

***In vivo* evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells**

WEI XIAO* AND LEONA SAMSON†

Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115

Communicated by Bruce N. Ames, November 19, 1992

ABSTRACT Three genes that participate in the repair of DNA alkylation damage were recently cloned from *Saccharomyces cerevisiae*: the *MGT1* O⁶-methylguanine DNA methyltransferase gene, the *MAG* 3-methyladenine DNA glycosylase gene, and the *APN1* apurinic/aprimidinic (AP) endonuclease gene. Altering the expression levels of these three genes produced significant changes in the *S. cerevisiae* spontaneous mutation rate. Spontaneous mutation increased in the absence of the *MGT1* DNA methyltransferase, presumably because unrepaired, spontaneously produced, O⁶-alkylguanine lesions mispair during replication. Moreover, changing the ratios of the *MAG* 3-methyladenine DNA glycosylase and the *APN1* AP endonuclease had profound effects on spontaneous mutation rates. In the absence of *APN1*, the overexpression of *MAG* increased spontaneous mutation, and the underexpression of *MAG* decreased spontaneous mutation. We infer that the *MAG* glycosylase acts upon spontaneously produced 3-alkyladenine and 7-alkylguanine DNA lesions to produce mutagenic abasic sites, and that if the repair of these abasic sites is not initiated by the *APN1* AP endonuclease they cause mutations during replication. Our results indicate that eukaryotic cells harbor endogenous metabolites that alkylate nuclear DNA at both oxygens and nitrogens.

Mutations are defined as spontaneous when they arise in populations that were not patently exposed to exogenous, xenobiotic mutagens. It has been argued that one of the early steps in the progression of human tumors is to elevate the rate of spontaneous mutation (i.e., to induce a mutator phenotype), and this argument is based upon the fact that the progression of many human tumors is accompanied by the accumulation of a large number of mutations (1-4). Thus, if changes in spontaneous mutation rates are involved in carcinogenesis, it is clearly important to define the pathways that influence spontaneous mutation rates in eukaryotic cells.

The characterization of *Escherichia coli* mutator and antimutator strains has defined several pathways that can limit spontaneous mutation rates, and has thus defined several ways that the *E. coli* genome can undergo spontaneous change. The potential causes of spontaneous genetic change in *E. coli* include (i) infrequent errors in DNA replication and the occasional failure to correct those errors (5, 6); (ii) spontaneous DNA bond breakage causing depurination, deamination, and other kinds of DNA damage (7-9); and (iii) the production of DNA damage and damaged DNA precursors by their interaction with reactive intermediary metabolites (10-15). Under some conditions, *E. coli* can apparently elevate the rate of spontaneous mutation for certain beneficial mutational events (16, 17), but the way in which this is accomplished is not understood.

The nature of the intermediary metabolites that cause DNA damage and the kinds of damage they produce are not fully understood. It was shown that (14, 18) *uvr*⁻ *E. coli*, unable

to repair lesions that cause DNA helical distortions, suffer an elevated rate of spontaneous mutation. The nature of the helix-distorting mutagenic DNA damage was not determined and could represent a collection of many different lesions. It is now clear that active oxygen species (by-products of aerobic metabolism) oxidize DNA and its precursors. The *E. coli* spontaneous mutation rate is known to be limited by the detoxification of active oxygen species (10, 11, 15), by the selective hydrolysis of certain oxidized DNA precursors (12), and by the specific repair of oxidatively damaged DNA (19-22). That the specific repair of DNA alkylation damage also contributes to limiting spontaneous mutation rates in *E. coli* (ref. 13; W. Mackay and L.S., unpublished work) suggests that, in addition to spontaneous oxidative damage, the bacterial genome also suffers spontaneous DNA alkylation damage from endogenous metabolic products. We now extend this observation to eukaryotic cells by showing that DNA alkylation repair influences the spontaneous mutation rate in *Saccharomyces cerevisiae*.

Our laboratory recently cloned and characterized two *S. cerevisiae* DNA repair genes whose products specifically repair DNA alkylation damage: the *MGT1* O⁶-methylguanine (O⁶MeG) DNA methyltransferase (MTase) gene (23-25) and the *MAG* 3-methyladenine (3MeA) DNA glycosylase gene (26-28). The *MGT1* MTase directly transfers the methyl group from the mutagenic O⁶MeG lesion [and possibly the mutagenic O⁴-methylthymine (O⁴MeT) lesion] to a cysteine residue in the *MGT1* protein itself, thereby preventing the alkylated bases from mispairing during DNA replication (23, 29). The *MAG* DNA glycosylase releases 3MeA and 7-methylguanine (7MeG) bases from alkylated DNA and provides resistance to the cell-killing effects of alkylating agents (27, 30). The abasic sites generated by the action of the *MAG* glycosylase are subject to cleavage by apurinic/aprimidinic (AP) endonucleases. The major *S. cerevisiae* AP endonuclease is encoded by the *APN1* gene, which was also recently cloned and characterized (31, 32); *apn1* null mutants are sensitive to killing by agents that induce both oxidative and alkylation DNA damage (32). *apn1* mutants also suffer an elevated spontaneous mutation rate, thought to originate from replication past unrepaired abasic sites which lack the correct coding information (32). The abasic sites could be derived, at least in part, from DNA that was either oxidized or alkylated by endogenous compounds.

