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A Single-Strand Specific Lesion Drives MMS-Induced Hypermutability at a Double-Strand Break in Yeast

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Abstract

Localized hypermutability (LHM) can be important in evolution, immunity, and genetic diseases. We previously reported that single-strand DNA (ssDNA) can be an important source of damage-induced LHM in yeast. Here, we establish that the generation of LHM by methyl methanesulfonate (MMS) during repair of a chromosomal double-strand break (DSB) can result in over 0.2 mutations/kb, which is ~20,000 fold higher than the MMS-induced mutation density without a DSB. The MMS-induced mutations associated with DSB-repair were primarily due to substitutions via translesion DNA synthesis at damaged cytosines, even though there are nearly 10 times more MMS-induced lesions at other bases. Based on this mutation bias, the promutagenic lesion dominating LHM is likely 3-methylcytosine, which is single-strand specific. Thus, the dramatic increase in mutagenesis at a DSB is concluded to result primarily from the generation of nonrepairable lesions in ssDNA associated with DSB-repair along with efficient induction of highly mutagenic ssDNA-specific lesions. These findings with MMS-induced LHM have broad biological implications for unrepaired damage generated in ssDNA and possibly ssRNA.

Keywords

localized hypermutability; double-strand break; single-strand DNA; DNA polymerase ζ ; methyl methanesulfonate

1. Introduction

Mutagenesis is one of the major driving forces of evolution and also contributes to carcinogenesis and genetic diseases in humans [1]. Continuous strong genome-wide mutability can be deleterious to species [2], while transient localized hyper-mutability (LHM) can provide opportunities for evolution without significantly increasing the load on fitness [3,4] and may increase the likelihood of carcinogenesis [5,6]. Somatic hypermutability in the immunoglobulin genes is a dramatic example of LHM that actually benefits an organism [7].

Recently, using the yeast *Saccharomyces cerevisiae* we reported that DNA damage can induce high levels of mutability in the regions near DSBs or at uncapped telomeres,

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

providing new insights into mechanisms of LHM [8]. The UV-induced LHM in a reporter gene exhibited strand-biased mutations toward changes of pyrimidines in the unresected strand used for recombinational repair of a DSB (herein referred to as the *template strand* because it provides the template for DNA synthesis associated with DSB-repair as illustrated in Fig. 1) as well as in the unresected strand that can arise transiently at uncapped telomeres. Therefore, the source of LHM was attributed to premutational lesions in ssDNA because the template strand likely appears as a transient ssDNA intermediate in the processing of ends for DSB-repair [8,9]. Since the ssDNA would not be subject to excision repair, lesions would have a much greater potential for mutation than if they occur in dsDNA. Although the earlier results can be explained by damage in ssDNA (Fig. 1a), it is also possible that lesions in dsDNA could give rise to strand-biased LHM (Fig. 1b), if the lesions were not repaired and the complementary strand was removed prior to completion of DSB-repair (Fig. 1c-d). Conversion of damaged dsDNA to damaged ssDNA prior to DSB-repair might occur, for example, in the case of an agent such as methyl methanesulfonate (MMS) that can generate clustered lesions in dsDNA leading to DSBs as well as cause single base damage in dsDNA and ssDNA [10]. Damage that is specific to ssDNA can be instrumental in assessing the relative contribution of ssDNA vs dsDNA to LHM in the vicinity of a DSB, as well as the density of lesions in ssDNA.

While the lesions produced by UV are mostly pyrimidine dimers or 6-4 photoproducts, MMS primarily induces single-base damage. Repair of either UV or MMS lesions involves excision and replacement of damaged nucleotides using the complementary strand as a template. Unlike for UV, an abasic intermediate is generated during base excision repair (BER) of MMS lesions. Removal of MMS damage from dsDNA as well as ssDNA also might occur through enzymatic reversal (*e.g.*, *E. coli* AlkB or its human homologues) [11-15]. There does not appear to be a difference in the kinds of lesions induced by UV in ssDNA or dsDNA [16,17], as suggested by similar mutation spectra in ssDNA and dsDNA [8,18]. However, as shown in Fig. 2, the distribution of lesions induced by MMS are different in ssDNA and dsDNA, both *in vitro* and within cells [14,19-23]. We, therefore, anticipated that mutational spectra might reveal marked differences in the *in vivo* mutational properties of MMS-induced lesions in ssDNA as compared to dsDNA.

In the present study we have found that MMS actually generates a class of lesions that lead to mutations specific to ssDNA and that the overall lesion generation in ssDNA may be much greater than in dsDNA, suggesting that ssDNA is much more vulnerable than dsDNA to alkylation damage and subsequent genome instability. The nearly 20,000-fold difference in the density of MMS-induced mutations associated with DSB-repair regions, as compared to no DSB, primarily results from a combination of increased induction of lesions in ssDNA, induction of lesions that are more mutagenic and lack of repair of single-strand damage prior to DSB-repair.

2. Materials and Methods

2.1 Yeast Strains

All yeast strains were constructed from a strain isogenic to CG379 with the following common markers *MATa ade5-1 his7-2 leu2-3,112 trp1-289 ura3Δ* which was used in our previous study [8]. The “no-DSB”, “DSB-*cen*” and “DSB-*tel*” strains are illustrated in Fig. 3.

2.2 Measurement of Mutation Frequency

Procedures were similar to those in the previous study [8]. Briefly, yeast strains were grown with shaking in rich liquid media (YPDA) for approximately 16 hours and then 1.5 ml

culture was diluted into 50 ml fresh 2% galactose synthetic complete media to induce Gal-I-*SceI* expression for generation of a site-specific DSB. After 6 hours, cells were spun down and washed once with 50 ml dH₂O, resuspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) (~10⁷ cells/ml) and treated with either 11.8 mM (0.1% v/v) MMS or no MMS for 30 or 15 minutes at room temperature with occasional shaking. MMS was inactivated by adding Na₂S₂O₃ to a final concentration of 5% (w/v) and incubating for 2 minutes. Cells were washed once with 50 ml dH₂O followed by transformation to Lys⁺ or Ura⁺, respectively, with a pair of complementary oligonucleotides as previously described [24-26]. DSB-*cen* and DSB-*tel* strains were transformed with a pair of oligonucleotides described previously [8]. The *can1* mutants were screened among Lys⁺ or Ura⁺ transformant colonies by replica plating onto medium with 60 mg/ml of L-canavanine as well as among all survivors by directly plating onto media with 60 mg/ml of L-canavanine. The “No-DSB” cells went through the same procedure without adding oligonucleotides, but *can1* mutants were only selected on media with 60 mg/ml of L-canavanine. Mutation frequencies of *CAN1* were measured in transformants and in total cell populations (Fig. 4 and Table S1).

