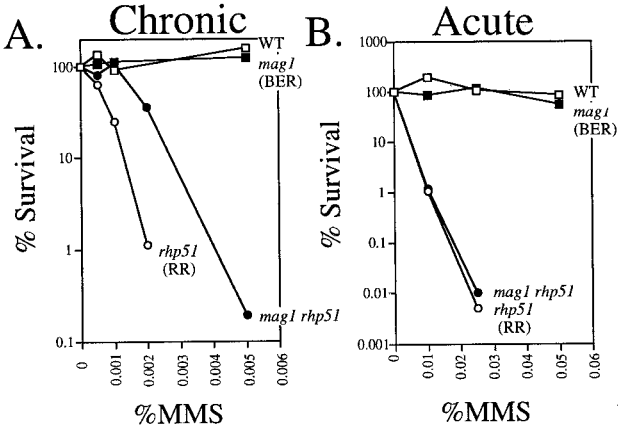

FIG. 5. MMS sensitivity of *S. pombe* mutant for *mag1* and recombinational repair. (A) Logarithmically growing *S. pombe* was diluted and plated onto solid agar medium containing the indicated doses of MMS. Colony-forming ability was determined. The data are from a representative experiment. Because RR-deficient strains are hypersensitive to MMS, slight variations in dose due to the reactivity of MMS have large effects on viability. Thus, there was significant between-experiment variability in all experiments using *rhp51 S. pombe* and its derivative strains. The between-experiment variability made it impossible to pool results and calculate values for the mean and standard error for each data point. However, the relationship between the strains was always the same, and similar results were observed in three independent experiments. (B) Logarithmically growing *S. pombe* was treated with the indicated dose of MMS for 1 h as described in Materials and Methods. Aliquots were collected and analyzed for colony-forming ability. The data shown are from a representative experiment; similar results were obtained with three independent experiments. Strains analyzed were wild-type (WT; TE 236), *mag1* (AM006), *rhp51* (TE792), and *mag1 rhp51* (AM013) *S. pombe*.

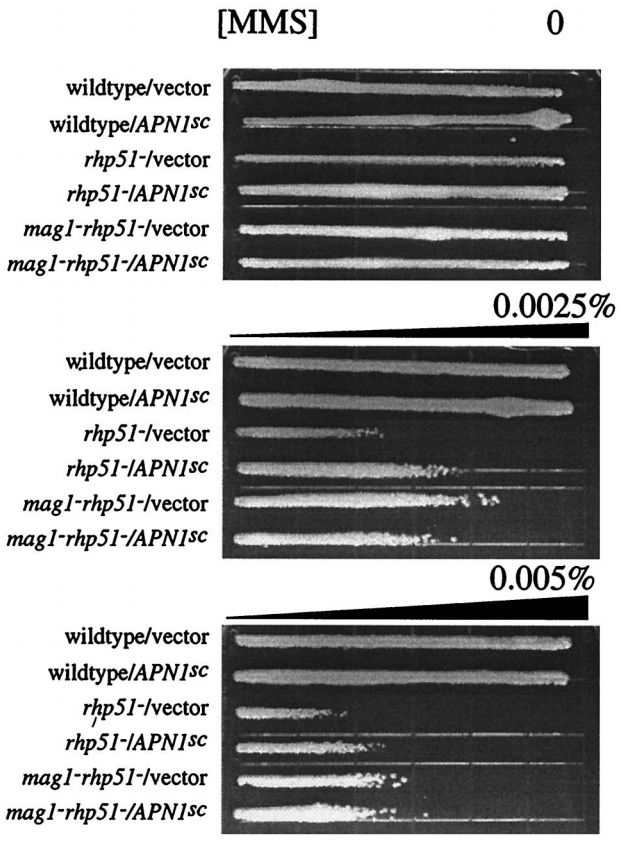

FIG. 6. MMS sensitivity of *S. pombe* overexpressing *S. cerevisiae* AP endonuclease. MMS gradient plate analysis was performed on wild-type, *rhp51*, and *mag1 rhp51 S. pombe* strains expressing either the pREP vector plasmid or the pREP-*APN*^{sc} plasmid expressing the *S. cerevisiae* AP endonuclease gene *APN1* as described in Materials and Methods. The MMS concentration (0.005%) reflects the concentration of the bottom layer of agar and corresponds to the highest concentration of the gradient. The strains and plasmids are as indicated. Similar results were obtained from three independent experiments using two independent transformants for each strain.