To further characterize the functional interaction of the *MGT1* DNA MTase, the *MAG* DNA glycosylase, and the *APN1* AP endonuclease in protecting cells against DNA alkylation damage, and in limiting spontaneous mutation rates, we created yeast strains with single and combined *mgt1*, *mag*, and *apn1* null mutations. The influence of these

Abbreviations: AP, apurinic/aprimidinic; MMS, methyl methane-sulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MTase, methyltransferase; 3MeA, 3-methyladenine; 7-MeG, 7-methylguanine; O⁶MeG, O⁶-methylguanine; O⁴MeT, O⁴-methylthymine.

*Present address: Department of Microbiology, University of Saskatchewan, Saskatoon, SK, S7N 0W0, Canada.

†To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

mutations on the *S. cerevisiae* spontaneous mutation rate provides *in vivo* evidence for the endogenous O-alkylation and N-alkylation of the eukaryotic genome.

MATERIALS AND METHODS

Yeast Strains. The *S. cerevisiae* strains used in this study are all derivatives of DBY747 (*MATa*, *his3-Δ1*, *leu2-3,112*, *ura3-52*, *trp1-289*). Mutants were created by homologous replacement of endogenous genes with *in vitro* deletion constructs (33). In JC8901 the endogenous *MAG* is replaced with the *mag-Δ1::URA3* cassette (27). WX1991 and WX9104 were made by disrupting genomic *MGT1* in DBY747 and JC8901, respectively, by using the $\Delta mgt1::LEU2$ cassette (24). Southern hybridization confirmed the integrity of disruptions at the *MAG* and *MGT1* loci (refs. 24 and 27; and data not shown). WX9105, WX9106, WX9107, and WX9108 were made by disrupting the endogenous *APN1* in DBY747, JC8901, WX1991, and WX9104, respectively, by replacement with the $\Delta apn1::HIS3$ cassette (32). The phenotype of each strain was confirmed by MTase, 3MeA DNA glycosylase, or AP endonuclease assays and by its alkylation-sensitive phenotype (24, 26, 31).

Plasmids. The construction of disruption cassettes for *MAG* (27), *MGT1* (24), and *APN1* (32) has been described. pYES2.0 (2μ -*STB*, *URA3*, *P_{GAL1}-TCYCI*) was purchased from Invitrogen (San Diego). The 2.65-kb *Spe I*-*HincII* fragment from plasmid pMGT1-Xb (25), which contains the entire *MGT1* gene plus a second gene of unknown function (referred to as *ORF2*) was cloned into pYES2.0 to replace the 1-kb *Spe I*-*Ssp I* fragment containing the *GAL1* promoter and fl origin region; the resulting plasmid is called YEpmGT1-ORF2. *ORF2* was removed from YEpmGT1-ORF2 by *Aat II* digestion and religation to generate YEpmGT1. YEpm13A was previously isolated from a yeast genomic YEpm13 (2μ -*STB*, *LEU2*) library and carries the *MAG* DNA glycosylase gene (26). Yeast cells were grown (34) and transformed with plasmid (35) as described. For targeted gene disruption, the plasmids carrying the deletion-selection cassettes ($\Delta mgt1::LEU2$, *mag-Δ1::URA3*, $\Delta apn1::HIS3$) were digested with the appropriate restriction enzymes to generate recombinogenic ends homologous to the target sequence; linear plasmids were transformed into yeast, and *Leu*⁺, *Ura*⁺, or *His*⁺ transformants were isolated and checked for the appropriate DNA repair deficiency.

Yeast Cell Extracts and MTase Assay. Extracts were prepared and MTase levels were determined by gel assay (23). Activities shown in Fig. 2A were in the linear range.

Cell Killing. Yeast cells were cultured overnight at 30°C in SD selective media (for strains carrying plasmids) or in YPD and then used to inoculate YPD at a dilution of 1:10 (from the SD overnight) or 1:25 (from the YPD overnight). Growth was continued for 4–6 hr to reach a density of 2×10^7 cells per ml. Cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methyl methanesulfonate (MMS) for various times, then washed, diluted, and plated onto YPD agar and incubated at 30°C for 3 days.

Fluctuation Test. The fluctuation test described by von Borstel (36) was used to measure the spontaneous mutation rate for *Trp*⁺ reversion in *S. cerevisiae*. The *trp1-289* allele in DBY747 bears an amber mutation (37) such that DBY747 can revert to the *Trp*⁺ phenotype by point mutations in *trp1-289* or by amber suppressor mutations; thus mutation to the *Trp*⁺ phenotype probably monitors several different mutational events. Two hundred forty individual 1-ml cultures were grown from 4×10^3 cells per ml to about $0.5\text{--}0.7 \times 10^7$ cells per ml in SD medium limited in tryptophan (1.5 μM), and the number of cultures that had no *Trp*⁺ revertants after 11 days at 30°C was used to calculate the mutation rate as described (36).