2.3 Selection of Mutants for Mutation Analyses

MMS-induced *can1* mutants associated with DSB-repair were collected from four independent experiments, two from DSB-*cen* strains and two from DSB-*tel* strains. However, in order to avoid a preferential selection on the pre-existing identical *can1* mutants in the culture because of lower induced mutability, we grew 40 independent No-DSB strains from 40 single colony isolates and confirmed that the *can1* mutation frequencies were all within the range. Among them, either 2 *can1* mutants or 1 *can1* mutant were selected from “+ MMS” and “– MMS” experiments, respectively. For the cases where 2 mutants were picked, only one pair among 32 pairs of mutants from the same cultures contained identical mutations (Table S3). All *can1* mutant colonies were streaked out for single colonies to avoid mixtures of cells, and the single colonies were prepared for sequencing. Each mutant was sequenced using standard Bigdye Terminator Kit (Applied Biosystems, Inc.). The sequence trace files of mutants were analyzed against the wild-type starting strain using Lasergene 8.0 (DNASTAR, Inc.) or CLC workbench 4.0 (CLC Bio). Similarly to the previous work [8], all mutations with 20 bases of flanking context in the coding sequence are presented in the Supplemental tables S2-S8 and an overall view of the mutation spectra is presented in Fig. 5 using Prism 5.0 (GraphPad Software).

3. Results

3.1 MMS Frequently Causes Multiple Mutations in the Vicinity of a Repaired DSB

Previously we showed that the MMS- and UV-induced mutagenesis at the *CAN1* gene, a reporter for forward mutations in yeast, was greatly increased if a DSB was created nearby prior to exposure to the mutagens [8]. As described in Fig. 3, the site-specific DSB is produced by galactose inducible I-*SceI* in *URA3* (DSB-*tel*) or *LYS2* (DSB-*cen*) that are close to a *CAN1* reporter. The *can1* mutation frequencies increased from around 10⁻⁵ and 10⁻⁴ in the entire population to as much as 3% and 7% of the cells that repaired a DSB after MMS and UV treatment, respectively ([8] and Fig. 4, Table S1). The UV-induced mutagenesis was attributed to robust translesion synthesis (TLS) past pyrimidine dimers in the template strand by DNA polymerase ζ . Over 30% of the DSB-associated, UV-induced LHM mutants contained widely-separated multiple mutations. We surmised, based on the incidence of multiple mutants, that the high levels of UV-induced LHM were due to a hypermutable subpopulation containing long stretches of damaged ssDNA associated with DSB-repair.

Based on the unique damage specificity of MMS (Fig. 2), we sought to address the relative contribution of damaged ssDNA and dsDNA to LHM (Fig. 1) and also to assess lesion

density in ssDNA. As shown in Tables 1, S2, and S3, there were only a few multiple *can1* mutants ($\leq 3\%$) recovered from the “No DSB” cells that were either treated or not treated with MMS (p-value is close to 1.0 for the comparison of “No DSB + MMS” and “No DSB – MMS”). This observation is in agreement with an earlier report on MMS-induced mutations in the *URA3* gene (Tables 1 and S4) [27]. These results contrast with those obtained from cells that experienced a DSB followed by MMS exposure and subsequent DSB-repair. With a 30-minute MMS exposure, the overall frequency of induced mutants was over 1000-fold greater than for the “No-DSB” cells (Fig. 4 and Table S1) and 21 to 31% of the mutants contained more than one mutation (Tables 1, S5-a and S6-a). The position of the DSB relative to the reporter (DSB-*tel* and DSB-*cen*) did not influence the likelihood of a multiple mutant ($p > 0.7$) while there was a strong difference in multiple mutants for cells that received a DSB ($p < 0.001$). Many of the mutations in the 12 multiple mutants were widely-spaced (Fig. 5A-B), with a mean of 328 bp and a range of 13-1107 bp between adjacent mutations (Tables S5-a and S6-b). With a shorter 15-minute MMS exposure, the frequency of induced *can1* mutants is about 2-fold less (Fig. 4 and Table S1) and there were 8 multiple mutants (Table 1, S5-b and S6-b and Fig. 5C-D). The incidence of multiple mutants was not significantly different between the two exposures ($p = 0.13$), while, the mutation density was reduced ~2 fold (see below). Among the total of 130 *can1* mutations there were only 3 that are complex mutations (*i.e.*, two mutations separated by ≤ 10 bp; Table S7), suggesting that the mechanism of complex mutation differs from that leading to multiple mutations.

As discussed previously [8], a high proportion of mutants that contain multiple mutations suggests a hypermutable population among the *Ura*⁺ or *Lys*⁺ cells in which the DSB had been repaired (cells that give rise to colonies on the selective media must have undergone DSB repair). The strong skewing towards mutants containing multiple mutations (*i.e.*, up to ~20 to 30% of MMS-induced mutants in the “+ DSB” protocol) implies that a large proportion of cells in the hypermutable population are, in fact, mutated in these experiments [8]. Furthermore, based on the biased mutation spectrum among the single as well as multiple mutants associated with DSB repair (see below), most *can1* mutants are concluded to originate from the hypermutable population. Assuming that the likelihood of mutation is the same throughout the hypermutable population, a minimal mutation density can be estimated simply by the frequency of additional mutations detected among the 45 *can1* mutants obtained under the “30-minute MMS + DSB” conditions (Table 1). The 8 double, 3 triple and 1 four-mutation mutants (Table 1 and S5-S6) correspond to a total of 17 additional mutations. Thus, the density of mutations within the group of 45 mutants is ~0.38 (17/45) mutations per *CAN1* gene or ~0.2 mutations/kb. This mutation density is ~20,000 times the level observed with the “No DSB + MMS” protocol where there were almost no multiple mutants ($\sim 1 \times 10^{-5}$ mutations/kb; Fig. 4 and Table S1). The actual frequency of mutations may be even higher, since not all *can1* mutations inactivate protein function [28]. Similarly, for a 15-minute MMS exposure, the minimal mutation density is estimated to be ~0.17 (10/58) mutations per *CAN1* gene or ~0.09 mutations/kb, suggesting that lesion induction was directly proportional to time of exposure.

