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Transcription Increases Methylmethane Sulfonate-Induced Mutations in *alkB* Strains of *Escherichia coli*

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Abstract

Methylmethane sulfonate (MMS) produces DNA base lesions, including 3-methylcytosine (m3C), more effectively in single-stranded DNA. The repair of m3C in *Escherichia coli* is mediated by AlkB through oxidative demethylation and in the absence of repair, m3C leads to base-substitution mutations. We describe here results of experiments that were designed to investigate whether transcription of a gene in *E. coli* affects the process of mutagenesis by MMS and the roles played by AlkB and lesion bypass polymerase PolV. Using a genetic reversion assay, we have confirmed that MMS mutagenesis is suppressed by AlkB, but is enhanced by PolV. High transcription of the target gene enhances reversion frequency in an orientation-dependent manner. When the cytosines that are the likely targets of MMS were in the non-template strand (NTS), transcription increased the MMS-induced reversion frequency several fold. This increase was dependent on the presence of PolV. In contrast, when the same cytosines were present in the template strand, transcription had little effect on reversion frequency induced by MMS. These data suggest that MMS creates 3-methylcytosine adducts in the NTS and are consistent with an idea proposed previously that transcription makes the NTS transiently single-stranded and more accessible to chemicals. We propose that this is the underlying cause of its increased sensitivity to MMS and suggest that transcriptionally active DNA may be a preferred target for the action of alkylating agents that prefer single-stranded DNA.

Keywords

AlkB; 3-methylcytosine; transcription; alkylation; methylmethane sulfonate

INTRODUCTION

Alkylation-induced mutagenesis and carcinogenesis result from the formation of DNA base adducts that are not repaired or incorrectly repaired by cellular mechanisms. A variety of different DNA base alterations can be produced depending upon the source of the donated alkyl group (for reviews see [1,2]). Nitrosoureas, for example, produce O-alkylations, such as O⁶-alkylguanine and O⁴-alkylthymine. In comparison, the methane sulfonates primarily produce N-alkylated bases including 7-alkylguanine, 3-alkyladenine, 1-alkyladenine and 3-alkylcytosine. As a result, the mutagenic consequences of different alkylating agents can be dramatically different. In addition, the cellular mechanisms that repair these different adducts can also be distinct. O⁶-alkylguanine and O⁴-alkylthymine may be repaired by

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methyltransferases, which transfer the alkyl group from the affected base to a cysteine residue within the protein [3]. Absence of this repair can lead to base substitution mutations, primarily transitions, during normal DNA replication. In contrast, the N-alkylated bases, such as 3-alkyladenine, are substrates for DNA glycosylases, which physically remove the affected base to produce an apurinic/apyrimidinic (AP) site [4]. Failure to repair some of these N-alkylated bases has a variety of consequences, principal amongst which is a block in DNA replication. DNA synthesis past many of these adducts is mediated by the error-prone Y-family polymerases, such as *E. coli* PolV or human polymerase η [5,6]. Such “error-prone” replication may result in a variety of different base substitution mutations including transitions and transversions.

One particularly novel form of DNA repair of alkylated bases is exemplified by the AlkB family of proteins [7–9]. Its prototype, AlkB from *E. coli*, and its human homologs, hABH2 and hABH3, employ a dioxygenase-type reaction mechanism and are capable of directly repairing base adducts including 1-methyladenine (m1A), 3-methylcytosine (m3C), 1-methylguanine (m1G), and 3-methylthymine (m3T) [10–13]. The enzymes utilize α -ketoglutarate, Fe^{+2} and molecular oxygen to remove the alkyl group via oxidation to restore the normal DNA base. An interesting property of the enzymes AlkB and hABH3 is that they prefer single-stranded DNA (ssDNA) substrates [14]. The preference for ssDNA is interesting because of the fact that the N-1 and N-3 positions are normally involved in base pairing interactions in double stranded DNA and are poorly accessible to alkylating chemicals. It has been suggested that efficient alkylation at these sites would occur in ssDNA regions as might be observed within replication forks or transcription bubbles [15]. By the same token, formation of the N-alkylated base prevents normal hydrogen bonding interactions and, hence, DNA replication and transcription would be expected to be blocked by 1-alkylpurine and 3-alkylpyrimidine lesions. Therefore, repair of such lesions is critical to cell survival.

