ABSTRACT

DNA methylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) are potent carcinogens; their carcinogenic effect is mainly due to the effect of production of O6-methylguanine (O6 MeG) on DNA. O6 MeG is not only mutagenic but also toxic to the cell because Mer−/Mex− cells unable to remove O6 MeG are very sensitive to killing by MNNG. It has been proposed that repeated futile mismatch correction of O6 MeG-containing bp is responsible for the genotoxicity of the O6 MeG lesion and that loss of mismatch repair activity results in cellular tolerance to O6 MeG, but the hypothesis has not been proved. We used yeast as a model to test this hypothesis and found that chromosome deletion of any known nuclear mitotic mismatch repair genes, including MLH1, MSH2, MSH3, MSH6, and PMS1, did not rescue mgt1Δ O6 MeG DNA repair methyltransferase-deficient cells from killing by MNNG. A large number of mgt1Δ, MNNG-tolerant revertants were isolated, among which one cell line, XS-14, has been found to carry a mutated allele of the MSH5 gene. The mutation also affected spore survival but did not increase the spontaneous mutation rate. We further demonstrated that a mutated form of the MSH5 gene, msh5Δ, not the msh5Δ-null mutation, is responsible for the cellular tolerance to MNNG in XS-14 cells. This observation offers an alternative model that may reconcile seemingly contradictory observations of yeast and mammalian cells.

INTRODUCTION

The genome of the cell is dynamic and reacts readily with physical and chemical agents, resulting in modifications to its molecular structure. DNA alkylating agents are present as one of the largest classes of environmental chemical carcinogens; some of the alkylating agents are also produced endogenously as a consequence of cellular metabolic processes. Challenging cells with S1-type methylating agents such as MNNG3 and N-methyl-N-nitroso-N-nitrosoguanidine mutagenic and carcinogenic lesions like O6 MeG and (to a lesser extent) O4 MeT, which, if not corrected by the DNA repair MTases, pair with thymine and guanine, respectively, resulting in transition mutagenesis (1, 2) and carcinogenesis in mammals (3).

In the absence of MTase, bacterial, yeast, and mammalian cells become extremely sensitive not only to mutagenesis but also to killing by MNNG, which cannot be explained simply on the basis of induced mutation rates (3–5). It is thus evident that persistence of O6 MeG and possibly O4 MeT lesions is genotoxic, although the mechanism of cell death is not yet clear. The MMR system has been implicated in the processing of O6 MeG-containing bp into lethal lesions, because recent studies with Mer−/Mex− mammalian cells lacking MTase activity (6, 7) suggest that alklation toxicity is accompanied by loss of normal MMR activities. This correlation was predicted by the abortive MMR hypothesis (8), which suggested that repeated futile attempts to correct mispairs during replication of a damaged template results in the accumulation of single-stranded nicks in DNA, which are ultimately lethal to the cell. This hypothesis is attractive not only because it provides an alternative to O6 MeG/O4 MeT genotoxicity but also because genetic defects in several human MMR genes have been linked to hereditary nonpolyposis colon cancer as well as other types of cancers (9–14). However, the abortive MMR hypothesis should be viewed with caution because the current supporting evidence with mammalian cells is not conclusive and a convenient mammalian system is not available to vigorously test the hypothesis.

The MMR system consisting of Escherichia coli MutS and MutL homologues has been extensively studied recently in yeast and human cells (reviewed in Refs. 14 and 15). Saccharomyces cerevisiae contains six MutS homologues (Msh) and three MutL homologues. Msh1 is responsible for mitochondrial DNA repair (16). Msh2, Mlh1, and Pms1 are required for the correction of various mismatches because mutation in any of the three genes results in a markedly enhanced spontaneous mutation rate or instability of simple repeats, which is not further increased in the double mutants (16–18). In contrast, Msh3 and Msh6 appear to form alternative pathways to correct 2–4-bp insertions and deletions or 1-bp mismatches, respectively (19, 20).

Msh4 (21) and Msh5 (22) have been shown to facilitate meiotic reciprocal recombination but are believed to play no role in the actual process of mismatch correction.