3.2 Most MMS-induced Mutations Are at Cytosines in the Template Strand Used for DSB-Repair

Single base substitutions account for > 85% of the LHM mutations (Table S7). Assuming that a single base substitution arises at the position of MMS-induced base damage, the spectrum of mutations corresponds to a combination of the likelihood of base damage and mutability. The *CAN1* coding sequence is the template strand for repair of the DSB in the DSB-*tel* construct and it is the complementary strand for the DSB-*cen* construct (Fig. 1 and 3). Since the mutation spectra are comparable between 15 and 30-minute MMS exposures (Table 2 and Fig. 5; $p > 0.7$) we combined the spectra in the analysis below. Assuming the

template strand for DSB-repair is the source of damage-induced single base substitutions (Fig. 1), damaged cytosines are the most frequent sources of mutations for either DSB motif: 88% and 75%, respectively (Table 2). Since there is no significant difference in the mutation spectra associated with the template strands between two DSB motifs ($p > 0.9$), the spectra have been merged. The average contribution of cytosine to mutations is 81% and mutations at adenine, guanine and thymine account for 10%, 5%, and 3%, respectively (derived values from Table 2). As seen in Fig. 5, the mutation spectra obtained from the “No DSB $\pm$ MMS” experiments are markedly different from those involving induction of a DSB ($p < 0.0001$ or $p < 0.05$, respectively, for “No DSB + MMS” vs “DSB-*tel* + MMS” or “DSB-*cen* + MMS”; Table 2). In addition, 75% (36/48) of the base substitutions in the 20 multiple mutants are associated with cytosines in the template strand (Fig. 5A-D and Tables S5-S6), consistent with multiple mutations being caused by multiple lesions.

MMS is a DNA monofunctional methylating agent that acts primarily at the 6 nitrogens associated with a double bond in adenine, cytosine and guanine (Fig. 2). The frequency of adenine lesions or guanine lesions among all lesions does not differ dramatically (< 2 -fold) between ssDNA and dsDNA as determined for lesions induced *in vitro* and within cells (Fig. 2 legend and references [14,19-22]). However, cytosine damage is unique to ssDNA and it is primarily 3-meC (Fig. 2). This specificity provides an opportunity to address the mutability of different MMS-damaged bases in ssDNA. Our observation that ~80% of the “DSB + MMS” induced mutations occurred at a cytosine, along with previous reports about chemical specificity of MMS base damage (Fig. 2), strongly supports the view that the LHM is primarily due to 3-meC. Assuming the frequencies of lesions presented in Fig. 2 where ~10% of MMS-induced lesions are 3-meC, the mutability of 3-meC is about 20 times that of adenine lesions and 100 times guanine lesions (or ~36 times the combined adenine and guanine lesions). This agrees with measurements of the mutability of 1-methylpurines and 3-methylpyrimidines in vector DNA transformed into AlkB-deficient *E. coli* [12] where 3-meC is ~30 fold more mutagenic than 1-meA. Our results are also consistent with a recent observation of MMS-induced mutation bias in a highly transcribed region where 3-meC was suggested as the promutagenic lesion generated in transient ssDNA during transcription [29]. In contrast 1-meA, which is also considerably enhanced in ssDNA (Fig. 2), is much less mutagenic than 3-meC based on our result and those of Delaney *et al.*, [12], possibly because it is capable of forming T(anti).1-meA(syn) Hoogsteen base pairs [30].

3.3 Polymerase ζ Dependent Bypass of 3-meC Generates Random Base Substitutions

Similar to UV-induced LHM, the MMS-induced LHM was found to be largely dependent on translesion synthesis (TLS) by the DNA polymerase ζ (*REV3*) and not on polymerase η (*RAD30*; Fig. 4, Table S1 and [8]). Among the 130 “DSB + MMS” induced mutations 117 were base substitutions and 95 of the substitutions occurred at cytosines (Table S7, S8, 2 and Fig. 2A-D). Since incorporation of adenine, cytosine, or thymine opposite the cytosines was comparable (Table S8; 18:7:21 and 16:14:19 for DSB-*tel* and DSB-*cen*, respectively), there is no strong specificity in DNA polymerase ζ dependent bypass of 3-meC, suggesting nonspecific base recognition.

4. Discussion

4.1 Mutability of MMS in Double vs Single-Strand DNA

We have demonstrated that not only does the combination of DSB plus DNA damage lead to high levels of LHM [8] but also that the LHM is primarily due to nonrepairable lesions caused by MMS in ssDNA. This conclusion is based on the unique spectrum of mutations induced by MMS that is largely attributed to the single-strand specific 3-meC. The finding that MMS efficiently induces multiple mutations only in the ssDNA generated by a site-

specific DSB but not in the absence of a DSB (Table 1) argues that mutations induced under normal conditions of MMS exposure are unlikely to occur in long stretches of ssDNA generated during DNA metabolic processes such as replication fork uncoupling or long resection at DSBs caused by MMS treatment [10,31]. It is also conceivable that mutations might arise from lesions in shorter ssDNA regions. However, they cannot account for the bulk of mutations under normal conditions. Although G/C base pairs account for the majority of mutations from the “No DSB + MMS” conditions (39 G/C : 16 A/T), the contribution of mutations in G/C pairs under “DSB + MMS” conditions is much greater (101 G/C : 16 A/T from the combined 15 and 30-minute treatments; $p = 0.02$) (Table 2).