Statistical Analysis. The experimental data were analyzed by a statistical analysis system (SAS) program, PROC GLM, for

multiple comparisons (38). The results of Duncan's multiple range test were expressed by Duncan groupings at certain *P* values (38).

RESULTS

Generation of *mgt1*, *mag*, and *apn1* Null Mutants. To determine the relative roles of the *MGT1*, *MAG*, and *APN1* genes in protecting cells against alkylation damage and in limiting spontaneous mutation, we constructed the set of isogenic DNA alkylation repair-deficient mutants described in *Materials and Methods*. Three nonrevertible DBY747 auxotrophic mutations (*ura3-52*, *leu2-3,112*, and *his3-Δ1*) were exploited to create disruptions in the *MAG* 3MeA DNA glycosylase gene (*mag-Δ1::URA3*), the *MGT1* O⁶MeG DNA MTase gene ($\Delta mgt1::LEU2$), and the *APN1* AP endonuclease gene ($\Delta apn1::HIS3$). 3MeA DNA glycosylase activity in the *mag-Δ1::URA3* strain was about 10% of the wild-type level, as described previously (27); O⁶-MeG DNA MTase activity in the $\Delta mgt1::LEU2$ strain was undetectable (24); and class II AP endonuclease activities in the $\Delta apn1::HIS3$ disruption strain was <2% of the wild-type level (32). These DNA repair deficiencies had no deleterious effects on normal growth, since all of the strains grew normally in both rich broth and minimal salts medium.

Epistatic Analysis of DNA Alkylation Repair-Deficient Mutants. We compared the alkylation sensitivities of the *mag*, *mgt1*, and *apn1* mutants, plus all the double mutant strains, in order to establish whether they act in the same or separate pathways—i.e., to establish their epistatic relationships. *MAG* and *APN1* were expected to fall in the same epistatic group (since they act sequentially in the base excision repair of 3MeA and 7MeG), and *MGT1* was expected to be epistatically distinct from both *MAG* and *APN1*. The results shown in Fig. 1 confirm these expectations. Fig. 1 A–C show the sensitivity of each strain to MMS; the *mag-Δ1::URA3* allele conferred the greatest MMS sensitivity, $\Delta apn1::HIS3$ intermediate sensitivity, and $\Delta mgt1::LEU2$ only slight sensitivity. As expected, the sensitivity of the *mag-Δ1::URA3* $\Delta apn1::HIS3$ double mutant did not exceed that of the *mag-Δ1::URA3* single mutant, indicating that *MAG* and *APN1* are epistatic (Fig. 1A). In contrast, the MMS sensitivities of the $\Delta mgt1::LEU2$ $\Delta apn1::HIS3$ and the $\Delta mgt1::LEU2$ *mag-Δ1::URA3* double mutant strains represents the sum of the sensitivities of the corresponding single mutant strains (Fig. 1 B and C), indicating that *MGT1* is epistatically distinct from *MAG* and *APN1*. Even though the small increase in MMS sensitivity conferred by the $\Delta mgt1::LEU2$ allele was highly reproducible, we further confirmed that *MGT1* and *MAG* are epistatically distinct by their MNNG sensitivities. Fig. 1D shows clearly that the alkylation sensitivities conferred by the two mutant alleles are additive, indicating that the *MAG* and *MGT1* repair proteins act in different DNA repair pathways and act on different types of DNA alkylation damage.

The *MGT1* MTase Limits the *S. cerevisiae* Spontaneous Mutation Rate. We previously reported that the MTase-deficient $\Delta mgt1::LEU2$ *S. cerevisiae* strain exhibits a higher spontaneous mutation rate than wild-type cells (25). However, in addition to the *MGT1* deletion, the $\Delta mgt1::LEU2$ cassette used for gene replacement also disrupts an adjacent open reading frame (referred to as *ORF2*) which is transcribed as a 1.2-kb mRNA (24) and which could encode a 33.4-kDa protein with 287 amino acids (unpublished data). To distinguish which mutated gene is responsible for the spontaneous mutation rate increase, we determined whether expression of the *MGT1* gene (in the absence of *ORF2*) could suppress the increased spontaneous mutation rate in $\Delta mgt1::LEU2$ cells. Two high-copy YEpm plasmids were constructed, YEpmGT1-ORF2, containing both *MGT1* and *ORF2*, and YEpmGT1, containing only *MGT1*. The