Methylmethane sulfonate (MMS) is a well established chemical agent that produces primarily N-alkylated DNA bases. In double-stranded DNA, 7-methylguanine (m7G) predominates among the MMS-induced lesions (about 85% of the total), followed by 3-methyladenine (m3A, about 10% of the total). With ssDNA, however, the percentages of m1A and m3C caused by MMS increase considerably to 10% and 8% of the total, respectively [1]. The structural analogs m1G and m3T may also be produced more frequently in ssDNA, albeit at much lower levels than m1A and m3C. The mutagenic consequences of these base adducts were studied recently in *E. coli* by Delany and Essigmann [16] and were found to be affected by AlkB and SOS functions. In the absence of SOS functions, only m3T illustrated substantial mutagenicity; about 30% of the bypass events resulted in T to A transversions. In the presence of SOS, but the absence of AlkB, m3C produced 35% C to T transitions, 30% C to A transversions and 5% C to G transversions; the remaining bypass events were non-mutagenic. The m1G adduct produced about 40% G to T transversions, 20% G to A transitions and a small number of G to C transversions. The mutagenicity of m3T was only slightly increased in the presence of SOS; about 40% of the bypass events were T to A transversions. Significantly, m1A was consistently non-mutagenic. In the absence of both SOS and AlkB, the number of mutations produced by m3C and m1G were considerably reduced, suggesting the involvement of PolV in replication bypass of these lesions. Several other reports [17,18] have also shown that C to T, C to A, and T to A base substitutions are enhanced in the absence of AlkB. Therefore, AlkB appears to play an important role in preventing mutations resulting from alkylation damage to DNA and in its absence, a characteristic set of DNA alterations and base substitutions are produced.

The preferred substrate for MMS and other $\text{S}_{\text{N}}2$ -type agents, ssDNA, is created transiently during replication and transcription. In the latter case, the non-template strand (non-transcribed strand, NTS) in the transcription bubble is accessible to the aqueous environment inside the cell and would be a preferred target. In this report, we have investigated the possibility that the

transcription bubble is accessible to MMS using a genetic assay that was used previously to show that a high level of transcription enhances deamination of cytosines to uracil and oxidation of guanine to 8-oxoguanine [19–21]. The intracellular agents that create such damage are thought to be water and reactive oxygen species, respectively, and in both cases, the genetic data are consistent with a preferential attack of the NTS by these agents [20,21]. The results reported herein clearly indicate that transcription also makes the NTS more susceptible to the mutagenic effects of MMS, and that the absence of AlkB-mediated repair greatly enhances these effects when DNA PolV is available.

MATERIALS AND METHODS

Plasmids

The plasmids employed in this research were pUP21-op75 [19] and pUP27-op75 [20], which contained a mutated version of the *kan-ble* operon from Tn5 transcribed from its natural promoter (P_{kan}). In addition, the plasmids contain the very strong *UP-tac* promoter that is repressed by the *lac* repressor (Figure 1). The inactive allele of *kan*, *kanS-94D*, harbors a missense mutation (TTG to CCA) that results in a kanamycin-sensitive phenotype; reversion to kanamycin resistance (Kan^R) can result from C to T, C to A or C to G base changes at either of the cytosines in the CCA sequence. The inactive allele of *ble*, *ble-op75*, harbors an opal (TGA) mutation that inactivates the gene, resulting in a bleomycin-sensitive phenotype. This phenotype was not used in the experiments described below and will not be discussed here. Furthermore, the plasmids pUP21-op75 and pUP27-op75 are hereafter referred to as pUP21 and pUP27, respectively, to keep the nomenclature simple. The difference between pUP21 and pUP27 is the orientation of the *kan-ble* operon with respect to the *UP-tac* promoter (Figure 1). In pUP21, the *UP-tac* promoter is oriented in the same direction as P_{kan} . In other words, induction of *UP-tac* with IPTG leads to transcription of the normally transcribed DNA strand of the operon. In pUP27, *UP-tac* is oriented in the opposite direction as P_{kan} such that induction of *UP-tac* with IPTG leads to transcription of the normally non-transcribed DNA strand of the operon. The differing consequences of transcription in pUP21 and pUP27 are illustrated in Figure 1.