4.2 Molecular Mechanisms of Mutagenesis Caused by MMS-induced Lesions in ssDNA

The high efficiency of *CAN1* inactivation by MMS-induced 3-meC is primarily dependent on error-prone TLS by polymerase ζ as are mutations associated with other lesions. We propose that all MMS damaged bases can be bypassed by yeast TLS during restoration of damaged ssDNA to dsDNA, but only TLS at 3-meC is highly mutagenic. This lesion is a block to *E. coli* DNA polymerase I but not RNA polymerase I *in vitro*, and to *E. coli* DNA polymerases *in vivo* [12,32,33]. The effect of 3-meC on DNA synthesis by yeast DNA polymerases remains to be determined.

It is conceivable that LHM is attributable to TLS at abasic sites that occur spontaneously or through BER, since abasic sites are highly mutagenic *in vitro* and *in vivo* [34,35]. If this were so, abasic sites would be expected to lead to significant numbers of mutations at bases other than cytosines, contrary to our observations. Alternatively, enzymes that act in a manner similar to AlkB in *E. coli* or its homologues in human cells to reverse alkylation lesions directly [11] could act on damaged bases other than 3-meC. However, 3-meC is a good substrate in ssDNA for AlkB [12,13], suggesting that if such enzymes exist in yeast, they would correct the promutagenic 3-meC. These considerations also lead us to conclude that yeast lack a system(s) for the efficient removal of MMS-damaged bases from ssDNA. This is in agreement with the finding [36] that among 3 yeast ORF sequences that could complement an AlkB defect in *E. coli*, none coded for AlkB homologues. Furthermore, the corresponding genes did not contribute to the MMS resistance in yeast. The absence of functional AlkB homologues suggests that heterologous AlkB from *E. coli* or its mammalian homologues might be useful in future studies of alkylation-induced LHM, although conditions of functional expression in yeast would need to be developed. Also, if AlkB functional expression could be accomplished, it would be better to use it with an LHM detection system that does not include a transformation step, because of the difficulty in optimizing conditions for AlkB expression along with maintaining high transformation efficiency.

The requirement for *REV3* in the creation of MMS-induced multiple mutations scattered over as much as 1 kb (Fig. 5) at damaged cytosines associated with DSB-repair suggests that bypass of 3-meC involves sequential actions of at least two polymerases, the replication polymerase which carries out most DNA synthesis plus polymerase ζ which provides lesion bypass to overcome stalled DNA synthesis [37]. It is interesting that while the frequency of MMS-induced lesions other than 3-meC in ssDNA is nine times the frequency of 3-meC (Fig. 2), they are much less mutagenic (see Results). This suggests that the other lesions in ssDNA are possibly more accurately bypassed and have less of a blocking effect on DNA synthesis [12,14,15] and/or are capable of correct base pairing [30,38]. Unlike polymerase ζ , polymerase η has no role in the mutations induced under the “DSB + MMS” conditions. This further supports the view that abasic sites are not generated in ssDNA since TLS at abasic sites by polymerase η would be mutagenic and would lead to a bias in base substitutions [39,40].

As schematically described in Fig. 6, the dramatic difference in frequency and spectra of MMS-induced mutations in ssDNA vs dsDNA is due to a combination of a) induction of a single-strand specific lesion that leads to efficient mutation-prone bypass synthesis, b) other lesions that appear to be less mutagenic and c) a general lack of repair of MMS-induced lesions in ssDNA. Except for 3-meC, the other lesions are also produced in dsDNA and would be subject to efficient repair and potentially different mechanisms of mutagenesis. The scheme presented is likely relevant to mutagenesis by other DNA damaging agents. While UV is also highly mutable in ssDNA [8], it does not create ssDNA specific premutational lesions.

As shown in Fig. 4, 64-90% of *can1* mutants appearing after “DSB + MMS” induction could be attributed to MMS lesions and the rest (36-10%) to “other” mutational events associated with just the appearance of a DSB (“DSB only”) [41,42]. Regardless of the time of MMS exposure, ~80% of base substitutions occurred at cytosines in the template strand (Table 2). Previously, we showed that in the DSB only protocol, ~67% of base substitutions occurred at cytosines in the template strand for DSB-repair, which also depends on the error-prone TLS involving polymerase ζ [8]. The similarity in bias suggests that cytosines may also be an important target for DSB associated LHM, even in the absence of mutagen treatment. Possibly they are due to endogenous methylation or deamination. Interestingly, the base substitutions at cytosines are strongly biased towards C:G $\rightarrow$ T:A (9 in 15) and C:G $\rightarrow$ G:C (6 in 15) changes [8], which suggests that the endogenously generated lesions differ from 3-meC.

4.3 Use of Mutational Analysis to Address Induction and Repair of MMS-induced Lesions

Our results provide the opportunity to estimate the overall lesion frequency in ssDNA of the hypermutable cells. As described above, the mutation density is at least 0.2 mutations/kb in the *CAN1* gene in the cells that experience MMS-induced LHM. Since ~80% of mutations in ssDNA occur at cytosines, there are 0.16 mutated cytosines/kb, corresponding to > 0.08% cytosines in the *CAN1* gene being damaged by 30-minute MMS treatment. Assuming that the estimate of ~10% of MMS damaged bases are cytosines (Fig. 2) applies to the single-strand *CAN1* gene, the minimal overall lesion frequency in all bases of *CAN1* is estimated to be 1.6/kb. This value differs considerably from our previous estimate of < 0.1 lesions/kb in dsDNA after the same MMS treatment of G1 yeast cells, as measured by quantitative PCR [10]. If the efficiency of lesion induction in ssDNA and dsDNA are comparable in the two sets of experiments, the estimated difference in lesion density at the time of TLS can be explained by fast BER of MMS-induced lesions in dsDNA during the 30-minute exposure to 0.1% MMS as compared to the absence of repair in ssDNA of logarithmically growing cells used in the present experiments. Assuming comparable levels of damage induction in ssDNA and dsDNA, ~94% of the lesions would need to be removed from the dsDNA during the MMS treatment to account for the difference in damage density. Alternatively, the discrepancy could be reconciled if the generation of lesions in dsDNA is actually much less efficient than in ssDNA, as is the case for 3-meC. This would suggest that the chemical accessibility to ssDNA *in vivo* may be another contributor to the dramatic mutational synergy between DSB induction and subsequent DNA damage (Fig. 6).