DISCUSSION

Here we demonstrate that although *S. pombe* employs the same range of DNA alkylation repair pathways as several other organisms, the relative importance of each pathway for providing resistance to each organism varies dramatically. In wild-type *S. pombe*, the Mag1 3MeA DNA glycosylase was not a major determinant of MMS sensitivity. In contrast to *S. pombe*, 3MeA DNA glycosylase-deficient *E. coli* was profoundly sensitive to MMS. MMS doses that had no effect on survival of the wild type allowed less than 0.01% survival of 3MeA DNA glycosylase-deficient *E. coli* (8). Similarly, in *S. cerevisiae*, doses of MMS that allowed greater than 10% survival in the wild type allowed less than 0.0001% survival in *mag1 S. cerevisiae* (9). The contribution of 3MeA DNA glycosylase activity to MMS sensitivity of mammalian cells was more modest but nevertheless significant; MMS doses that allowed approximately 10% survival of the wild type caused less than 1% survival of 3MeA DNA glycosylase-deficient Aag$^{-/-}$ null cells (12). Note that there was no detectable 3MeA DNA glycosylase activity in cell extracts made from Aag$^{-/-}$ embryonic stem cells, suggesting that the presence of another 3MeA DNA glycosylase with redundant activity is unlikely (12).

NER and RR were shown to play major roles in *S. pombe* MMS resistance. NER-deficient *rad13 S. pombe* strains are significantly MMS sensitive and RR-deficient *rhp51* strains are profoundly MMS sensitive compared to the wild type. Similarly, *S. cerevisiae* strains mutant for NER genes are modestly MMS sensitive, whereas *S. cerevisiae* mutant for genes involved in RR are extremely MMS sensitive (49). In contrast, NER-deficient mammalian cells are not sensitive to MMS (20, 43). Moreover, it appears in *S. pombe* that BER plays a secondary role in DNA methylation repair, which is revealed in the absence of NER.

We have also shown a role for *S. pombe* FEN1 (Rad2) in MMS resistance. *rad2 S. pombe* strains were identified by virtue of their UV sensitivity. In contrast, mutants in the *S. cerevisiae rad2* homologue, *rth1* (also known as *rad27*), are not sensitive to either UV or γ irradiation, but they do display an elevated mitotic recombination, elevated spontaneous mutation, temperature sensitivity, and MMS sensitivity (41, 44). Here we show that *rad2 S. pombe* is moderately sensitive to MMS. The nuclease activity of FEN1 is known to be important in mammalian cells for processing of Okazaki fragments during DNA lagging-strand synthesis, for UVDE-mediated excision repair, and also for the long-patch subpathway of BER. Our data do not indicate which function of FEN1 (Rad2) is important for MMS resistance.

It is still formally possible that BER plays a significant role in providing alkylation resistance to *S. pombe*, initiated by glycosylases other than Mag1. In support of this notion, a predicted ORF for a second enzyme with sequence similarity to *E. coli* AlkA and *S. pombe* Mag1 has been identified in the *S. pombe* genome, representing a putative second *S. pombe* 3MeA DNA glycosylase. Additionally, two separate *S. pombe* ORFs bearing sequence similarity to the major AP endonucleases in *E. coli*, ExoIII and EndoIV, have been identified (5). Although the structural genes for BER enzymes exist in *S. pombe*, 3MeA DNA glycosylase activity and AP endonuclease activity were undetectable in crude *S. pombe* extracts, suggesting that these genes may not be expressed at very high levels (reference 29 and data not shown).

The interaction between *S. pombe* BER and NER was shown to be synergistic; i.e., a deficiency in both BER and NER produced much more MMS sensitivity than that predicted from an additive contribution of each repair pathways. Synergism is observed between DNA repair genes when nei-

