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Mismatch repair proteins collaborate with methyltransferases in the repair of O^6 -methylguanine

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Abstract

DNA repair is essential for combatting the adverse effects of damage to the genome. One example of base damage is O^6 -methylguanine (O^6 mG), which stably pairs with thymine during replication and thereby creates a promutagenic O^6 mG:T mismatch. This mismatch has also been linked with cellular toxicity. Therefore, in the absence of repair, O^6 mG:T mismatches can lead to cell death or result in G:C→A:T transition mutations upon the next round of replication. Cysteine thiolate residues on the Ada and Ogt methyltransferase (MTase) proteins directly reverse the O^6 mG base damage to yield guanine. When a cytosine is opposite the lesion, MTase repair restores a normal G:C pairing. However, if replication past the lesion has produced an O^6 mG:T mismatch, MTase conversion to a G:T mispair must still undergo correction to avoid mutation. Two mismatch repair pathways in *E. coli* that convert G:T mispairs to native G:C pairings are methyl-directed mismatch repair (MMR) and very short patch repair (VSPR). This work examined the possible roles that proteins in these pathways play in coordination with the canonical MTase repair of O^6 mG:T mismatches. The possibility of this repair network was analyzed by probing the efficiency of MTase repair of a single O^6 mG residue in cells deficient in individual mismatch repair proteins (Dam, MutH, MutS, MutL, or Vsr). We found that MTase repair in cells deficient in Dam or MutH showed wild-type levels of MTase repair. In contrast, cells lacking any of the VSPR proteins MutS, MutL, or Vsr showed a decrease in repair of O^6 mG by the Ada and Ogt MTases. Evidence is presented that the VSPR pathway positively influences MTase repair of O^6 mG:T mismatches, and assists the efficiency of restoring these mismatches to native G:C base pairs.

Keywords

Mismatch repair; Methyltransferase repair; O^6 -Methylguanine; Very short patch repair

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2007.09.003.

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1. Introduction

Alkylating agents can produce a host of DNA lesions by reacting at different nucleophilic atoms on the DNA bases. The net biological impact of a lesion depends on the balance between the ability of the cell to repair the lesion and the mutagenic and toxic properties of the lesion. One particularly critical type of DNA alkylation damage is *O*⁶-methylguanine (*O*⁶mG), which has been shown to be both mutagenic [1–4] and, when cells cannot distinguish nascent from parental DNA strands, toxic [5–7]. Extensive *in vivo* mutagenesis studies done in our laboratory and others established that *O*⁶mG codes as adenine during replication in *E. coli* nearly 100% of the time [1,8]. As a result, initial replication past the lesion results in a promutagenic *O*⁶mG:T mismatch, and the following round of replication yields a G:C→A:T transition mutation (Fig. 1). The toxicity of this lesion is thought to arise from the action of methyl-directed mismatch repair (MMR) on *O*⁶mG:T mismatches [9–11]. It is hypothesized that recursive and futile attempts to replace the mismatched thymine with a base that does not recreate a substrate for another round of MMR, may lead to persistent repair gaps and toxic double strand breaks [12]. Therefore, the repair of *O*⁶mG is paramount to avoiding the lethality of alkylating agents.

In *E. coli*, repair of *O*⁶mG (Fig. 1) is the responsibility of the methyltransferase (MTase) proteins Ada and Ogt [6]. Both of these proteins can repair *O*⁶mG directly by transferring the methyl group to the sulfhydryl group of an active site cysteine residue [13–16]. In the case where MTase repair of *O*⁶mG occurs after one round of replication, a mismatched G:T base pair results. To prevent mutation, this mismatch must be processed further before replication.

The G:T mismatch is a substrate for two different types of mismatch repair (MR), very short patch repair (VSPR) [17] and MMR [18]. Both systems are stimulated by the activities of the MutS and MutL mismatch recognition proteins, although the downstream mechanisms of DNA strand incision and replacement are quite different.