Based on the association of mutations with damaged cytosines, the 20,000-fold increase in MMS-induced mutations is largely due to efficient induction of the promutagenic 3-meC lesion in ssDNA. Therefore, contributions to strand-biased LHM by unrepaired lesions induced by MMS in dsDNA that is a subject to strand removal during DSB repair (Fig. 1c-d) must be small. Nevertheless, unrepaired lesions induced by other agents (such as UV) in such dsDNA could contribute significantly to LHM.

5. Implications

The unique pattern of MMS-induced multiple mutations for ssDNA involving multiple cytosines can help dissect the contribution of resection to repair of various kinds of induced DSBs in yeast. Along this line, alkylation damage could give rise to DSBs at clustered sites [10,43] as well as lesions in the subsequent resected ends. Also, the DSB repair system that we employ may be useful in addressing the presence of endogenous alkylating agents and their potential for contributing to LHM [41,42]. As found for MMS treatment, regions of DNA that are rich in cytosine might be especially prone to mutational changes by combinations of DSBs and alkylating agents. Also, multiple mutations in CpG islands could impact epigenetic responses [21,44] in mammalian cells if there is insufficient AlkB repair.

Since most species of RNA are single-stranded, our findings with ssDNA suggest that RNA is also a sensitive target for MMS [20,45]. Lesions in RNA could reduce translation, alter coding, and affect posttranscriptional processing of RNAs such as splicing and degradation [46]. For organisms that contain RNAi, MMS treatment could also impact its function although the target is much smaller. In light of this, genome-wide screening in yeast has identified genes involved in RNA metabolism and protein translations that are required for tolerance of MMS [47-49]. Although AlkB and its homolog are efficient in the repair of RNA alkylation lesions [11,13,22,46], they are absent in yeast. Possibly there are other mechanisms for toleration, repair or translational bypass of lesions in RNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LHM	localized hypermutability
ssDNA	single-strand DNA
dsDNA	double-strand DNA
MMS	methyl methanesulfonate
DSB	double-strand break
TLS	DNA translesion synthesis
BER	base excision repair
HR	homologous recombination
NER	nucleotide excision repair

References

1. Friedberg EC. DNA damage and repair. *Nature*. 2003; 421:436–440. [PubMed: 12540918]
2. Giraud A, Matic I, Tenaillon O, Clara A, Radman M, Fons M, Taddei F. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science*. 2001; 291:2606–2608. [PubMed: 11283373]

3. Drake JW, Bebenek A, Kissling GE, Peddada S. Clusters of mutations from transient hypermutability. *Proc. Natl. Acad. Sci. U S A.* 2005; 102:12849–12854. [PubMed: 16118275]
4. Drake JW. Too many mutants with multiple mutations. *Crit. Rev. Biochem. Mol. Biol.* 2007; 42:247–258. [PubMed: 17687667]
5. Chen Z, Feng J, Buzin CH, Sommer SS. Epidemiology of doublet/multiplet mutations in lung cancers: evidence that a subset arises by chronocoordinate events. *PLoS One.* 2008; 3:e3714. [PubMed: 19005564]
6. Wang J, Gonzalez KD, Scaringe WA, Tsai K, Liu N, Gu D, Li W, Hill KA, Sommer SS. Evidence for mutation showers. *Proc Natl Acad Sci U S A.* 2007; 104:8403–8408. [PubMed: 17485671]
7. Odegard VH, Schatz DG. Targeting of somatic hypermutation. *Nat. Rev. Immunol.* 2006; 6:573–583. [PubMed: 16868548]
8. Yang Y, Sterling J, Storici F, Resnick MA, Gordenin DA. Hypermutability of damaged single-strand DNA formed at double-strand breaks and uncapped telomeres in yeast *Saccharomyces cerevisiae*. *PLoS Genet.* 2008; 4:e1000264. [PubMed: 19023402]
9. Mimitou EP, Symington LS. Nucleases and helicases take center stage in homologous recombination. *Trends Biochem Sci.* 2009; 34:264–272. [PubMed: 19375328]
10. Ma W, Panduri V, Sterling JF, Van Houten B, Gordenin DA, Resnick MA. The transition of closely opposed lesions to double-strand breaks during long-patch base excision repair is prevented by the coordinated action of DNA polymerase delta and Rad27/Fen1. *Mol Cell Biol.* 2009; 29:1212–1221. [PubMed: 19075004]
11. Aas PA, Otterlei M, Falnes PO, Vagbo CB, Skorpen F, Akbari M, Sundheim O, Bjoras M, Slupphaug G, Seeberg E, Krokan HE. Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. *Nature.* 2003; 421:859–863. [PubMed: 12594517]
12. Delaney JC, Essigmann JM. Mutagenesis, genotoxicity, and repair of 1-methyladenine, 3-alkylcytosines, 1-methylguanine, and 3-methylthymine in alkB *Escherichia coli*. *Proc Natl Acad Sci U S A.* 2004; 101:14051–14056. [PubMed: 15381779]
13. Westbye MP, Feyzi E, Aas PA, Vagbo CB, Talstad VA, Kavli B, Hagen L, Sundheim O, Akbari M, Liabakk NB, Slupphaug G, Otterlei M, Krokan HE. Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. *J Biol Chem.* 2008; 283:25046–25056. [PubMed: 18603530]
14. Sedgwick B. Repairing DNA-methylation damage. *Nat Rev Mol Cell Biol.* 2004; 5:148–157. [PubMed: 15040447]
15. Sedgwick B, Bates PA, Paik J, Jacobs SC, Lindahl T. Repair of alkylated DNA: Recent advances. *DNA Repair (Amst.).* 2007; 6:429–442. [PubMed: 17112791]
16. Doetsch PW, Chan GL, Haseltine WA. T4 DNA polymerase (3'-5') exonuclease, an enzyme for the detection and quantitation of stable DNA lesions: the ultraviolet light example. *Nucleic Acids Res.* 1985; 13:3285–3304. [PubMed: 2987881]
17. Chan GL, Doetsch PW, Haseltine WA. Cyclobutane pyrimidine dimers and (6-4) photoproducts block polymerization by DNA polymerase I. *Biochemistry.* 1985; 24:5723–5728. [PubMed: 4084488]
18. Madzak C, Sarasin A. Mutation spectrum following transfection of ultraviolet-irradiated single-stranded or double-stranded shuttle vector DNA into monkey cells. *J Mol Biol.* 1991; 218:667–673. [PubMed: 1902520]
19. Beranek DT, Weis CC, Swenson DH. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. *Carcinogenesis.* 1980; 1:595–606. [PubMed: 11219835]
20. Singer, B.; Grunberger, D. *Molecular biology of mutagens and carcinogens.* Plenum Press; 1983. monofunctional alkylating agents; p. 55-78.
21. Beranek DT. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res.* 1990; 231:11–30. [PubMed: 2195323]
22. Drablos F, Feyzi E, Aas PA, Vaagbo CB, Kavli B, Bratlie MS, Pena-Diaz J, Otterlei M, Slupphaug G, Krokan HE. Alkylation damage in DNA and RNA--repair mechanisms and medical significance. *DNA Repair (Amst.).* 2004; 3:1389–1407. [PubMed: 15380096]