Bacterial Strains

The bacterial host strain employed in this research was *E. coli* B/r FX-10 [22]. This strain was auxotrophic for tyrosine (*tyrA14*, a UAA nonsense defect) and leucine (*leu308*, a UAG nonsense defect), and was excision repair defective (*uvrA115*). The excision repair defective strain was used to prevent the possible removal of DNA base adducts and potential strand bias effects produced by transcription-coupled DNA repair. Strain FX-10 was also *gyrA*⁺. Strain FX-10 *alkB* was constructed by P1 transduction from *E. coli* HK82 [23] into FX-10 with selection for the closely linked *nalA* (*gyrA*) allele, conferring nalidixic acid resistance. The AlkB[−] phenotype was confirmed by enhanced sensitivity to methylmethane sulfonate (MMS). Strain FX-10 *alkBΔumuDC* was constructed by P1 transduction of the *umuDC595::cat* allele from strain RW82 [24] into FX-10 *alkB*. The UmuDC[−] phenotype was confirmed by loss of ultraviolet (UV) light mutability. All three strains, FX-10, FX-10 *alkB* and FX-10 *alkBΔumuDC* were transformed with pUP21 and pUP27, and transformants were selected on LB-ampicillin plates.

Mutagenesis Assay

The mutagenesis assays were conducted as follows. Overnight cultures of the plasmid-harboring FX-10 strains were grown in LB broth supplemented with ampicillin (100 μg/ml). The next day, the cultures were diluted 1:100 into 10 ml of the same medium. Cells were grown at 37°C with shaking until the OD₄₅₀ reached 0.25. Then, the cultures were divided into two tubes of 5 ml each. IPTG was added to one tube to give a final concentration of 1 mM. The

tubes were then incubated with shaking for 30 min at 37°C to induce transcription from the UP-*tac* promoter. Then, 1 ml from each tube was removed to two 1.5 ml microcentrifuge tubes. MMS was added to one of the tubes to give a final concentration of about 12 mM and the cells were placed at 37°C for 15 min. Next, the cells were harvested by centrifugation and 1 ml of LB broth plus or minus IPTG (1 mM) was added. Following an incubation period of 60 min at 37°C to allow expression of the mutant phenotype, dilutions of the cells were plated onto LB-kanamycin plates (50 µg/ml) to assay for mutagenesis or LB-ampicillin plates (100 µg/ml) to assay viable cell counts. All plates were incubated at 37°C overnight and the number of colonies appearing on the plates were counted. The frequency of kanamycin-resistant revertants (Kan^R) was determined by dividing the number of colonies on the LB-kanamycin plates by the number of colonies on the LB-ampicillin plates and correcting for the dilutions employed.

DNA Sequence Analysis

A large collection of independent Kan^R revertants was isolated as described above with a few changes to the procedure. Cells were grown to an OD₄₅₀ of 0.25, the culture was divided in half and IPTG was added to one of the tubes. Following a 30 min incubation, the cells were divided into multiple 1.5 ml centrifuge tubes and exposed to 0 or 12 mM MMS. Following a 15 min incubation, the cells were centrifuged, resuspended in LB broth with or without IPTG and 100 µl was transferred to individual wells of a 96-well dish. The dishes were incubated for 1 hour at 37°C to allow expression of the mutant phenotype and 20 µl from each well was plated onto LB plates with kanamycin (50 µg/ml). After overnight incubation of the agar plates, a single mutant colony was picked from each separate well and restreaked on a plate with kanamycin.

To sequence the revertants, a 1.5 ml culture was grown from each independent colony and plasmid DNA was isolated using BioRobot 9600 (Qiagen, Valencia, CA) from 96 cultures per batch. DNA sequencing was performed using the primer 5'-ATGATTGAACAAGATGG (SIGMA-Genosys, The Woodlands, TX) at the University of Michigan Sequencing Core (Ann Arbor, MI). The sequencing primer was specific for a part of the T7 RNA polymerase promoter and the sequencing runs typically were of ~700 nt. A ~500 nt stretch of DNA in the coding region of the *kan* gene from several revertants (typically ~20) were aligned with the sequence of the *kanS-94D* allele using MacVector software (v. 9.0.2, MacVector Inc., Cary, NC). Mismatches in the alignment were considered to be potential mutations and any sequencing ambiguities were resolved by inspecting the original chromatograms using Chromas Software (Technelysium Pty. Ltd., Australia).

Statistical analyses to compare different mutation frequencies or types of base substitutions utilized the Student's *t*-test. The test performed a two-tailed analysis using the assumption that the population samples had the same variance. A probability value less than 0.01 was used to determine significant differences between the two samples tested.