The abortive MMR hypothesis has been tested in yeast, which is relevant to mammalian systems in that all MTase and MMR proteins are highly conserved between the two. It was found that none of the msh2Δ, msh3Δ, msh5Δ, or pms1Δ-null mutations could rescue mgt1Δ strains from MNNG-induced killing and that MMR defects were not required for the selection of MNNG-tolerant clones (23). The conflict between the observations of yeast and human cells suggests that either the abortive MMR hypothesis is not universal or O6 MeG genotoxicity is the result of recognition/repair of O6 MeG by a mechanism other than MTase that interferes with DNA replication and results in cell death. The former possibility seems unlikely, because in the absence of MTase the lesion is equally toxic in all organisms studied to date (3–5, 24). It also remains possible that O6 MeG toxicity is due to the involvement of yet to be identified MutHLS homologues or to the specific mutations of known MutHLS homologous genes.

We report here an attempt to further investigate the mechanism of alkylation toxicity and its relationship to MMR. We have isolated and characterized a large number of MTase-deficient, MNNG-tolerant cell lines; one of the extensively studied mutants appears to carry a mutation in the MSH5 gene that is associated with the MNNG resistant phenotype.

MATERIALS AND METHODS

Plasmids and Yeast Transformation. Plasmid pWX1149 carries the MGT1 gene in a single-copy YCP vector. YCP-MLH1 (17) was from Dr. M. Liskay (Oregon Health Sciences University, Portland, OR), pll-2 (YCP-MSH2; Ref. 25) was obtained from Dr. R. Kodol (Harvard Medical School, Cambridge, MA), pWBK3-PMS1 (YCP-PMS1; Ref. 26) from Dr. W. Kramer (Georg-August University, Göttingen, Germany), YCP-MSH3 (27) and YCP-MSH6 from Dr. O. Crouse (Emory University, Atlanta, GA) and YCP-MSH4 (21) was from Dr. S. Roeder (Yale University, New Haven, CT). Plasmid pNH189–2 (22) was a gift from Dr. N. Hollingsworth (State University of New York, Stony Brook, NY). A 4.5-kb BamHI/HindIII fragment containing
MSH5 MUTATION AND ALKYLATION TOLERANCE

Table I S. cerevisiae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WX97102</td>
<td>MATa ura3-52 trpl-Δ35 leu2-Δ1 mig1Δ::LEU2 GAL4</td>
<td>Ref. 54</td>
</tr>
<tr>
<td>XS-803-2C</td>
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<td>D. Gietz</td>
</tr>
<tr>
<td>XS-803-3A</td>
<td>MATa leu2-3,112 ura3-52 his1-2 trp1-1 20 mm</td>
<td>D. Gietz</td>
</tr>
<tr>
<td>2Cmg1Δ</td>
<td>Like XS-803-2C but with mg1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>3Amg1Δ</td>
<td>Like XS-803-3A but with mg1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>2Cmg1Δmsh5Δ</td>
<td>Like XS-803-2C but with msh5::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>3Amsh5</td>
<td>Like XS-803-3A but with msh5::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>3AmsΔ</td>
<td>Like XS-803-3A but with msΔ::ura3</td>
<td>This study</td>
</tr>
<tr>
<td>2CmsΔΔ</td>
<td>Like XS-803-2C but with msΔ::URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>2Cmg1ΔmshΔΔ</td>
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<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>XS-14</td>
<td>MNNG-resistant mutant of 2Cmg1Δ::LEU2</td>
<td>This study</td>
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</table>

XS-803-2C but with mshΔ::URA3 This study
XS-803-2C but with mshΔ::hisG-URA3-hisG This study
XS-803-2C but with mshΔ::hisG-URA3-hisG This study
XS-803-2C but with mshΔ::hisG-URA3-hisG This study
XS-803-2C but with mshΔ::hisG-URA3-hisG This study
XS-803-2C but with mshΔ::hisG-URA3-hisG This study

The entire MSH5 gene was cloned into plasmids YCplac33 and Yplac211 (28) to form YCP-MSH5 and Ypl-MSH5, respectively. Yeast transformation was as described previously (23). Plasmid pmg1Δ::LEU2 (5) was used to create mgt1Δ strains. pmsh6Δ::hisG-URA3-hisG was constructed by removing a 0.84-kb Clal-EcoRV fragment from the MSH5 coding region and inserting a 3.8-kb BamHI-BglII hisG-URA3-hisG fragment from pNKY51 (29) at the unique BglII site.

