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

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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/apyrimidinic (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-methylguanine 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) ura- 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 O6-methylthymine (O6MeT) 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/apyrimidinic (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/apyrimidinic; MMS, methyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; Mtase, methyltransferase; 3MeA, 3-methyladenine; 7MeG, 7-methylguanine; O6MeG, O6-methylguanine; O6MeT, O6-methylthymine.
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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-D1, leu2-3,112, ura3-52, trpl-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-Δ::URA3 cassette (27). WX9191 and WX9104 were made by disrupting genomic MGT1 in DBY747 and JC8901, respectively, by using the Δmgt1::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, WX9191, and WX9104, respectively, by replacement with the Δapnl::HIS3 cassette (32). The phenotype of each strain was confirmed by MTase, 3MeA DNA glycosylase, or AP endonuclease assays and by its alkylating-sensitivities 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, Pgal::TryC1) was purchased from Invitrogen (San Diego). The 2.65-kb Spe I–HincII fragment from plasmid pMT1-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 GALI 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 YEpl3 (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 (Δmgt1::LEU2, mag-Δ::URA3, Δapnl::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 × 10^6 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.

#### Limiting-Enzyme 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 × 10^5 cells per ml to about 0.5–0.7 × 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 apnl Null Mutants

To determine the relative roles of the MGT1, MAG, and APN1 genes in protecting cells against alkylating damage and in limiting spontaneous mutations, we constructed the set of isogenic DNA alkylation repair-deficient mutants described in Materials and Methods. Three nonrevertible DBY747 auxotroph mutations (ura3-52, leu2-3,112, and his3-D1) were exploited to create disruptions in the MAG 3MeA DNA glycosylase gene (mag-Δ::URA3), the MGT1 O-3MeG DNA MTase gene (Δmgt1::LEU2), and the APN1 AP endonuclease gene (Δapnl::HIS3). 3MeA DNA glycosylase activity in the mag-Δ::URA3 strain was about 10% of the wild-type level, as described previously (27); O-3MeG DNA MTase activity in the Δmgt1::LEU2 strain was undetectable (24); and class II AP endonuclease activities in the Δapnl::HIS3 disruption strain was <25% 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 alkylating sensitivities of the mag, mgt1, and apnl 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. 1A–C show the sensitivity of each strain to MMS; the mag-Δ::URA3 allele conferred the greatest MMS sensitivity, Δmgt1::HIS3 intermediate sensitivity, and Δapnl::LEU2 only slight sensitivity. As expected, the sensitivity of the mag-Δ::URA3 Δapnl::HIS3 double mutant did not exceed that of the mag-Δ::URA3 single mutant, indicating that MAG and APN1 are epistatic (Fig. 1A). In contrast, the MMS sensitivities of the Δmgt1::LEU2 Δapnl::HIS3 and the Δapnl::LEU2 mag-Δ::URA3 double mutant strains represents the sum of the sensitivities of the corresponding single mutant strains (Fig. 1B and C), indicating that MGT1 is epistatically distinct from MAG and APN1. Even though the small increase in MMS sensitivity conferred by the Δmgt1::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 alkylating 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 Δmgt1::LEU2 *S. cerevisiae* strain exhibits a higher spontaneous mutation rate than wild-type cells (25). However, in addition to the MGT1 deletion, the Δmgt1::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 Δmgt1::LEU2 cells. Two high-copy *YEp* 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 MNN9-induced killing. (A) MMS-induced killing of wild-type (×), mag (○), apnl (□), and mag apnl (△) cells. (B) MMS-induced killing of wild-type (×), mgtl (●), apnl (△), and mgtl apnl (□) cells. (C) MMS-induced killing of wild-type (×), mag (○), mgtl (●), and mag mgtl (△) cells. (D) MNN9-induced killing of wild-type (×), mag (○), mgtl (●), and mag mgtl (△) cells. Results were the average of at least two experiments and each showed similar pattern. ×, DBY747 (wild type); ○, JC8901 (mag-AJ::URA3); ●, WX1991 (Δmgtl::LEU2); □, WX9105 (Δapnl::HIS3); △, WX9110 (mgtl::URA3, Δapnl::HIS3); ○, WX9114 (mgtl::URA3, Δmgtl::LEU2); □, WX9107 (Δmgtl::LEU2, Δapnl::HIS3).

Δmgtl::LEU2 strain containing either YEpMGT1-ORF2 or YEpMG T1 had 5- to 7-fold more MTase activity than wild-type DBY747 cells (Fig. 2A), and as expected both plasmids conferred increased MNN9 resistance upon wild-type cells (Fig. 2B). Moreover, each plasmid reduced the spontaneous mutation rate in the Δmgtl::LEU2 strain to wild-type cells (Fig. 2C). We thus infer that the MTase deficiency, and not the ORF2 deficiency, in the Δmgtl::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 oxygen and nitrogenous bases in DNA, but various agents produce different proportions of Nalkyl and O-alkyl lesions. All simple alkylating agents (e.g., MMS and MNN9) 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., MNN9) can also efficiently produce O-alkylation, with OMeG being produced at about the same level as 3MeA (39, 40). We reasoned that an endogenous metabolite 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 mgtl 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 Δmgtl::LEU2 MTase-deficient cells (Fig. 4).

MAG DNA Glycosylase Deficiency Partially Suppresses the Elevated Spontaneous Mutation Rate in Δapnl::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 spontaneous...

Fig. 2. Phenotypic analysis of WX1991 (Δmgtl::LEU2) containing the YEpMGT1-ORF2 and YEpMGT1 plasmids. (A) Gel assay of MTase activity. MTase activity in WX1991/YEpMGT1-ORF2 (●) and WX1991/YEpMGT1 (○) cells was compared with that in wildtype DBY747 cells (△). Molecular mass of the MTase peaks was estimated at 23–25 kDa. (B) Cell killing induced by MNN9 (40 μg/ml) in the same strains (averaged from two experiments).

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ous mutation rates. \textit{apnl} 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 \textit{Δapnl::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 \textit{Δapnl::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 \textit{Δapnl::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 \textit{Δapnl::HIS3} cells, one would predict that increased N-alkylation repair might elevate the spontaneous 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 \textit{S. cerevisiae} (26). We examined the spontaneous mutation rates in YEp13A transformants of wild-type and \textit{Δapnl::HIS3} cells, and the results were striking (Fig. 6). A 10-fold overexpression of the MAG glycosylase in \textit{Δapnl::HIS3} cells dramatically increased the spontaneous mutation rate to 12-fold more than the wild-type rate and 4-fold more than that in \textit{Δapnl::HIS3} cells. The overexpression of MAG in the \textit{Δapnl::HIS3} strain does not confer extra resistance to the lethal effects of alkylation in \textit{apnl} (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 \textit{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 nitration 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 O^6-MeG and O^4-MeT 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 O^6-MeG 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 this is true for all alkyl groups are transferred to both nitrogens and oxygens in DNA.

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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 O^6-MeG (and possibly O^4-MeT) 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 O^6-MeG 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.

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