In the case of MMR, the MutS and MutL proteins recruit the MutH endonuclease to create a single-stranded incision at the nearest canonical MutH cleavage sequence [d(GATC), either 5' or 3' to the mismatch] not yet fully methylated by Dam [19]. It has been shown that the distance between the incision and the damage sites can be thousands of nucleotides [20]; and therefore significant DNA resynthesis is necessary. Subsequent to incision, removal of the error-containing strand is accomplished through the concerted efforts of many proteins including DNA helicase II, single-stranded DNA binding protein, and Exo I, Exo VII, Exo X, or Rec J [21]. DNA Polymerase III resynthesizes the error-containing strand and DNA Ligase seals the nick [22].

In the case of VSPR, the MutS and MutL proteins recruit the Vsr endonuclease, which creates a single-stranded nick adjacent to the mismatched thymine on its 5' side [23]. Pol I then removes and replaces a small number of bases (<10) 3' to the nick utilizing its 5' → 3' exonuclease and polymerase activities [24]. DNA Ligase seals the nick and completes the restoration of the native G:C pairing.

The primary purpose of VSPR is believed to be the repair of G:T mismatches that arise through the deamination of the Dcm product, 5-methylcytosine [25]. The Dcm protein methylates the 5-carbon of the second cytosine of the sequence 5'-CCWGG-3' (where W is an A or T) [26]. Therefore, Vsr most commonly initiates repair on G:T mismatches in the 5'-CTWGG-3' sequence context (where T is mismatched with guanine). It is noteworthy, however, that Vsr also has significant endonuclease activity for G:T mismatches in a variety of sequence contexts [27,28]. Therefore, VSPR may also play a role in repairing G:T mismatches that arise through MTase repair of *O*⁶mG:T mismatches.

Proper restoration of O^6 mG:T mismatches to G:C base pairs requires that mismatch repair follows methyltransferase repair, and we hypothesize that this process may be accomplished by coordination between MR and MTase proteins. To investigate this possibility, a matrix set of mutant *E. coli* defective in MTase activities (Ada, Ogt, or both) and a single MR protein (Dam, MutH, MutS, MutL, and Vsr) was constructed. We then studied the efficiency of O^6 mG repair *in vivo* by MTases using an assay that has been previously described [29]. Our assay uses the genome of phage M13 into which O^6 mG has been inserted site-specifically to model the processing of that lesion, in the presence or absence of the aforementioned proteins, in chromosomal DNA. In total, 24 cell strains were assayed, allowing for a comprehensive view of how MR proteins affect MTase repair of O^6 mG. Cells deficient in Dam and MutH were observed to repair O^6 mG as efficiently as their parental counterparts, indicating that the efficiency of MTases is not affected by a deficiency in the MMR pathway. In contrast, strains deficient in MutS, MutL, and/or Vsr showed a decrease in O^6 mG repair by Ada and Ogt, revealing that MTase repair, in order to be maximally efficient, relies on the VSPR pathway. These data show that MTase repair *in vivo* is significantly affected by MR proteins, and assist in our understanding of how seemingly unrelated repair systems collaborate to maximize efficient repair and protect genome integrity.

2. Materials and methods

2.1. Strains

Construction and characterization of all strains is described in the supplementary information. Briefly, parental C215 (wt), C216 (*ogt::kan*), C217 (*ada,alkB::cam*), and C218 (*ogt::kan, ada,alkB::cam*) strains have been described previously (as FC215 derivatives) [8,30–32], and were transduced using mutant alleles produced by PCR mediated gene replacement [33]. Alleles provided insertion deletion mutants in which the majority of the target MR gene was replaced by a marker for tetracycline resistance. All strains were characterized genotypically by sequencing the PCR products obtained using primers that amplified regions within or near the *ada*, *dam*, *mutH*, *mutL*, *mutS*, *ogt*, and *vsr* genes.