RESULTS

Effects of MMS and IPTG on Cell Survival

The effect of high transcription of the *kan-ble* genes on cell survival with or without MMS treatment was examined. Table 1 shows the survival of FX-10 and its derivatives containing pUP21 or pUP27 grown under different conditions. For the pUP21 harboring strains, addition of 1 mM IPTG to the growth media in the absence of MMS treatment caused only a small reduction (10–30%) in survival. In comparison, when cells were exposed to 12 mM MMS for 15 min, survival was reduced in a strain-dependent manner. In the *alkB*⁺ strains, survival was reduced to about 20–30 percent. In the *alkB* strains, survival was reduced to about 15 percent

of the untreated cells and in the *alkBΔumuDC* strains, survival was reduced to roughly 10 percent. Addition of IPTG prior to MMS treatment did not seem to significantly affect survival in any of the strains. Comparable results were seen with cells containing pUP27, except that these cells were slightly less sensitive to the effects of addition of IPTG to the growth media. These results show that while the addition of MMS had significant effects on cell survival when AlkB was missing, addition of IPTG did not have a dramatic effect on survival with or without MMS treatment in the three genetic backgrounds tested. It should be noted that the survival value observed for the FX-10 *alkB* strains was very similar to that observed by Nieminuszczy et al. [17]. In contrast, the *alkB*⁺ FX-10 strain was more sensitive to MMS than the *alkB*⁺ strain (AB1157) used in the previous study [17].

Spontaneous Mutations in *alkB* Cells

Spontaneous Kan^R revertants may result from a variety of mechanisms including endogenous alkylation damage. To test the effect of AlkB and active transcription on the production of spontaneous mutations, cells containing pUP21 or pUP27 were grown in the presence or absence of IPTG and Kan^R revertants were selected. Overall, the spontaneous Kan^R mutation frequencies were relatively low and similar to one another (Table 1). In the case of pUP21, the average frequency of revertants in the *alkB* strain was somewhat greater than the average frequency in the *alkBΔumuDC* or the wild-type strain. Regardless, these differences were not statistically significant. Addition of IPTG to the growing cultures affected the frequency in all strains only slightly and again, the differences were not significant. Therefore, spontaneous mutations did not appear to be dramatically affected by increased transcriptional activity of the *kan* gene or AlkB-mediated repair.

Frequencies of MMS-induced Kan^R Mutants

Treatment of cells with 12 mM MMS increased the frequency of Kan^R revertants in all three genetic backgrounds tested (Table 1). This was true for both the plasmids regardless of the state of transcription of the *kan* gene. As expected, AlkB suppressed the mutagenicity of MMS. For example, in the absence of IPTG induction, MMS treatment increased the Kan^R frequency 4-fold in pUP21 in the *alkB*⁺ background. In comparison, the Kan^R frequency was enhanced by MMS about 14-fold for the same plasmid in the *alkB* strain (compare rows 1 and 3 in Table 1). Presence of the SOS-inducible DNA polymerase, PolV, was also important for the mutagenicity of MMS. In the *alkBΔumuDC* strain, MMS-caused mutations in pUP21 during growth without IPTG at about the same level as in the *alkB*⁺ strain (row 3, Table 1). Furthermore, the mutagenic effects of MMS in pUP27 when transcription of the *kan* gene was at a low level were similar to those in pUP21 (compare rows 5 and 7 in Table 1). These data show that MMS was mutagenic in this genetic system and that its mutagenicity was reduced greatly by AlkB-mediated repair. Additionally, much of the MMS mutagenesis required PolV-mediated translesion synthesis. It should be noted that, in the absence of IPTG, the absolute frequency for the MMS-induced mutations in the pUP21 *alkB* strain was much greater than the frequency in the pUP27 *alkB* strain, but that the fold increase due to MMS was roughly equivalent (14-fold vs 12-fold, respectively). The greater frequency in the pUP21 strain may reflect a lack of complete repression of the UP-*tac* promoter in the absence of IPTG.