Strains and Cell Culture. The S. cerevisiae strains used in this study are listed in Table 1. All mg1Δ::LEU2 mutants were created as described (5). The mshΔ mutants were obtained by Spht + EcoRI digestion of plasmid pmshΔ::hisG-URA3-hisG, constructed by Dr. W. Kramer. Two mshΔ disruption alleles were created. The MSH5 gene was interrupted in mshΔ::URA3 at the unique BglII site in pWH190 as described (22). The mshΔ5A mutants were obtained by HpaI + EcoRI digestion of pmshΔ5::hisG-URA3-hisG. Cells containing the mshΔ5::hisG-URA3-hisG allele were cultured on 5-fluorocytosinic acid plates (30) to select for ura3 deletions (mshΔ5::hisG). All of the targeted gene disruption mutants were created by enzyme digestion of the plasmid carrying the disruption cassette prior to yeast transformation, and the genomic structure of the deletion mutants was confirmed by Southern hybridization. Culture media used in this study were YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) supplemented with amino acids and bases as recommended (31). To make plates, 2% Bacto-agar was added. All incubations were carried out at 30°C.

Mutant Isolation and Cell Killing. MNNG was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solution of 1 mg/ml was made in 100 mM acetate buffer, pH 5.0, aliquoted, frozen, and thawed only once. For both mutant isolation and cell killing, the yeast cells were cultured overnight in 2 ml of YPD at 30°C. Five ml of YPD were inoculated with 200 μl of the overnight culture and allowed to grow for 4—6 h to reach a cell density of 2 · 10^8 cells/ml. For mutant isolation, the cells were treated with 1 μg/ml MNNG for 20 min, washed, resuspended, and plated on SD plates containing 2 μg/ml MNNG. Cell survival was monitored by plating an appropriate dilution of the treated cells on SD medium into a tilted, square Petri dish. After solidification, the plate was printed across the gradient using microscope slides, and the plates were incubated for 3 days. The length of growth across the gradient was measured, and relative growth was expressed as a percentage of full-length growth on the plate without MNNG.

Spontaneous Mutagenesis Assay. Overnight cultures of XS-803-2C and its derivatives were inoculated into five tubes containing 10 ml of YPD each, to a final concentration of 20 cells/ml. The tubes were incubated at 30°C until a concentration of 2 · 10^8 cells/ml was reached. The cells were harvested, washed, resuspended, and plated on SD medium lacking threonine and methionine to score Hom+ revertants from hom3—10 mutants and on YPD for total cell survival.

Quantitation of Cellular Glutathione. Crude yeast cell extract was prepared as described previously (32). Glutathione content was estimated by the method of Hissin and Hinf (33).

RESULTS

The msh6Δ Mutant Does Not Rescue mg1Δ from Killing by MNNG. Our previous results had shown that deletion of any of the then known MMR genes (i.e., PMS1, MLH1, MSH2, and MSH3) could not rescue the mg1Δ mutant from MNNG-induced killing. The MSH6 gene has since been isolated from both humans (34, 35) and yeast (19, 20). This gene is particularly interesting because hMS6 was characterized as a G:T mismatch binding protein, GTBP (34), and a similar activity has been shown to recognize O6 MeG and O4 MeT-containing mismatches (36). Furthermore, a mutation in each hMSH6 allele was reported in the MNNG-tolerant cell line MT1 (37), suggesting that Msh6 may be involved in O6 MeG toxicity. To test this hypothesis in yeast, Msh6 was deleted in a MTase-deficient background. We observed that the msh6Δ mutant behaves like other MMR mutants in that the msh6Δ single mutant did not confer MNNG sensitivity, and loss of Msh6 function did not result in tolerance of the mg1Δ cells to MNNG (data not shown).