2.2. Construction of O^6 mG-containing M13 viral genome

Genomes were constructed on a 24 pmol scale, essentially as described previously [29]. Single-stranded M13mp7 (L2) DNA (2.4 pmol/ μ L) was linearized by cleavage with 70 units (U) of *Eco*RI (New England Biolabs, NEB) for 8 h at 23 °C in a total of 50 μ L, using the supplier's buffering conditions. Cleavage occurred at a hairpin containing an *Eco*RI site [29]. The linearized M13mp7 (L2) DNA was annealed to 1.25 equivalents of the scaffolds 5'-GGT CTT CCA CTG AAT CAT GGT CAT AGC-3' and 5'-AAA ACG ACG GCC AGT GAA TTG GAC GC-3' which partially complemented the 5' and 3' sides of the insert and vector termini. Annealing was accomplished by heating the mixture to 50 °C for 5 min and cooling linearly to 0 °C over 50 min. The O^6 mG lesion-containing insert, 5'-GAA GAC CT O^6 mG GGC GTC C-3' was synthesized as described previously [29], and phosphorylated on the 5'-terminus with T4 PNK (10 U, NEB) using the supplier's buffering conditions supplemented with an excess of adenosine triphosphate (ATP, Roche). The phosphorylated insert (30 pmol), the annealed scaffold mixture (24 pmol), 1 μ L of 100 mM ATP (Roche), and 2 μ L of T4 DNA Ligase (800 U, NEB) were mixed in a final reaction volume of 55 μ L and incubated at 16 °C for 20 h to recircularize the genome [29]. The scaffold DNA was removed by supplementing the mixture with 3 μ L of T4 DNA polymerase (10.5 U, NEB) and incubating the mixture at 37 °C for 6 h, as previously described [34,35]. The genome construct mixture was brought to a final volume of 100 μ L with water, extracted twice with 100 μ L phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v, Invitrogen), and desalted using G-50 Sephadex Quick Spin Columns (Roche). Typical yields were in the range of 5% (1.2

pmol) and enough for approximately 200 electroporations in which at least 10,000 individual transformation events occurred.

2.3. Determination of O^6 mG repair efficiency

To prepare cells for electroporation, an over-night culture grown in Luria-Bertani (LB) media [36] (containing 50 μ g/mL kanamycin, 25 μ g/mL chloroamphenicol, or 12 μ g/mL tetracycline where appropriate) was diluted 1:50, in quadruplicate, in 10 mL LB and grown to mid-log phase ($OD_{600} \sim 0.6$). Cells were transferred into 15 mL tubes, harvested by centrifugation ($6000 \times g$, 10 min), washed twice with 15 mL 4 °C water, and resuspended in 75 μ L 10% glycerol. The following day, 100 μ L of competent cells ($\sim 2 \times 10^9$ cells) and 5 μ L of diluted O^6 mG-M13 viral genome (~ 5 fmol) were added to chilled electrocuvettes for transformation. Cell/genome mixtures were electropo-rated (2.5 kV, 129 Ω in a 0.2-cm cuvette) using a Electro Cell Manipulator 600 electroporation system (Bantex), transferred to 10 mL LB, and vortexed briefly. Fractions of this mixture (typically 20, 75, and 200 μ L) were transferred individually to tubes containing 3.75 mL overlay mix at 51 °C (see below), which were then briefly vortexed and spread over the surface of a B-broth plate (per L; 10 g tryptone, 8 g NaCl, 20 g bacto agar) [37]. Overlay mix was prepared by adding 375 μ L of a solution containing, per overlay, 300 μ L plating bacteria (NR9050 over-night diluted 1:5 in $2 \times$ YT and grown on a roller drum at 37 °C for 1.5 h), 25 μ L 1% thiamine (Sigma), 10 μ L of 100 mM IPTG (Roche), and 40 μ L of 40 mg/mL X-gal (Roche) in DMF to 3 mL B-broth soft agar (per L; 10 g tryptone, 8 g NaCl, 6 g bacto agar) [37] just before use.

After completion of all genome electroporations and cell platings (Fig. 2), the soft agar mix on the top of each plate was allowed to cool for an additional 10 min on the bench. Plates were then put at 37 °C, upside down. After incubation for 16–20 h, the plate from each replicate that contained the highest number of discernable plaques was scored for the number of light blue and dark blue plaques.