23. Bodell WJ, Singer B. Influence of hydrogen bonding in DNA and polynucleotides on reaction of nitrogens and oxygens toward ethylnitrosourea. *Biochemistry*. 1979; 18:2860–2863. [PubMed: 476059]
24. Storici F, Durham CL, Gordenin DA, Resnick MA. Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. *Proc. Natl. Acad. Sci. U S A*. 2003; 100:14994–14999. [PubMed: 14630945]
25. Storici F, Lewis LK, Resnick MA. *In vivo* site-directed mutagenesis using oligonucleotides. *Nat. Biotechnol*. 2001; 19:773–776. [PubMed: 11479573]
26. Storici F, Resnick MA. The *delitto perfetto* approach to *in vivo* site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. *Methods Enzymol*. 2006; 409:329–345. [PubMed: 16793410]
27. Lee GS, Blonsky KS, Van On DL, Savage EA, Morgan AR, von Borstel RC. Base alterations in yeast induced by alkylating agents with differing Swain-Scott substrate constants. *J Mol Biol*. 1992; 223:617–626. [PubMed: 1542109]
28. Lang GI, Murray AW. Estimating the per-base-pair mutation rate in the yeast *Saccharomyces cerevisiae*. *Genetics*. 2008; 178:67–82. [PubMed: 18202359]
29. Fix D, Canugovi C, Bhagwat AS. Transcription increases methylmethane sulfonate-induced mutations in alkB strains of *Escherichia coli*. *DNA Repair (Amst)*. 2008; 7:1289–1297. [PubMed: 18515192]
30. Yang H, Zhan Y, Fenn D, Chi LM, Lam SL. Effect of 1-methyladenine on double-helical DNA structures. *FEBS Lett*. 2008; 582:1629–1633. [PubMed: 18435925]
31. Sogo JM, Lopes M, Foiani M. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*. 2002; 297:599–602. [PubMed: 12142537]
32. Saffhill R. Differences in the promutagenic nature of 3-methylcytosine as revealed by DNA and RNA polymerising enzymes. *Carcinogenesis*. 1984; 5:691–693. [PubMed: 6373042]
33. Larson K, Sahm J, Shenkar R, Strauss B. Methylation-induced blocks to *in vitro* DNA replication. *Mutat. Res*. 1985; 150:77–84. [PubMed: 4000169]
34. Gibbs PE, McDonald J, Woodgate R, Lawrence CW. The relative roles *in vivo* of *Saccharomyces cerevisiae* Pol eta, Pol zeta, Rev1 protein and Pol32 in the bypass and mutation induction of an abasic site, T-T (6-4) photoadduct and T-T cis-syn cyclobutane dimer. *Genetics*. 2005; 169:575–582. [PubMed: 15520252]
35. Pages V, Johnson RE, Prakash L, Prakash S. Mutational specificity and genetic control of replicative bypass of an abasic site in yeast. *Proc Natl Acad Sci U S A*. 2008; 105:1170–1175. [PubMed: 18202176]
36. Wei YF, Chen BJ, Samson L. Suppression of *Escherichia coli* alkB mutants by *Saccharomyces cerevisiae* genes. *J Bacteriol*. 1995; 177:5009–5015. [PubMed: 7665478]
37. Takata K, Wood RD. Bypass specialists operate together. *Embo J*. 2009; 28:313–314. [PubMed: 19225445]
38. Lee S, Bowman BR, Ueno Y, Wang S, Verdine GL. Synthesis and structure of duplex DNA containing the genotoxic nucleobase lesion N7-methylguanine. *J Am Chem Soc*. 2008; 130:11570–11571. [PubMed: 18686953]
39. Zhao B, Xie Z, Shen H, Wang Z. Role of DNA polymerase eta in the bypass of abasic sites in yeast cells. *Nucleic Acids Res*. 2004; 32:3984–3994. [PubMed: 15284331]
40. Haracska L, Unk I, Johnson RE, Johansson E, Burgers PM, Prakash S, Prakash L. Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. *Genes. Dev*. 2001; 15:945–954. [PubMed: 11316789]
41. Strathern JN, Shafer BK, McGill CB. DNA synthesis errors associated with double-strand-break repair. *Genetics*. 1995; 140:965–972. [PubMed: 7672595]
42. Holbeck SL, Strathern JN. A role for *REV3* in mutagenesis during double-strand break repair in *Saccharomyces cerevisiae*. *Genetics*. 1997; 147:1017–1024. [PubMed: 9383049]
43. Ma W, Resnick MA, Gordenin DA. Apn1 and Apn2 endonucleases prevent accumulation of repair-associated DNA breaks in budding yeast as revealed by direct chromosomal analysis. *Nucleic Acids Res*. 2008; 36:1836–1846. [PubMed: 18267974]