When transcription from the UP-*tac* promoter was induced with IPTG, MMS caused different levels of mutation depending on the orientation of the *kan* gene. In all three genetic backgrounds, combining IPTG-induction of the UP-*tac* promoter in pUP21 with MMS treatment increased the Kan^R frequencies more than MMS treatment alone (compare rows 3 and 4 in Table 1). The greatest frequency was observed in the *alkB* strain with both MMS and IPTG (Table 1, row 4, *alkB* revertant frequency, highlighted in bold text). While the magnitude of the difference between the high and low transcription conditions was different in different backgrounds, the ratio of mutation frequencies with or without IPTG was ~3 to 4. The

enhancement of mutations by PolV in both the absence and presence of IPTG suggests that the mechanism of mutagenesis under the two conditions was similar.

A different pattern of Kan^R revertants was seen with the plasmid pUP27 in response to transcription from the UP-*tac* promoter. In contrast to pUP21, induction of UP-*tac* failed to increase Kan^R frequencies for pUP27 (compare lines 7 and 8 in Table 1). Together, the results with pUP21 and pUP27 plasmids demonstrate that the mutagenic effects of MMS were greatly enhanced by transcription when the cytosine residues within the CCA missense codon were located in the non-transcribed DNA strand but not in the transcribed strand. Since MMS-induced damage occurs more frequently in single-stranded DNA and also because AlkB repairs single stranded DNA more effectively, the results strongly suggest a role for damaged cytosine residues in the production of these revertants. To confirm this point, DNA sequence analysis was employed to examine the nature of mutations in the Kan^R revertants.

Relative Frequencies of Different Classes of Mutations

DNA sequence analysis was performed on a total of 806 revertants and this analysis revealed that all three possible base changes at the first two positions of the CCA codon could be recovered among the revertants. No mutations outside codon 94 of *kan* were obtained in a ~500 bp window in any of the revertants. The distribution of the changes in codon 94 is listed in Table 2 and Supplemental Table 1.

One way to analyze the mutational data is to look at the types of base substitutions within codon 94 of *kan* under each genetic and growth condition (Table 2). In both wild-type and *alkBΔumuDC* backgrounds, C to T transitions dominated the spectra and ranged from 74%–96%. This was true for either plasmid and regardless of whether the cells were grown in the presence of IPTG or treated with MMS. However, C to T transitions did not dominate the mutations in the *alkB* background under some conditions; instead, C to A transversions formed a significant proportion of the total mutations (Table 2), particularly when transcription was induced with IPTG and cells were treated with MMS. For example, in pUP21, the percentage of C to A mutations increased from 10% to 29% due to IPTG alone, and 10% to 49% due to MMS treatment (Table 2). Under the combined conditions of growth in the presence of IPTG and MMS treatment, this class of mutations was approximately half of all the mutations for either plasmid used in the *alkB* background (Table 2). Thus C to A transversions increase substantially when MMS attacks a highly transcribed gene and these may be more characteristic of the type of mutations prevented by AlkB than C to T transitions. The percentage of C to G transversions was also somewhat higher in the *alkB* background compared to wild-type or *alkBΔumuDC* strains and these numbers were higher in MMS-treated cells (Table 2).

Transcription and PolV Dependence of Different Classes of Mutations

Another way to look at these data is to compare the frequencies of transition or transversion mutations for each of the two mutable positions in codon 94 of *kan* under different experimental conditions. The numbers, percentages and frequencies at the two positions for mutations are listed in Supplemental Table 1; the MMS-induced mutation frequencies are shown in Figure 2. One conclusion that can be drawn from this analysis is that, in the *alkB* strain, the frequency of C to T and C to A mutations caused at either position increased significantly for the *kan* gene in pUP21 when transcription was induced with IPTG. For example, the frequency of C to T mutations at the second codon position increased from 6.1×10^{-6} to 23.0×10^{-6} , while the frequency of C to A mutations at the same position increased from 2.8×10^{-6} to 21.3×10^{-6} (Figure 2 and Supplemental Table 1B). The variation within these data is small (Supplemental Table 1B) and the differences are statistically significant.

As mentioned earlier, the increases in Kan^R reversion frequency due to MMS treatment with IPTG were not significant in the *alkBΔumuDC* background and the predominant class of mutations in this case was a C to T change. The C to T mutations at each of the two codon positions reflect this lack of IPTG dependence. For example, in this genetic background C to T mutation at the first position increased only from $(0.9 \pm 0.8) \times 10^{-6}$ to $(2.8 \pm 1.8) \times 10^{-6}$ when IPTG was included in the growth media (Supplemental Table 1C). Thus the IPTG-dependent increases in C to T mutations caused by MMS also depended on the presence of PolV in cells.