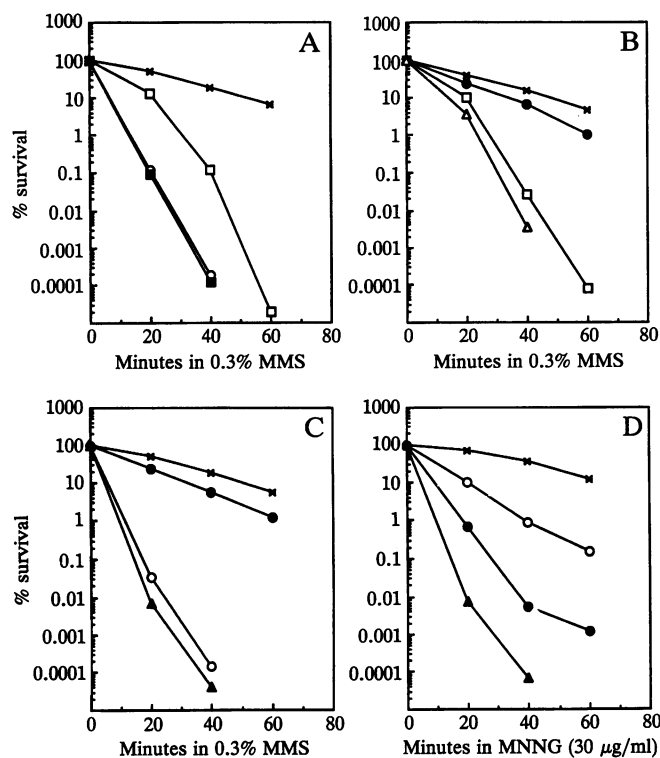


FIG. 1. Epistatic relationship of the *MGT1*, *MAG*, and *APN1* genes for MMS- and MNNG-induced killing. (A) MMS-induced killing of wild-type (\times), *mag* (\circ), *apn1* (\square), and *mag apn1* (\blacksquare) cells. (B) MMS-induced killing of wild-type (\times), *mgt1* (\bullet), *apn1* (\square), and *mgt1 apn1* (\triangle) cells. (C) MMS-induced killing of wild-type (\times), *mag* (\circ), *mgt1* (\bullet), and *mag mgt1* (\triangle) cells. (D) MNNG-induced killing of wild-type (\times), *mag* (\circ), *mgt1* (\bullet), and *mag mgt1* (\triangle) cells. Results were the average of at least two experiments and each showed similar pattern. \times , DBY747 (wild type); \circ , JC8901 (*mag*- $\Delta 1$::*URA3*); \bullet , WX1991 (*Δ mgt1*::*LEU2*); \square , WX9105 (*Δ apn1*::*HIS3*); \blacksquare , WX9106 (*mag*- $\Delta 1$::*URA3*, *Δ apn1*::*HIS3*); \triangle , WX9104 (*mag*- $\Delta 1$::*URA3*, *Δ mgt1*::*LEU2*); \triangle , WX9107 (*Δ mgt1*::*LEU2*, *Δ apn1*::*HIS3*).

Δ mgt1::*LEU2* strain containing either YEpMGT1-ORF2 or YEpMGT1 had 5- to 7-fold more MTase activity than wild-type DBY747 cells (Fig. 2A), and as expected both plasmids conferred increased MNNG resistance upon wild-type cells (Fig. 2B). Moreover, each plasmid reduced the spontaneous mutation rate in the *Δ mgt1*::*LEU2* strain to about the wild-type level (Fig. 3). We thus infer that the MTase deficiency, and not the ORF2 deficiency, in the *Δ mgt1*::*LEU2* strain was responsible for the observed increase in the rate of spontaneous mutation. These results indicate that, like bacteria, eukaryotic cells harbor endogenous compounds that produce DNA O-alkylation. Interestingly, increasing the *S. cerevisiae* MTase level above that seen in wild-type cells did not produce a lower-than-wild-type spontaneous mutation rate. Thus the wild-type level of 100–150 MTase molecules per cell (23) appears to be sufficient for the repair of the endogenously produced mutagenic O-alkyl lesions.

MAG DNA Glycosylase Deficiency Does Not Affect the Wild-Type *S. cerevisiae* Spontaneous Mutation Rate. Simple monofunctional alkylating agents alkylate many of the oxygens and nitrogens in DNA, but various agents produce different proportions of N-alkyl and O-alkyl lesions. All simple alkylating agents (e.g., MMS and MNNG) produce abundant N-alkylation, with 7MeG and 3MeA being the most common products (comprising about 65–80% and 10% of the total lesions, respectively); a subset of simple alkylating agents (e.g., MNNG) can also efficiently produce O-alkylation, with O⁶MeG being produced at about the same level as 3MeA (39, 40). We reasoned that an endogenous metabolite

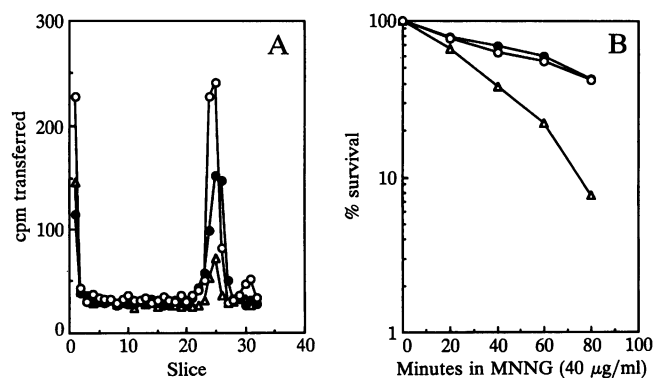


FIG. 2. Phenotypic analysis of WX1991 (*Δ mgt1*::*LEU2*) containing the YEpMGT1-ORF2 and YEpMGT1 plasmids. (A) Gel assay of MTase activity. MTase activity in WX1991/YEpMGT1-ORF2 (\bullet) and WX1991/YEpMGT1 (\circ) cells was compared with that in wild-type DBY747 cells (\triangle). Molecular mass of the MTase peaks was estimated at 23–25 kDa. (B) Cell killing induced by MNNG (40 μ g/ml) in the same strains (averaged from two experiments).