44. Tommasi S, Denissenko MF, Pfeifer GP. Sunlight induces pyrimidine dimers preferentially at 5-methylcytosine bases. *Cancer Res.* 1997; 57:4727–4730. [PubMed: 9354431]
45. Feyzi E, Sundheim O, Westbye MP, Aas PA, Vagbo CB, Otterlei M, Slupphaug G, Krokan HE. RNA base damage and repair. *Curr Pharm Biotechnol.* 2007; 8:326–331. [PubMed: 18289040]
46. Ougland R, Zhang CM, Liiv A, Johansen RF, Seeberg E, Hou YM, Remme J, Falnes PO. AlkB restores the biological function of mRNA and tRNA inactivated by chemical methylation. *Mol Cell.* 2004; 16:107–116. [PubMed: 15469826]
47. Jelinsky SA, Samson LD. Global response of *Saccharomyces cerevisiae* to an alkylating agent. *Proc Natl Acad Sci U S A.* 1999; 96:1486–1491. [PubMed: 9990050]
48. Ravi D, Wiles AM, Bhavani S, Ruan J, Leder P, Bishop AJ. A network of conserved damage survival pathways revealed by a genomic RNAi screen. *PLoS Genet.* 2009; 5:e1000527. [PubMed: 19543366]
49. Rooney JP, George AD, Patil A, Begley U, Bessette E, Zappala MR, Huang X, Conklin DS, Cunningham RP, Begley TJ. Systems based mapping demonstrates that recovery from alkylation damage requires DNA repair, RNA processing, and translation associated networks. *Genomics.* 2009; 93:42–51. [PubMed: 18824089]

Fig. 1. Lesions in ssDNA as well as unrepaired lesions in dsDNA can lead to strand-biased LHM around a repaired DSB

An inducible DSB (double red lines) resides in the DNA sequence (grey lines) that is deleted at the end of DSB-repair by oligonucleotides. A mutation reporter is located at either the telomere (green thick lines) or centromere (blue thick lines) side of the DSB. In our DSB-repair experiments, the template strands used for repair are indicated by arrowheads. The DSB repair is initiated by a pair of oligonucleotides (magenta arrows) with homology to sequences on both sides of the DSB. The oligonucleotide-mediated DSB-repair is described in terms of the “template model” which involves DNA synthesis (dotted lines) leading to deletion of the I- *SceI* containing cassette and restoration of the *LYS2* or *URA3* gene (see Fig. 3); this process is referred to as *delitto perfetto* in Storici *et al.*, [24-26]. Within our experimental scheme [8], which involves asynchronous induction of a DSB by I-*SceI*, there will be varying amounts of 5' to 3' resection prior to acute DNA damage and the subsequent DSB repair. Lesions in the template and in the complementary strands are indicated by red and blue stars, respectively. The amounts and types of lesions in ssDNA vs dsDNA could differ (as described in Fig. 2). Strand-biased mutations in the reporter could arise by the following scenarios after induction of a DSB: a) lesions are induced in the template strand (*i.e.* ssDNA) remaining after resection; b) lesions are induced in dsDNA that is resected before lesions are repaired or c) lesions are induced in dsDNA that is subject to strand unwinding and degradation in association with DNA synthesis (area indicated by brackets in (d)). The resulting mutation spectrum in the nascent reporter strand (green or blue dots) would be due solely to translesion DNA synthesis (TLS) across unrepaired lesions in the template strand, thereby leading to strong mutational bias. Once lesions are bypassed, they are likely to be repaired by excision repair prior to the next round of DNA replication. As a result mutant sequence appears in both strands (not shown). Lesions in dsDNA in which both strands are retained during the course of DSB repair would not lead to a strand-biased spectrum (not presented).

Fig. 2. Frequency (%) of MMS-induced base lesions among all dsDNA or ssDNA lesions

The reactive methyl group of MMS can attack cellular targets including DNA by nucleophilic substitution through SN2 mechanism. The G/C and A/T base pairs are illustrated using Jmol (an open-source Java viewer for chemical structures in 3D. <http://www.jmol.org/>) and a published X-ray structure of DNA (PDB ID: 1D66). The six nitrogens with a double bond are the primary DNA targets of methylation by MMS and are labeled and highlighted with arrows. Atoms are distinguished by colors (Nitrogen: blue; Carbon: grey; Oxygen: red; Hydrogen: white) and are presented at their 25% *Van der Waals* radii and bonds are shown in 0.1 Å. Based on its position within a structure, N3 of cytosine is not readily accessible to MMS in dsDNA due to hydrogen bonds [23] and base stacking (not shown); N1 of adenine is partially accessible. The frequencies of individual lesions among all lesions were adapted from the summary in [20] where some frequencies are averages of multiple measurements so that the “sum” may exceed 100%. Also, the “sum” may be less than 100% since in some measurements, some lesions are not distinguishable. The numbers are largely consistent with data summarized in a more recent report [21]. For *in vitro* experiments, M13 phage DNA was the source of ssDNA and DNA from salmon sperm and other cells were the sources of dsDNA. For *in vivo* experiments, transfected single-strand vector DNA or nuclear DNA from human cells treated with MMS were isolated and analyzed in a manner similar to the *in vitro* samples.

Fig. 3. Yeast strains used to address the impact of DSBs on MMS-induced mutations

The strains used to assess MMS-induced LHM were described previously [8]. Presented is the portion of yeast chromosome V containing *URA3*, *CAN1*, and *LYS2* ORFs is shown along with the telomere (triangle) and centromere (oval). A self-generating DSB cassette [24-26] containing a hygromycin resistance gene, the I-SceI endonuclease ORF under the control of the inducible *GAL1*-promoter and the I-SceI cut site was placed either in *LYS2* (DSB-*cen*) or *URA3* (DSB-*tel*). The “no-DSB” control cassette placed in *LYS2* contained a noncleavable I-SceI half-site (not shown). The *CAN1* mutation reporter is located within either 3.4 kb (DSB-*cen* and no DSB) or 4.4 kb (DSB-*tel*) from the I-SceI sites. *URA3* and *LYS2* were inserted 30 kb and 34 kb away from the telomere, respectively, in the DSB-*tel* and DSB-*cen* constructs.