Finally, a few C to G transversions were obtained in pUP21 at the first codon position in the *alkB* background (Table 2, Figure 2 and Supplemental Table 1B). While the number of these revertants was small, the pattern was consistent; mutation frequencies due to MMS were greatest in the *alkB* pUP21 strain following treatment with IPTG. The frequency of these mutations was also lower in *alkBΔumuDC* background (Supplemental Table 1C).

In contrast to the pUP21 results, MMS-induced mutation frequencies produced in plasmid pUP27 in the absence or presence of IPTG did not show any strain to strain variation, nor was there a significant difference between the two positions within the CCA sequence (Figure 2 and Supplemental Table 1). These results show that transcription did not affect the susceptibility of DNA to damage in pUP27 as much as it did in pUP21.

DISCUSSION

The results described in this paper shed light on several interesting aspects of MMS mutagenesis and DNA repair by AlkB. To begin with, we investigated whether spontaneous revertants of the *kanS-94D* allele might result from endogenous alkylation damage that was susceptible to AlkB-mediated repair or transcriptional activity. In our experiments, no effect on spontaneous mutagenesis due to transcription was observed in the absence of AlkB or DNA PolV. This contrasts with spontaneous hydrolytic deamination of C to U (causing C to T mutations) or oxidation of guanine to 8-oxoguanine (G to T mutations). In appropriate DNA repair-deficient backgrounds the frequency of spontaneous cytosine deamination and guanine oxidation was increased substantially by transcription of the reporter gene [20,21]. The lack of such an increase in spontaneous mutations due to IPTG in the *alkB* background suggests that, during normal growth, *E. coli* DNA suffers little alkylation damage involving G:C base pairs that is promoted by transcription and is repairable by AlkB.

Our results also demonstrate several interesting points regarding MMS-induced DNA damage that gives rise to Kan^R revertants. In particular, these points apply to MMS damage that produces C to T and C to A mutations. First, this damage is susceptible to AlkB-mediated repair. Second, mutagenic processing by PolV is required to fix the lesions as mutations. Third, the damage or its conversion to a mutation is enhanced by transcription. Fourth, the mutagenic effects of MMS are sequence-dependent, especially when the target gene is being transcribed. Fifth, the transcription-dependence of MMS is seen when the CCA codon is in the NTS.

While the small amount of O⁶-methylguanine created by MMS could cause C to T transitions, this adduct is not susceptible to AlkB nor does it require PolV to produce a mutation. In contrast, MMS produces a large amount of m7G, which could produce primarily C to A transversions via an apurinic site intermediate [25]. This process would require PolV but would not be expected to be susceptible to AlkB. Based on these considerations, and results from Delany and Essigmann [16], the DNA adduct responsible for the AlkB-susceptible PolV-dependent mutations is likely to be m3C or m1G. The use of IPTG to induce high-level transcription from the UP-*tac* promoter helped to identify the DNA adduct responsible.

IPTG significantly enhanced the MMS-induced C to T, C to A and C to G mutations in the absence of AlkB and presence of PolV, but only in the pUP21-harboring strain. The m3C adduct, and probably the m1G adduct, are produced better in single stranded DNA [1,2] because of the accessibility of the N-3 (pyrimidine) or N-1 (purine) position in the absence of base pairing interactions. Since transcription initiated from the UP-*tac* promoter in plasmid pUP21 places the cytosine residues of the target CCA sequence in the non-transcribed DNA strand, which would be expected to be in a more single stranded state, it seems clear that the lesion responsible for the increased mutagenesis must be m3C. If m1G contributed significantly to the observed mutations, the frequencies in the *alkB* pUP27 strain would have also been expected to increase, and this was not the case.

Our results also suggest that several factors including sequence context and the state of transcription determine the mutation spectrum of MMS. Delany and Essigmann [16] found that in an *alkB* background, m3C caused 50% C to T transitions, 43% C to A transversions and 7% C to G transversions. Nieminiusz et al. [18], found a different distribution of mutations (72%, 18% and 9%, respectively). We found that this dominance of C to T mutations was observed at the second position in CCA, but not the first (Supplemental Table 1B). At the first position cytosine in the absence of IPTG, C to T transitions only accounted for 12% of the total mutations at that site, while C to A transversions predominated (71%). With inclusion of IPTG in the growth media, the frequency of all mutations increased but the percentage of C to T transitions remained essentially unchanged (14%); the percentage of C to A transversions was reduced to 50% of the total and the percentage of C to G transversions increased from 18% to 36%.