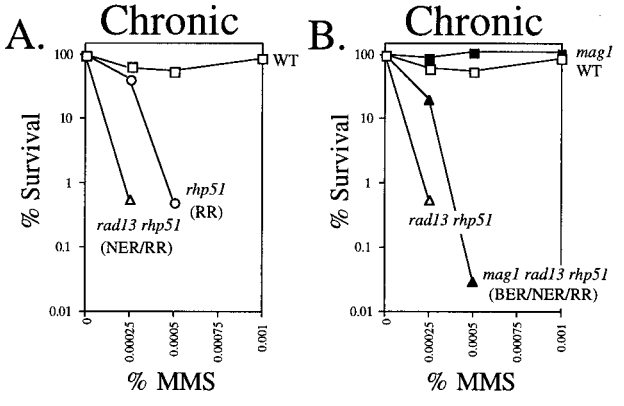

FIG. 7. MMS sensitivity of *S. pombe* mutant for *mag1*, NER, and RR. Logarithmically growing *S. pombe* was diluted and plated onto solid agar medium containing the indicated doses of MMS. Colony-forming ability was determined. The data shown are from a representative experiment. Similar results were observed in three independent experiments. Strains analyzed were wild-type (WT; TE236), *rhp51* (TE792), and *rad13 rhp51* (AM019) (A) and wild-type (TE236), *mag1* (AM006), *rad13 rhp51* (AM019) and *mag1 rad13 rhp51* (AM022) (B) *S. pombe*.

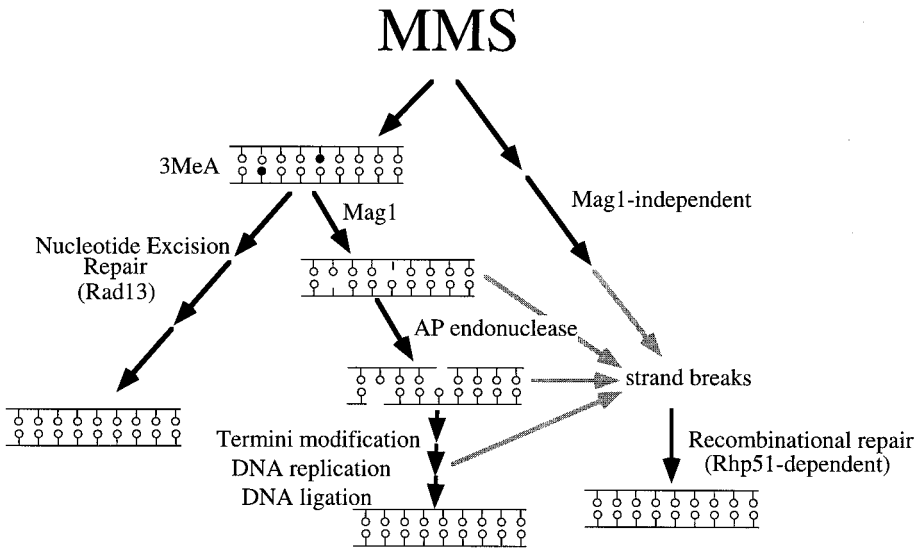

FIG. 8. Model for MMS-induced strand breaks and DNA repair in *S. pombe*. As diagrammed, BER and NER can repair potentially lethal 3MeA lesions. BER generates DNA strand breaks during the repair process, and these can be substrates for Rhp51-dependent recombinational repair. Alternatively, MMS can induce DNA strand breaks by a mechanism whose molecular details are unknown.

ther DNA repair pathway is operating to its full capacity in wild-type cells. Thus, when one pathway is absent, the other can at least partially compensate, and in some cases a DNA damage-sensitive phenotype is avoided altogether (20). Synergism between *S. pombe* BER and NER indicates several points. First, it confirms that Mag1-initiated BER does indeed function in *S. pombe*, albeit in a supportive role, for the repair of DNA methylation damage. Furthermore, these data indicate that insertion of *ura4* into the *mag1* ORF did disrupt the biological activity of Mag1, as predicted. The synergism also indicates that while the *S. pombe* BER and NER pathways are distinct, they can act on the same methylated DNA lesions, most likely 3MeA (20), and that at least one of these lesions (again, most likely 3MeA) is lethal in *S. pombe* if left unrepaired.

Although a similar synergistic interaction between BER and NER was observed for *S. cerevisiae*, for this organism, BER predominates over NER (48). Further, NER does not seem to play any role in MMS resistance in mammalian fibroblasts (43). While the list of known in vitro substrates for NER has expanded from just DNA helix-distorting lesions to include O^6-methylguanine, AP sites, N^6-methyladenine, and some mismatched bases, it is not yet known whether 3MeA is repaired by NER (17, 22). Given the biological evidence presented here and similar studies in *S. cerevisiae*, it seems highly likely that 3MeA can be repaired by NER, at least in *S. pombe* and *S. cerevisiae* (48). It is worth noting that XPG/ERCC5, the human homologue of the *S. pombe rad13* gene studied here, has been reported to stimulate the excision of thymine glycol DNA lesions by the human thymine glycol DNA glycosylase and can thus act in an accessory role for BER (6, 27). It is not known whether *S. pombe rad13* has a similar stimulatory effect on thymine glycol DNA glycosylase or whether XPG and its homologues stimulate other DNA glycosylases.