Fig. 4. Mutation frequencies of *CAN1*

Cells of DSB-*cen* and DSB-*tel* constructs (Fig. 3) were transformed to Lys⁺ or Ura⁺, respectively, using a process of *delitto perfetto* described by Storici *et al.* [24-26] and with a pair of complementary oligonucleotides described in [8]. The *can1* mutant frequencies were determined among Lys⁺ (DSB-*cen*) or Ura⁺ (DSB-*tel*) transformants that arose after DSB induction and repair by the oligonucleotides. The mutation frequencies in the “no-DSB” controls were determined in cells containing the Hyg-Gal-I-*SceI* insert (Fig. 3). The I-*SceI* half site in the *LYS2* position served as a “No-DSB” control. The medians of mutation frequencies obtained in several independent transformation experiments are presented as log₁₀ (mutation frequency × 10⁴). The data from the previous work [8] and this work were pooled together in this figure, but clearly identified in Table S1, where median frequencies, ranges of variation and numbers of repeats are provided. Numbers inside the grey bars represent frequencies of MMS-induced mutants, whereas numbers inside the white bars are frequencies of spontaneous mutations from the “no damage” controls. The mutants picked for sequencing analysis, as described in Materials and Methods, were summarized in Table S1. Each mutant was sequenced using standard Bigdye Terminator Kit (Applied Biosystems, Inc.). The sequence trace files of mutants were analyzed against the wild-type starting strain using Lasergene 8.0 (DNASTAR, Inc.) or CLC workbench 4.0 (CLC Bio). Similarly to the previous work [8], all mutations with 20 bases of flanking context in the coding sequence are presented in the Supplemental tables S2-S8 and an overall view of the mutation spectra is presented in Fig. 5 using Prism 5.0 (GraphPad Software).

Fig. 5. Mutated bases of *can1* alleles induced by MMS

Every *can1* allele is given an arbitrary mutant number (as shown on the Y- axis). The mutated bases of *can1* alleles are distinguished by colored symbols (green – thymine, blue – guanine, red – cytosine, and black – adenine) and by positions in the *CAN1* ORF. The mutated bases are in the coding sequence of *CAN1* and correspond to the template strand for repair of a DSB-*tel* and the complementary strand for repair of a DSB-*cen*. Open symbols represent single deletions and filled symbols represent single base substitutions. Multiple mutations appearing in the same mutant are connected by brown lines. To provide easy readability, grey lines separate groups of 5 mutants. Mutants were grouped by the mutated bases, and then mutations were ordered from lower to higher positions in *CAN1* when possible. The number (n) of sequenced mutations is shown and the details are summarized in Table S8. (A, C) The DSB-*cen* and (B, D) DSB-*tel* strains were incubated in galactose to induce a DSB for 6 hours and then treated with 0.1% (v/v) MMS for 30 and 15 minutes, respectively. “No-DSB” strains were incubated in galactose for 6 hours and then half the cells were exposed to MMS (E) and half were not (F).

Fig. 6. Lack of repair and selective error-prone TLS of base damage specific to ssDNA leads to LHM

Upon exposure to a DNA damaging agent, lesions (colored triangle, squares and circles) are generated that may differ between ssDNA and dsDNA. The triangle corresponds to ssDNA specific lesions (for example, 3-meC and 1-meA in the case of MMS) which are frequent sources of mutations (identified as thick vertical ticks). As discussed in the text, it is also possible that in addition to ssDNA specific lesions, the rate of lesion generation could be greater in ssDNA.

Table 1

Distribution of mutants containing 1, 2 or ≥ 3 mutations.

Mutation Reporter	DSB ^a	0.1% MMS (minutes)	Percent (number) of mutants			Total Mutants	Mutations/Mutant
			1 mutation	2 mutations	≥3 mutations		
CAN1	No	Ø	100 (31)	0	0	31	1
		30	97 (61)	3 (2)	0	63	1.0
	Cen	15	83 (25)	13 (4)	3 (1)	30	1.2
			86 (24)	14 (4)	0 (0)	28	1.1
	Cen	30	69 (18)	19 (5)	12 (3)	26	1.4
			79 (15)	16 (3)	5 (1)	19	1.3
URA3 ^b	No	60	100 (48)	0	0	48	1

^aThe I-*scel* inducible DSB cassette is at the centromere (*Cen*) side or the telomere (*Tel*) side of *CAN1* (Fig. 3).

^bResults adapted from reference (27), where yeast cells were treated with 0.2% MMS.

Table 2

Distribution of single base substitutions at four bases of the coding strand.

Mutation Reporter	DSB	0.1% MMS (minutes)	Percent (number) ^a				Total ^b
			C	G	A	T	
	No	Ø	54 (12)	27 (6)	14 (3)	5 (1)	71 (22)
		30	26 (14)	45 (25)	7 (4)	22 (12)	85 (55)
CAN1 ^c	Tel	15	90 (27)	3 (1)	3 (1)	3 (1)	94 (30)
	Cen		6 (2)	74 (23)	3 (1)	16 (5)	86 (31)
	Tel	30	86 (19)	5 (1)	9 (2)	0 (0)	88 (22)
	Cen		6 (2)	76 (26)	6 (2)	12 (4)	92 (34)
	Tel	(15 + 30) ^d	88 (46)	4 (2)	6 (3)	2 (1)	91 (52)
	Cen		6 (4)	75 (49)	5 (3)	14 (9)	89 (65)
URA3 ^e	No	60	18 (8)	64 (28)	11 (5)	7 (3)	92 (44)

^a Percent (number) of single base substitutions occurred at a given base in the coding strand.

^b Percent (number) of single base substitutions in all *can1* mutations.

^c The *CAN1* coding strand is the bottom 3'-5' strand (Fig. 3). It is the template strand for repairing the DSB-*tel* or the complementary strand to the template strand for repairing the DSB-*cen* due to 5' to 3' resection (Fig. 1).

^d Results from 15 and 30 minutes were pooled together.

^e Results adapted from the reference (27), where yeast cells were treated with 0.2% MMS.