Several factors could account for the differences between the previously reported MMS mutation spectra [16–18] and what is described above. First, the strains we employed were nucleotide excision repair (NER) defective (*uvrA*), unlike the strains used in other experiments. We used the *uvrA* derivatives to prevent the possible removal of DNA base adducts and potential strand bias effects produced by transcription-coupled NER of lesions. It is possible that some lesions created by MMS are subject to repair by NER and thus lack of NER changes the mutation spectrum. Second, the local sequence context may influence the production and repair of MMS-induced lesions. This would be less of a problem in the single-adduct experiments [16] but may play a role in the other mutagenesis studies [17,18]. For example, sequence-dependent differences in the level of alkylation have been observed for the O⁶-alkylguanine adduct; the guanine of a 5'-PuG sequence is more susceptible to alkylation and mutagenesis [26–29]. It is not known if such influences affect the formation of m3C. In addition, the CCA target sequence in the *kanS-94D* allele is contained within a CCAGG palindrome, the position of activity by the *E. coli dcm* gene product (Dcm, DNA-cytosine methylase), which normally methylates the second cytosines on both DNA strands to produce 5-methylcytosine [30]. Possibly, the presence of 5-methylcytosine alters the level of m3C within the target sequence. It is also possible that very short patch (VSP) repair [31,32], a mismatch repair process that acts within this context, affects the mutational outcome. Third, the DNA sequence at the site of the adduct may influence base selection by DNA polymerase and this could play a role in the type of substitution that results. Such "template-directed" effects have been repeatedly observed (for examples see [33,34]). Obviously, there is not one single model to explain these differences. Rather, multiple cellular processes interact to produce the final outcome.

These results provide important new insights into the role of transcription in MMS mutagenesis. Specifically, they show that MMS-induced Kan^R mutations are significantly increased when the affected cytosines are located in the non-transcribed strand. It is useful to note that we also have data regarding reversion of the nonsense mutation within the *ble-op75* allele by MMS that confirm this suggestion and extend to alkylation of adenine and thymine residues (D. Fix,

unpublished results). Furthermore, preliminary results also suggest that mutagenesis by the S_N1 -type agents methylnitrosourea (MNU) and ethylnitrosourea (ENU) is not strongly dependent on transcription. These agents react readily with double-stranded DNA and produce a much greater percentage of O-alkylated bases that contribute to mutations rather than N-alkylated bases [1,2]. Together these results argue that the pattern of alkylation (and other types of DNA damage) at positions that are less accessible within duplex DNA would be strongly influenced by the state of transcription of DNA.

Previous work in *E. coli* [19–21,35,36] and yeast [37–40] has shown that increased transcription promotes “spontaneous” DNA damage and results in mutations. The work presented here extends this observation to certain types of alkylation damage to DNA in *E. coli*. MMS is a classic alkylating chemical used to study the response of cells to alkylating agents and has a similar mode of action to clinically important agents such as busulfan [41]. Therefore, a better understanding of MMS mutagenesis may lead to a greater understanding in a variety of cells how the damage to DNA is targeted to specific sites and bases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Transcription of the *kanS-D94 ble-op75* operon in pUP plasmids in the presence or absence of IPTG induction. The black lines indicate the DNA, while the gray lines indicate the mRNA. The CCA missense codon is shown. In plasmid pUP21, the resident weak promoter for the *kan-ble* operon (*P_{kan}*) is oriented in the same direction as the strong *UP-tac* promoter, while in plasmid pUP27, the orientation is reversed. In the absence of IPTG, only the *P_{kan}* promoter is expressed, resulting in transcription of the normally transcribed strand in both plasmids. When IPTG is present, high level expression of the normally transcribed strand occurs in pUP21, while high level expression of the normally non-transcribed strand occurs in pUP27.

Figure 2. Mutation frequencies ($\times 10^{-6}$) for MMS-induced Kan^R revertants. The base changes resulting in Kan^R are shown on the right. Treatment with or without IPTG for each strain (*alkB*⁺, *alkB* or *alkBΔumuDC*) harboring either pUP21 or pUP27 is shown along the bottom.