which produces DNA O-alkylation (as evidenced above) must also produce DNA N-alkylation. The *MAG* gene encodes a 3MeA DNA repair glycosylase known to remove both 3MeA and 7MeG from alkylated DNA (refs. 26, 27, and 30; unpublished data). 3MeA and 7MeG are not thought to cause base mispairing and are thus not themselves mutagenic (41). However, the abasic sites produced by the action of the *MAG* glycosylase on 3MeA and 7MeG are known to cause mutation, and this is thought to be because abasic sites preferentially pair with adenine during replication (8). We therefore predicted that a *MAG* glycosylase deficiency would actually decrease the rate of spontaneous mutation due to a putative decrease in the flux of spontaneous abasic sites in the yeast genome. However, whereas the *mgt1* mutation produced about a 3-fold spontaneous mutation rate increase, the *mag* mutation neither increased nor decreased the spontaneous mutation rate in either wild-type cells or *Δ mgt1*::*LEU2* MTase-deficient cells (Fig. 4).

MAG DNA Glycosylase Deficiency Partially Suppresses the Elevated Spontaneous Mutation Rate in *Δ apn1*::*HIS3* *S. cerevisiae*. The lack of an effect of the *MAG* glycosylase deficiency on spontaneous mutation rates could be explained by the efficient repair of AP sites preventing their contribution to spontaneous mutation; if AP sites do not normally contribute to spontaneous mutation because they are efficiently repaired, a decrease in their flux would not affect spontane-

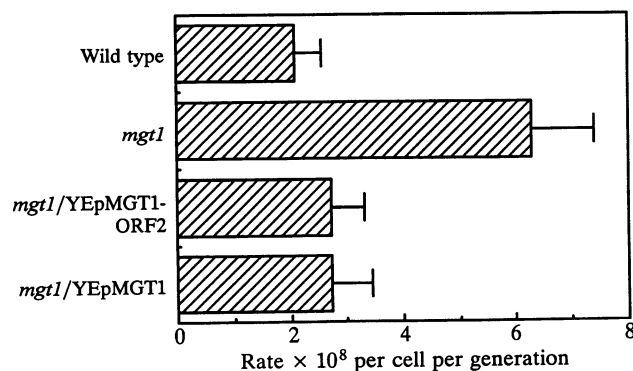


FIG. 3. Spontaneous mutation rates in strains expressing different levels of the *MGT1* MTase. Rate was measured for DBY747 (wild type), WX1991 (*Δ mgt1*::*LEU2*), WX1991/YEpMGT1-ORF2, and WX1991/YEpMGT1. Values are the average of four experiments in which all four strains were tested simultaneously, and error bars indicate standard deviation. The rate in WX1991 (*Δ mgt1*::*LEU2*) is significantly higher than the rate in the other strains at $P = 0.01$.

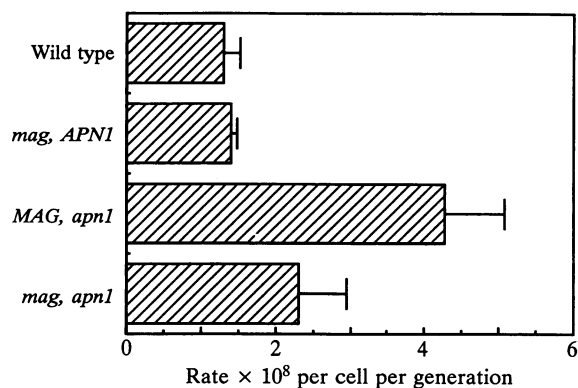


FIG. 4. Spontaneous mutation rates in strains expressing different levels of the MAG glycosylase and the MGT1 MTase. Rate was measured for DBY747 (wild type), WX1991 ($\Delta mgt1::LEU2$), JC8901 ($mag\text{-}\Delta 1::URA3$), and WX9104 ($mag\text{-}\Delta 1::URA3, \Delta mgt1::LEU2$). Values are the average of three experiments in which all four strains were tested simultaneously, and error bars indicate standard deviation. The rate in the two strains carrying the $\Delta mgt1::LEU2$ allele is significantly higher than that in the other strains at $P = 0.01$.

ous mutation rates. *apn1* mutants, which are almost totally deficient in type II AP endonuclease activity, suffer an elevated spontaneous mutation rate (32), presumably because unrepaired AP sites pair with adenine during replication (8). We reasoned that if glycosylase-mediated repair of spontaneous N-alkylation does indeed contribute to the spontaneous flux of AP sites, then a MAG DNA glycosylase deficiency in the $\Delta apn1::HIS3$ strain would decrease the spontaneous mutation rate. Fig. 5 clearly shows that at least half of the increase in the spontaneous mutation rate seen in $\Delta apn1::HIS3$ cells is suppressed in the absence of MAG DNA glycosylase activity. We infer that this decrease in the spontaneous mutation rate is due to 3MeA and 7MeG not being converted to mutagenic abasic sites by the MAG glycosylase. Moreover, we infer that about half of the spontaneous AP sites produced in these $\Delta apn1::HIS3$ cells may be derived from spontaneous DNA alkylation damage.