Isolation and Characterization of MTase-deficient, MNNG-tolerant Mutants. Because none of the MMR mutants resulted in tolerance to MNNG, we decided to isolate MNNG-tolerant mutants from mg1Δ cells. Strains XS-803-2C and XS-803-3A were chosen for this study due to their ability to sporulate well. The 2Cmg1Δ strain was used for the isolation of MNNG-resistant clones, 55 of which retained their resistant phenotype upon repeated subculturing. Crossing each of these mutants with the 3Amgt1Δ strain and comparing MNNG resistance of the diploids with control crosses (XS-803-2C X 3Amgt1Δ and 2Cmg1Δ X 3Amgt1Δ) identified 10 recessive, 2 codominant, and 43 dominant mutants. We were unable to use the recessive resistant strains described above for complementation analysis as planned, because although the diploids of some representative mutants with 3Amgt1Δ of opposite mating type had high level of sporulation (>80%), spore survival was extremely low.

Because alklylation tolerance was thought to be a result of faulty MMR processes, some of the recessive resistant mutants were subjected to a spontaneous mutagenesis assay. The assay, which scored reversion of hom3—10 auxotrophy, was performed to detect the mutator phenotype characteristic of pms1, mlh1, or msh2 MMR mutants (16, 17, 26). As shown in Table 2, the mg1Δ strain shows a 4-fold increase in spontaneous mutation rate over the wild-type level, whereas the mlh1Δ mutant increases the mutation rate by about 1000-fold. Of the four MNNG recessive resistant strains tested, only...
Table 2 Spontaneous mutation rates of S. cerevisiae strains

All the strains are derivatives of XS-803-2C carrying the hom3-10 mutation. The spontaneous mutation rate was calculated according to the method of Williamson et al. (55). The results were an average of three sets of experiments except for XS-64 and XS-53, which were from one experiment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Key alleles</th>
<th>Mutation rate (× 10⁻⁶)</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>XS-803-2C</td>
<td>Wild-type</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>2Cmgt1Δ</td>
<td>mgt1Δ</td>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>2Cmgt1Δmsh1Δ</td>
<td>mgt1Δ, msh1Δ</td>
<td>430.0</td>
<td>1078</td>
</tr>
<tr>
<td>XS-23</td>
<td>mgt1Δ, MNNGΔ</td>
<td>940.0</td>
<td>235</td>
</tr>
<tr>
<td>XS-64</td>
<td>mgt1Δ, MNNGΔ</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>XS-53</td>
<td>mgt1Δ, MNNGΔ</td>
<td>3.3</td>
<td>8.3</td>
</tr>
<tr>
<td>XS-14</td>
<td>mgt1Δ, MNNGΔ</td>
<td>2.1</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Fig. 1. Relative resistance of XS-803-2C and its derivatives to 10 μg/ml MNNG on a gradient plate. The gradient plates were incubated at 30°C for 72 h. Each experiment was repeated with at least three independent transformants, and a typical result is presented. Transformernts with single-copy plasmids containing different MMR genes are shown.

XS-23 exhibited a mutator phenotype. However, transformation of XS-23 with any of the known nuclear MMR genes (MSH2, MSH3, MSH6, MLH1, and PMS1) did not significantly alter its MNNG-resistant phenotype (data not shown).

Phenotype of XS-14. The XS-14 recessive resistant strain was selected for detailed study because it was the most MNNG resistant among the recessive mutants isolated and it did not exhibit the mutator phenotype. As a matter of fact, XS-14 was more resistant to killing by MNNG than its wild-type strain (Figs. 1 and 3). It also exhibited a slow-growth phenotype with a doubling time of 3.83 h, compared to 1.99 h for the parental strain 2Cmgt1Δ. It has been shown that the alkylating activity of MNNG is potentiated in the presence of thiols; thus, reduced intracellular levels of acid-soluble thiols, such as glutathione, may also be responsible for enhancing the cellular resistance to MNNG (38). To determine whether the resistance of XS-14 is a consequence of the above mechanism, intracellular glutathione content of XS-14 was measured and found to be comparable to that of the wild-type and mgt1-deleted strain from which it was isolated (data not shown). Thus, the alkylate tolerance of XS-14 is not due to altered MNNG metabolism. Furthermore, transformation of XS-14 with a wild-type copy of the MGT1 gene enhanced its resistance to MNNG, indicating that the mutated gene in XS-14 and MGT1 belong to different epistatic groups. Hence the XS-14 mutation confers resistance by a pathway distinct from MTag.