Surviving fractions and mutation frequencies ($\times 10^{-6}$) for Kan^R revertants. Three independent experiments were performed with each strain/plasmid combination and the results were averaged. Surviving fractions are presented relative to the untreated cells (minus IPTG, minus MMS, rows 1 and 5), plus or minus the standard error of the mean (SEM). Kan^R revertant frequencies ($\times 10^{-6}$) are presented plus or minus the SEM. The most significant frequencies are highlighted in bold.

Table 1

Plasmid	Treatment		Surviving Fractions			Revertant Frequency ($\times 10^{-6}$)			Row #
	IPTG	MMS	<i>alkB</i> ⁺	<i>alkB</i>	<i>alkB</i> Δ <i>umuDC</i>	<i>alkB</i> ⁺	<i>alkB</i>	<i>alkB</i> Δ <i>umuDC</i>	
pUP21	–	–	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.49 ± 0.24	1.12 ± 0.90	0.31 ± 0.09	1
	+	–	0.76 ± 0.05	0.89 ± 0.06	0.67 ± 0.02	0.74 ± 0.20	1.61 ± 1.12	0.47 ± 0.07	2
	–	+	0.23 ± 0.03	0.15 ± 0.01	0.07 ± 0.01	2.11 ± 0.21	15.8 ± 0.4	2.08 ± 1.82	3
	+	+	0.19 ± 0.02	0.11 ± 0.01	0.05 ± 0.01	6.03 ± 2.23	67.2 ± 4.8	9.13 ± 5.93	4
pUP27	–	–	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.81 ± 0.36	0.33 ± 0.15	0.18 ± 0.04	5
	+	–	0.99 ± 0.07	0.95 ± 0.08	0.92 ± 0.10	0.87 ± 0.36	0.29 ± 0.07	0.34 ± 0.09	6
	–	+	0.24 ± 0.05	0.14 ± 0.00	0.08 ± 0.01	2.88 ± 1.01	3.94 ± 1.03	1.41 ± 0.94	7
	+	+	0.36 ± 0.05	0.16 ± 0.01	0.10 ± 0.01	1.66 ± 0.71	2.87 ± 1.26	2.97 ± 2.97	8

Table 2

Mutation specificity of Kan^R revertants. The actual number of Kan^R revertants sequenced is listed, followed by the percentage of the total (in parentheses) for each of the strains, plasmids and conditions employed.

Strain FX-10 <i>alkB</i> ⁺ Plasmid	Treatment		Number and Percentage of Sequenced Mutations			
	IPTG	MMS	C to T	C to A	C to G	Total
pUP21	-	-	34 (85)	6 (15)	0 (0)	40 (100)
	+	-	31 (74)	8 (19)	3 (7)	42 (100)
	-	+	36 (86)	5 (12)	1 (2)	42 (100)
	+	+	37 (88)	4 (10)	1 (2)	42 (100)
pUP27	-	-	22 (88)	3 (12)	0 (0)	25 (100)
	+	-	22 (79)	5 (18)	1 (4)	28 (100)
	-	+	33 (80)	8 (20)	0 (0)	41 (100)
	+	+	30 (77)	9 (23)	0 (0)	39 (100)
Strain FX-10 <i>alkB</i>						
pUP21	-	-	37 (88)	4 (10)	1 (2)	42 (100)
	+	-	28 (67)	12 (29)	2 (5)	42 (100)
	-	+	17 (44)	19 (49)	3 (8)	39 (100)
	+	+	16 (39)	20 (49)	5 (12)	41 (100)
pUP27	-	-	39 (95)	2 (5)	0 (0)	41 (100)
	+	-	34 (85)	6 (15)	0 (0)	40 (100)
	-	+	27 (66)	12 (29)	2 (5)	41 (100)
	+	+	17 (40)	22 (51)	4 (9)	43 (100)
Strain FX-10 <i>alkBΔumuD</i> C						
pUP21	-	-	20 (91)	2 (9)	0 (0)	22 (100)
	+	-	18 (78)	5 (22)	0 (0)	23 (100)
	-	+	22 (96)	0 (0)	1 (4)	23 (100)
	+	+	18 (78)	3 (13)	2 (9)	23 (100)
pUP27	-	-	19 (86)	3 (14)	0 (0)	22 (100)
	+	-	19 (90)	1 (5)	1 (5)	21 (100)
	-	+	20 (91)	2 (9)	0 (0)	22 (100)
	+	+	21 (88)	3 (13)	0 (0)	24 (100)