MAG Overexpression Dramatically Increases the Spontaneous Mutation Rate in APN1-Deficient Cells. Since the reduced repair of DNA N-alkylation damage lowered the spontaneous mutation rate in $\Delta apn1::HIS3$ cells, one would predict that increased N-alkylation repair might elevate the spontaneous

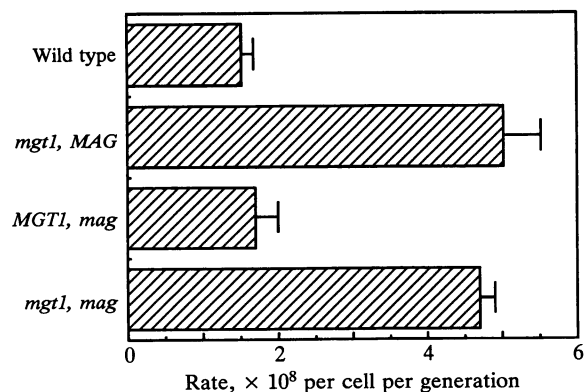


FIG. 5. Spontaneous mutation rates in strains with different levels of the MAG glycosylase and the APN1 AP endonuclease. Rate was measured for DBY747 (wild type), JC8901 ($mag\text{-}\Delta 1::URA3$), WX9105 ($\Delta apn1::HIS3$), and WX9106 ($mag\text{-}\Delta 1::URA3, \Delta apn1::HIS3$). Values are the average of three experiments in which all four strains were tested simultaneously, and error bars indicate standard deviation. The rate in WX9105 is significantly different from that in the other three strains at $P = 0.05$, and the rate in WX9106 is significantly different from DBY747 and JC8901 at $P = 0.1$.

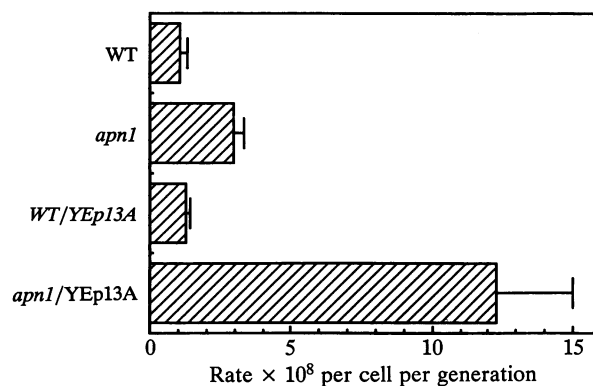


FIG. 6. Spontaneous mutation rates in cells expressing high levels of the MAG glycosylase. Rate was measured for DBY747 (wild type, WT), WX9105 ($\Delta apn1::HIS3$), DBY747/YEp13A, and WX9105/YEp13A. Values are the average of three experiments in which all four strains were tested simultaneously, and error bars indicate standard deviation. The rate in WX9105/YEp13A is significantly different from that of the three other strains at $P = 0.01$, and, as in Fig. 5, the rate in WX9105 is significantly different from that of wild-type cells at $P = 0.01$.

mutation rate in these cells. YEp13A is a multicopy plasmid carrying the *MAG* gene, and its presence produces a 10-fold increase in 3MeA DNA glycosylase levels in *S. cerevisiae* (26). We examined the spontaneous mutation rates in YEp13A transformants of wild-type and $\Delta apn1::HIS3$ cells, and the results were striking (Fig. 6). A 10-fold overexpression of the MAG glycosylase in $\Delta apn1::HIS3$ cells dramatically increased the spontaneous mutation rate to 12-fold more than the wild-type rate and 4-fold more than that in $\Delta apn1::HIS3$ cells. The overexpression of *MAG* in the $\Delta apn1::HIS3$ strain does not confer extra resistance to the lethal effects of alkylation in *apn1* (data not shown), and thus the appearance of extra mutants cannot be explained by a higher survival of cells destined to become mutant cells. The results in Figs. 5 and 6 support the notion that the *S. cerevisiae* genome harbors spontaneous DNA N-alkylation damage and suggest that wild-type glycosylase levels are not sufficient for the removal of all endogenously produced N-alkyl lesions. In contrast, *MAG* overexpression in wild-type cells had no effect on the spontaneous mutation rate, presumably because the resident APN1 can efficiently handle the increased flux of AP sites.