The XS-14 mutant was crossed with the 3Amgt1Δ strain of opposite mating type to conduct genetic analysis. The XS-14/3Amgt1Δ diploid sporulated well, forming a high percentage of four-spore tetrads. Upon dissection, however, the majority of spores were inviable. A series of diploids were created in various mutation backgrounds to determine whether reduced spore survival was due to strain background, deletion of MGT1, or the mutation in XS-14. Table 3 demonstrates that reduced spore survival accompanies XS-14. Thus, in addition to MNNG resistance, XS-14 also acquires a defect in meiosis.

A Mutant Allele of MSH5 in XS-14, msh5-14, Is Responsible for MNNG Tolerance. Because mutations in most MMR genes indeed affect meiotic phenotype (15), we reasoned that if XS-14 contains a mutation in one of these MMR genes, transforming XS-14 with one of the known MMR genes will make XS-14 sensitive to killing by DNA alkylating agents. As shown in Fig. 1, introduction of a single copy of the wild-type PMS1, MLH1, MSH2, MSH3, or MSH6 gene into XS-14 had no effect on its resistance. To our surprise, MSH4 partially reduced the MNNG tolerance, but maximum functional complementation was conferred by transformation of the MSH5 gene. The presence of a single copy MSH5 in the XS-14 strain significantly reduced its alkylaion tolerance compared to the untransformed strain. This result was confirmed by repeated nonselective culturing of XS-14/YCp-MSH5 for plasmid loss, which was accompanied by XS-14 regaining its resistant phenotype.

We created the msh5-null mutation in mgt1Δ strains and did not observe an increased MNNG resistance (Fig. 2, Lanes 2–4). Thus, it was important to determine whether the phenotype of XS-14 was due to a mutation within the MSH5 structural gene or to a secondary (suppressor) mutation. We designed the following experiments to address this question.

First, it was known that the wild-type strain and the MSH5 gene were able to suppress MNNG tolerance in XS-14. If the XS-14 mutation was within the MSH5 gene, one would expect a lack of complementation by the msh5-null mutant. Indeed, the mgt1Δ single mutant was able to complement the mutation in XS-14, whereas the

Table 3 Spore viability of control and XS-14 crosses

<table>
<thead>
<tr>
<th>Diploid</th>
<th>Viable spores/total spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>XS-803-2C × XS-803-3A</td>
<td>20/20</td>
</tr>
<tr>
<td>2Cmgt1Δ × XS-803-3A</td>
<td>20/20</td>
</tr>
<tr>
<td>2Cmgt1Δ × 3Amgt1Δ</td>
<td>20/20</td>
</tr>
<tr>
<td>XS-803-2C × WXY9102</td>
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</tr>
<tr>
<td>2Cmgt1Δ × WXY9102</td>
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</tr>
<tr>
<td>XS-14 × 3Amgt1Δ</td>
<td>0/40</td>
</tr>
<tr>
<td>XS-14 × WXY9102</td>
<td>1/40</td>
</tr>
</tbody>
</table>

Fig. 2. Relative resistance of different haploid and diploid strains to 10 μg/ml MNNG on a gradient plate. The gradient plates were incubated at 30°C for 72 h. Strain 1, XS-803-2C; strain 2, 2Cmgt1Δ; strain 3, 2Cmgt1Δmsh5Δ; strain 4, 3Amgt1Δmsh5; strain 5, 2Cmgt1Δ × 3Amgt1Δ; strain 6, 2Cmgt1Δ × 3Amgt1Δmsh5; strain 7, 2Cmgt1Δmsh5 × 3Amgt1Δmsh5; strain 8, XS-14 × 3Amgt1Δ; strain 9, XS-14 × 3Amgt1Δmsh5. Each strain was tested with three individual colonies. The XS-14 × 3Amgt1Δ strain appears to be slightly less resistant than other mgt1Δ diploids (Strains 5–7) on the gradient plate, probably due to its slow growth observed with the XS-14 haploid.
mgt1Δmsh5Δ double mutant failed to do so. As shown in Fig. 2, the relative sensitivity of diploids homozygous for mgt1Δ was the same regardless of the number (0, 1, or 2) of the MSH5 gene copies (Lanes 5–7). Although the diploid MSH5/SX-14 was sensitive to MNNG (Lane 8), the corresponding diploid msh5/SX-14 (Lane 9) was clearly more resistant than other diploids studied. These results indicate that the phenotype of the diploid is a manifestation of a mutation in the MSH5 structural gene.