Analysis of the MMS sensitivity of single and double mutants for *mag1* and *rhp51* revealed an unexpected genetic relationship. The observation that *rhp51 S. pombe* is more sensitive to MMS than *mag1 rhp51 S. pombe* is consistent with a model for MMS-induced single-strand DNA breaks accumulating as BER intermediates. As diagrammed in Fig. 8, the successive action of 3MeA DNA glycosylase and AP endonuclease creates a DNA break in one strand whose repair is completed by termini modification (by either deoxyribose phosphodiesterase or FEN1), DNA replication, and DNA ligation. If any of the latter three steps of BER were rate limiting, DNA strand breaks would accumulate in the genome and BER intermediates (either singly or closely opposed to one another), could act as substrates for RR involving *S. pombe* Rhp51. Alternatively, AP sites could give rise to DNA strand breaks due to their inherent chemical instability or their ability to block DNA replication. A stalled replication fork on the leading strand with continued DNA synthesis on the lagging strand could produce extended regions of single-stranded DNA that are potential substrates for RR. Indeed, heterologous expression of an *S. cerevisiae* AP endonuclease gene, *APN1*, in *mag1 rhp51 S. pombe* reverses the contribution of *mag1* to MMS sensitivity, suggesting that Mag1-induced DNA strand breaks are due to unrepaired AP sites (Fig. 6). The absence of Mag1-initiated BER may reduce the number of RR substrates, making RR-deficient cells more MMS resistant. Both of these models indicate that 3MeA DNA lesions are less lethal to *S. pombe* than DNA strand breaks. It is worth noting that the absence of Mag1 only partially reverses the MMS-sensitive phenotype of *rhp51 S. pombe*, suggesting that a small but significant portion of MMS-induced DNA strand breaks are attributable to Mag1-generated BER intermediates. It is unclear how the remaining MMS-induced DNA strand breaks are produced.

The genetic interaction between *mag1* and *rhp51* was observed only when *S. pombe* was treated chronically with MMS, not when *S. pombe* was treated acutely. Although *rhp51 S. pombe* was still extremely sensitive to acute MMS exposure, there was no difference in the MMS sensitivity of *rhp51* and *mag1 rhp51 S. pombe*. In the model presented, the contribution of BER to DNA strand breaks requires that at least one of the downstream components of BER be limiting, such that BER intermediates accumulate. Perhaps these components are induced under acute but not chronic exposure to MMS, such that BER intermediates are processed more efficiently under acute than under chronic MMS exposure. Our previous studies

showed that acute exposure of *S. pombe* to MMS did not affect *mag1* transcript levels (32). However, preliminary results show that chronic MMS exposure may moderately induce *mag1* transcript levels. Whether other components of the BER pathway are differentially expressed under various treatment conditions remains to be determined.

The studies described here illustrate an important point: a single DNA repair enzyme or a single DNA repair pathway does not solely determine sensitivity to DNA-damaging agents. Rather, the interactions between different DNA repair pathways are clearly very important for cell survival and thus can have both positive and negative outcomes. The data presented here demonstrate that a single type of DNA repair defect can have no effect, a positive effect, or a negative effect on cell survival of DNA damage, depending on the constellation of other repair pathways in the cell. We have also demonstrated that DNA repair intermediates from one DNA repair pathway can be substrates for a second DNA repair pathway. It is highly likely that the balance and interactions between DNA repair pathways differ among organisms and even among different tissues within the same organism. DNA-damaging agents are used in the clinic to treat cancers, and several gene therapy approaches are being developed to alter DNA repair capacity such that the efficacy of cancer chemotherapy is increased. It is therefore very important to explore potential interactions among DNA repair pathways in order to understand their influences on DNA damage susceptibility and to manipulate DNA repair pathways as effectively as possible.

ACKNOWLEDGMENTS

This research was supported by grants NCI CA55042 and NIEHS P01-ES03926. A.M. was supported by a fellowship from the Pharmaceutical Research and Manufacturers of America Foundation. L.S. is a Burroughs Wellcome Toxicology Scholar.

We thank Charles Hoffman for the *S. pombe ura4* plasmid, Tamar Enoch for *S. pombe* strains, and Dieter Wolf for the *S. pombe* expression plasmid. We are also grateful to Tamar Enoch and Bruce Demple for their thoughtful and constructive advice during this research and Brian Glassner and Lauren Posnick for helpful comments on the manuscript.

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