DISCUSSION

Spontaneous mutation rates must balance an organism's need for genetic stability and for evolutionary change. The rate of spontaneous genetic change is extremely low for most organisms (42, 43), and it is clear that enormous energy is invested to maintain those low rates. The final rate of spontaneous mutation reflects a balance between the induction of physical alterations in the genome and how well the cell repairs those alterations before they are fixed into permanent mutations; clearly, spontaneous mutation rates could be increased or decreased by changing either one of these parameters. Here we show that alterations in the rate of DNA alkylation repair can significantly change the rate of spontaneous mutation in eukaryotic cells.

It was reported many years ago that *S*-adenosylmethionine acts as a weak alkylating agent, under physiological conditions, to nonenzymatically methylate proteins and DNA (44–46). In addition, reactive methylating species have been postulated to arise *in vivo* from lipid peroxidation reactions (47) and from the endogenous nitrosation of amines (48, 49). Moreover, there have been numerous reports on the detection of naturally occurring 7MeG in the genomic DNA of various organisms (50–53). Taken together, these studies

suggested that the DNA of some organisms may suffer spontaneous alkylation damage which could contribute to spontaneous mutation. However, it was only recently shown that spontaneous DNA alkylation-induced mutation does indeed occur *in vivo* (13).

E. coli cells that are unable to repair the mutagenic O⁶MeG and O⁴MeT DNA lesions suffer an elevated spontaneous mutation rate (13). Moreover, the majority of these extra spontaneous mutations are G·C → A·T transitions (W. MacKay and L.S., unpublished work), which are known to result from replication past O⁶MeG lesions (3). One might imagine that prokaryotic genomes would be more prone to endogenous alkylation than eukaryotic genomes, because they are not protected from the cytoplasm by a nuclear membrane. However, our analysis of spontaneous mutation rates in *S. cerevisiae* DNA alkylation repair mutants demonstrates that the eukaryotic genome is also subject to significant spontaneous DNA alkylation. The particular reactive metabolites responsible for DNA alkylation are not known, nor is it clear exactly what type of alkyl group is transferred. However, it is clear that alkyl groups are transferred to both nitrogens and oxygens in DNA.

Overexpression of the MAG 3MeA DNA glycosylase increased spontaneous mutation in *apn1* AP endonuclease-deficient yeast cells, but not in wild-type cells. We interpret this to mean that all the MAG-induced abasic sites are quickly repaired in wild-type cells, but in *apn1* cells a significant number of these abasic sites escape repair and are replicated. Thus, the APN1 AP endonuclease in wild-type cells appears to be maintained at a level high enough to deal with an increased flux of abasic sites. However, it is unclear whether the APN1 levels adjust according to the number of spontaneous abasic sites arising in the genome, or whether this enzyme is always present at levels able to deal with excess abasic sites. The fact that MAG overexpression in an *apn1*⁻MAG⁺ background increases spontaneous mutation indicates that the wild-type level of the MAG DNA glycosylase is insufficient to release all the available spontaneously produced N-alkylated bases in the *S. cerevisiae* genome. Since MAG releases 3MeA much more efficiently than 7MeG (refs. 26 and 30; unpublished data), it is possible that wild-type levels of MAG are insufficient for the release of all the spontaneously produced 7MeG, but at 10 times the wild-type level, MAG might release significant quantities of this alkylated base.

Our experiments suggest that spontaneous DNA alkylation can act as a source of spontaneous mutation in at least two different ways: when 3MeA and 7MeG are efficiently removed to produce mutagenic abasic DNA sites which escape subsequent repair (as discussed above), and when O⁶MeG (and possibly O⁴MeT) mutagenic lesions are left unrepaired. One would therefore predict that (i) the extra spontaneous mutations in the MGT1 MTase-deficient yeast strain would be mainly G·C → A·T transitions produced by O⁶MeG pairing with thymine during replication and (ii) the extra mutations in the *apn1* strain overexpressing the MAG glycosylase would be mainly A·T → T·A and G·C → T·A transversions produced by the insertion of adenine opposite the abasic sites left by the repair of 3MeA and 7MeG.

We thank Bruce Demple for helpful discussion and plasmid pSCP19A, containing the $\Delta apn1::HIS3$ disruption cassette. We thank Dindial Ramotar for AP endonuclease assays. This work was supported by American Cancer Society Research Grant CN-71 and National Institutes of Health Grant CA55042. L.S. was supported by an American Cancer Society Faculty Research Award, and W.X. by a Markey Foundation Toxicology Postdoctoral Fellowship.

- Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J. & Vogelstein, B. (1990) *Mol. Cell. Biol.* **10**, 5772–5781.