Second, if the alkylation resistance in XS-14 is due to a mutation within the MSH5 gene and disruption of the MSH5 gene does not show the same phenotype, we would expect that disruption of the MSH5 gene in XS-14 results in loss of its MNNG resistant phenotype. Indeed, disruption of the MSH5 gene from XS-14 converted it into an MNNG sensitive strain indistinguishable from the 2Cmgt1Δ strain from which it was isolated (data not shown).

Finally, we reasoned that if the msh5—14 allele from XS-14 carries the mutation responsible for the resistant phenotype, it would also confer MNNG tolerance in an msh5 deletion strain. We designed experiments using the strategy of targeted integration and position cloning to isolate the msh5—14 allele from the XS-14 mutant. As depicted in Fig. 3A, YIp lac211 plasmid carrying the wild-type copy of the MSH5 gene was integrated at the HpaI site upstream of the genomic msh5—14 allele. Digestion of the genomic DNA isolated from the above integrant with HindIII resulted in a linear molecule containing the YIp lac211 vector and the msh5—14 allele, which was recovered by self-ligation and transformation into E. coli cells. The resulting plasmid, Ylp-msh5—14, was again allowed to integrate into the 2Cmgt1Δmsh5Δ strain by targeted integration at the SnBl site downstream of the msh5Δ::hisG allele, resulting in Ylp lac211 being flanked by msh5Δ::hisG and msh5—14 at the MSH5 locus (Fig. 3B). This chromosome structure was confirmed by Southern hybridization, and independent integrants were used for phenotypic analysis. As expected, the 2Cmgt1Δ and 2Cmgt1Δmsh5Δ strains were equally sensitive to MNNG-induced killing. However, upon integration of the msh5—14 allele, the 2Cmgt1Δmsh5Δ strain exhibited an alkylation-tolerant phenotype indistinguishable from the original XS-14 mutant (Fig. 3C).

All stable recessive MNNG-tolerant revertants (including those listed in Table 2) were transformed with plasmid YCp-MSH5 to determine whether other independent msh5 mutations result in a phenotype similar to XS-14. The MSH5 gene was unable to suppress MNNG-tolerance in all of the strains examined, nor were other known MMR genes (data not shown).

DISCUSSION

The lesion O6 MeG is primarily responsible for the mutagenic and lethal effects of the Sω1-type methylating carcinogens (3). Hence, any hypothesis attempting to explain the genotoxicity of O6 MeG is also relevant to understanding the mechanism of spontaneous and environmental carcinogenesis. The relationship between alkylation tolerance and MMR as proposed by the abortive MMR hypothesis (6–8) is of great interest. First, a significant percentage (20–30%) of naturally occurring human tumor cell lines and 60% of SV40-transformed cell lines are deficient in MTase activity (MerIMex) and exhibit an increased sensitivity to killing by methylating agents that produce O6 MeG residues (3, 39). Thus, loss of MTase activity can be a hallmark of tumor development. Second, because eukaryotic cells undergo a significant amount of endogenous alkylation damage (40, 41), impaired MMR would make these cells tolerant of endogenous damage, thereby conferring a growth advantage, and in turn enhancing spontaneous mutagenesis and carcinogenesis. However, the abortive mismatch hypothesis is not conclusive for several reasons. First, the Mer'/Mex− phenotype of the mammalian cell lines used for establishing support of this hypothesis is due to transcriptional down-regulation of, rather than mutations within, the hMGMT gene (42). Actually, the Mex+/Mex− states in a human lymphoblastoid cell line were found to be interconvertible (43). Second, because alkylation tolerance is achieved only after several rounds of selection under mutagenic conditions (8), the phenotype is probably the manifestation of more than one mutation. Third, these cell lines were derived from tumors and may carry some preexisting mutations that could contribute to their cellular sensitivity to or tolerance of DNA alkylating agents. Indeed, one of the tolerant mutants (7) was readily revertible.
to an alkylaton-sensitive phenotype. Finally, Koi et al. (44) reported that the alkylaton-tolerant human colon cancer cell line HCT116, which is homozygous for the hMLH1 mutation, was rendered sensitive by transformation with human chromosome 3 carrying a wild-type copy of the MLH1 gene. De Wind et al. (45) reported that transgenic mice defective in the hMSH2 gene become more resistant to MNNG when endogenous MTase is depleted. In both experiments, however, cells were MTase proficient, and the altered level of alkylation sensitivity/resistance after genetic manipulation differs by orders of magnitude from those previously reported (7, 8).

We have previously demonstrated that null mutation of known MMR genes MLH1, MSH2, MSH3, and PMS1 did not rescue yeast MTase-deficient cells from killing by DNA methylating agents (23). In this study, we showed that null mutation of two other MSH genes, MSH5 and MSH6, also did not confer a MNNG-tolerant phenotype. It is thus concluded that simple loss of MMR activity in yeast is not associated with loss of O° MeG/O° MeT genotoxicity. However, we found that a recessive mutation within the MSH5 gene, but not the msh5Δ-null mutation, is able to make mgt1Δ cells tolerant of MNNG. The Xs-14 cells carrying the msh5—14 mutation also display a meiotic defect but not a mutator phenotype. This phenomenon is reminiscent of alkylaton-tolerant clones isolated from HeLa cells (46), which also appear to fall into two groups: one group with a mutator phenotype and another that does not display an increased spontaneous mutation rate. Furthermore, it appears that although DNA repair MTase and MMR both recognize DNA methyl adducts such as O° MeG and O° MeT, they also have different specificities to other types of DNA adducts. A recent study (47) found that although MTase activity correlated with cellular resistance to both the methylation agent 5,6-bis(2-chloroethyl)-1-nitrosoarene, the alkylaton-tolerant MMR-defective cell lines did not confer resistance to 1,3-bis(2-chloroethyl-1-nitrosoarene). Conversely, several alkylaton-tolerant MMR-defective cell lines also became resistant to cisplatin (48, 49), an agent that causes DNA cross-links not recognized by MTases.

The inability of Xs-14 to produce viable spores in crosses is consistent with the mutant phenotype of two meiotic-specific genes MSH4 and MSH5. The mutation in Xs-14, however, appears to be dominant or codominant with regard to its meiotic phenotype. Careful examination indicates that its alkylaton tolerance is also somewhat codominant, because transformation with MSH5 did not restore alkylaton sensitivity to the level of the mgt1Δ mutant. The mitotic function of the MSH4 and MSH5 genes has not been reported. Our results indicate that at least the mutated form of the MSH5 gene is probably able to participate in the repair of O° MeG lesion. Our results also suggest that some allele-specific mutations, rather than loss-of-function mutations, in the MutS homologues may be involved in alkylaton tolerance observed in mammalian cultures.

The involvement of Msh5 in alkylaton tolerance and possibly MMR should come as no surprise because MMR deficiency in mammals is known to affect meiotic events like chromosome pairing and crossing over. The different phenotypes exhibited by mismatch-deficient mice with respect to meiosis [e.g., male and/or female sterility associated with pms2 (50) and mlh1 (51, 52) defects and the fertility of msh2 mice (45)] raise the possibility that other MutS-like proteins, such as Msh4 and Msh5, could interact with Mlh1 and Pms2 during meiosis. From our results, the reverse can also be hypothesized; namely, that Msh4 and Msh5 proteins can be involved directly or indirectly in MMR. Because yeast and mammalian cells contain a number of MDM2 and MutS homologues, other repair complexes involving various MutL- and MutS-like proteins are also possible.

Our observations may have clinical significance, as a recent study on hereditary nonpolyposis colon cancer patients from different kindreds (53) reported that 30% lacked mutations in the coding region of five previously identified MMR genes (hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6/GTBP). It is therefore anticipated that mutations in additional genes yet to be discovered may act as potential causes of hereditary cancers, and some of them may be new human homologues of MutS, MutL, or other components of the MMR system. We have determined the entire nucleotide sequence of the msh5—14 coding region and have identified five nucleotide substitutions, four of which result in amino acid substitutions (Q454R, L498F, L524F, and Y823H). We are in the process of determining which mutation or combination of mutations is actually responsible for the MNNG-tolerant phenotype observed in the Xs-14 strain.

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A Mutation in the \textit{MSH5} Gene Results in Alkylation Tolerance

Sonya Bawa and Wei Xiao


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