2. Fearon, E. R. & Vogelstein, B. (1990) *Cell* **61**, 759–767.
3. Loeb, L. A. (1991) *Cancer Res.* **51**, 3075–3079.
4. Strauss, B. S. (1992) *Cancer Res.* **52**, 249–253.
5. Echols, H. & Goodman, M. F. (1991) *Annu. Rev. Biochem.* **60**, 477–511.
6. Modrich, P. (1991) *Annu. Rev. Genet.* **25**, 229–253.
7. Lindahl, T. & Nyberg, B. (1972) *Biochemistry* **11**, 3610–3618.
8. Loeb, L. A. & Preston, B. D. (1986) *Annu. Rev. Genet.* **20**, 201–230.
9. Miller, J. H. & Brooks Low, K. (1984) *Cell* **37**, 675–682.
10. Farr, S. B., D'Ari, R. & Touati, D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8268–8272.
11. Greenberg, J. T. & Demple, B. (1988) *EMBO J.* **7**, 2611–2617.
12. Maki, H. & Sekiguchi, M. (1992) *Nature (London)* **355**, 273–275.
13. Rebeck, G. W. & Samson, L. (1991) *J. Bacteriol.* **173**, 2068–2076.
14. Sargentini, N. J. & Smith, K. C. (1981) *Carcinogenesis* **2**, 863–872.
15. Storz, G., Christman, M. F., Sies, H. & Ames, B. N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8917–8921.
16. Cairns, J., Overbaugh, J. & Miller, S. (1988) *Nature (London)* **335**, 142–145.
17. Foster, P. (1992) *J. Bacteriol.* **174**, 1711–1716.
18. Smith, K. C. (1992) *Mutat. Res.* **277**, 139–162.
19. Cabrera, M., Nghiem, Y. & Miller, J. H. (1988) *J. Bacteriol.* **170**, 5405–5407.
20. Michaels, M. L., Cruz, C., Grollman, A. P. & Miller, J. H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7022–7025.
21. Michaels, M. L., Pham, L., Cruz, C. & Miller, J. H. (1988) *Nucleic Acids Res.* **19**, 3629–3632.
22. Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P. & Nishimura, S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4690–4694.
23. Sassanfar, M. & Samson, L. (1990) *J. Biol. Chem.* **265**, 20–25.
24. Xiao, W., Derfler, B., Chen, J. & Samson, L. (1991) *EMBO J.* **10**, 2179–2186.
25. Xiao, W. & Samson, L. (1992) *Nucleic Acids Res.* **20**, 3599–3606.
26. Chen, J., Derfler, B., Maskati, A. & Samson, L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7961–7965.
27. Chen, J., Derfler, B. & Samson, L. (1990) *EMBO J.* **9**, 4569–4575.
28. Chen, J. & Samson, L. (1991) *Nucleic Acids Res.* **23**, 6427–6432.
29. Sassanfar, M., Dosanjh, M., Essigmann, J. M. & Samson, L. (1991) *J. Biol. Chem.* **266**, 2767–2771.
30. Berdal, K. G., Bjoras, M., Bjelland, S. & Seeberg, E. (1990) *EMBO J.* **9**, 4563–4568.
31. Popoff, S. C., Spira, A. I., Johnson, A. W. & Demple, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4193–4197.
32. Ramotar, D., Popoff, S. C., Gralla, E. B. & Demple, B. (1991) *Mol. Cell. Biol.* **11**, 4537–4544.
33. Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202–211.
34. Sherman, F., Fink, G. R. & Hicks, E. B. (1983) *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
35. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168.
36. von Borstel, R. C. (1978) *Methods Cell Biol.* **20**, 1–24.
37. Gralla, E. B. & Valentine, J. S. (1991) *J. Bacteriol.* **173**, 5918–5920.
38. Milton, J. S. & Tsokos, J. O. (1983) *Statistical Methods in the Biological and Health Sciences* (McGraw-Hill, New York).
39. Beranek, D. T. (1990) *Mutat. Res.* **231**, 11–30.
40. Day, R., III, Babich, M. A., Yarosh, D. B. & Scudiero, D. A. (1987) *J. Cell Sci. Suppl.* **6**, 333–353.
41. Lindahl, T., Sedgwick, B., Sekiguchi, M. & Nakabeppu, Y. (1988) *Annu. Rev. Biochem.* **57**, 133–157.
42. Drake, J. W. (1969) *Nature (London)* **221**, 1132.
43. Drake, J. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7160–7164.
44. Barrows, L. R. & Magee, P. N. (1982) *Carcinogenesis* **3**, 349–351.
45. Paik, W. K., Lee, H. W. & Kim, S. (1975) *FEBS Lett.* **58**, 39–42.
46. Rydberg, B. & Lindahl, T. (1982) *EMBO J.* **2**, 211–216.
47. Vaca, C. E., Wilhelm, J. & Harms-Ringdahl, M. (1988) *Mutat. Res.* **195**, 137–149.
48. Calmels, S., Ohshima, H., Crespi, M., Leclerc, H., Cattoen, C. & Bartsch, H. (1987) in *The Relevance of N-nitroso Compounds to Human Cancer*, eds Bartsch, H., O'Neill, I. & Schulte-Hermann, R. (IARC Scientific, Lyon, France), pp. 391–395.
49. Tsimis, J. & Yarosh, D. B. (1990) *Environ. Mol. Mutat.* **15**, 69–70.
50. Park, J.-W. & Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7467–7470.
51. Park, J.-W. & Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9508.
52. Tan, B. H., Bencsath, A. & Gaubatz, J. W. (1990) *Mutat. Res.* **237**, 229–238.
53. Shukar, D. E. G. & Farmer, P. B. (1992) *Chem. Res. Toxicol.* **5**, 450–460.