As described elsewhere [29], the color of each plaque indicates if lesion repair does (dark blue) or does not (light blue) occur before the viral DNA serves as a genetic code. If the lesion is repaired to guanine before transcription, the viral DNA will code properly for the alpha fragment of *lacZ*. In this case, the resulting alpha peptide of β -galactosidase will complement the omega peptide of the same protein (which is expressed from an F' episome within the cells), promote the hydrolysis of the chromogenic substrate X-gal, and subsequently lead to the formation of a dark blue color. In contrast, if the lesion is not repaired before the viral DNA codes, only a small amount of alpha peptide will be expressed, complementation to form active β -galactosidase will be infrequent, less X-gal will be hydrolyzed, and a light blue color will result. Therefore, this assay provides a simple method by which to quantify and compare the efficiency of O^6 mG repair to guanine for cell strains with different genetic backgrounds. Operationally, we define percent repair as dark blue plaques divided by total plaques (dark blue + light blue) $\times 100$.

Each plate we scored contained from ~ 100 to 800 plaques total, and averaged 397. The repair efficiency for each cell type was calculated in triplicate (at least), and the average and standard deviation of the replicates are reported. The number of individual transformation events was determined by dividing the number of plaques observed by the fraction of total volume plated. On average, the repair efficiency for each replicate was calculated from $\sim 40,000$ individual transformation events.

3. Results

3.1. Rates of methyltransferase repair in parental strains

The 24 cell strains assayed for their relative rates of O^6 mG repair showed a wide range of MTase activity. Firstly, the data from the C215, C216, C217, and C218 parental strains (Fig. 3, black columns) demonstrate good correlation between percent repair of O^6 mG and MTase status of the cell. As expected, the C215 wt strain that expresses both functional methyltransferases (Ada and Ogt) shows a high amount of lesion repair ($97.3 \pm 0.4\%$). The C216 strain, which lacks the Ogt protein (but still has Ada activity), shows significantly less net MTase activity ($68.4 \pm 2.9\%$), while the C217 strain, which lacks the Ada protein, shows near wt levels of MTase repair ($94.9 \pm 0.7\%$). The asymmetry in the results for these mutants was not surprising as the normal *E. coli* cell contains roughly thirty Ogt molecules [15] and only one molecule of Ada [38]. Therefore, cells that lack the predominant MTase under normal conditions, Ogt, were expected to display the more significant decrease in MTase activity. Accordingly, the C218 double mutant displayed results consistent with very poor repair of O^6 mG ($6.3 \pm 1.0\%$).

3.2. Rates of methyltransferase repair in *dam*[−] and *mutH*[−] mutants

Results for the strains deficient in *dam* (Fig. 3, gridded columns) and *mutH* (Fig. 3, light gray columns) did not deviate dramatically from the values observed in the parental strains. The percent repair of O^6 mG in the *dam*[−] strains C215D, C216D, C217D, and C218D were $96.0 \pm 0.4\%$, $79.7 \pm 0.6\%$, $96.3 \pm 0.7\%$, and $10.0 \pm 2.1\%$, respectively. The percent repair data for C215H, C216H, C217H, and C218H *mutH*[−] strains were $96.9 \pm 0.6\%$, $70.4 \pm 1.5\%$, $96.3 \pm 1.5\%$, and $6.3 \pm 0.8\%$, respectively. These data indicate that the Dam and MutH proteins do not impact MTase repair of O^6 mG.

3.3. Rates of methyltransferase repair in *mutS*[−], *mutL*[−], and *vsr*[−] mutants

In contrast to *dam*[−] and *mutH*[−] strains, those deficient in *mutS*, *mutL*, and *vsr* displayed levels of O^6 mG repair significantly lower than those for the C215, C216, and C217 parental strains (Fig. 4). The C215S, C216S, C217S, and C218S *mutS*[−] strains (Fig. 4, white columns) exhibited percent repair efficiencies of $68.0 \pm 1.0\%$, $43.1 \pm 4.2\%$, $76.2 \pm 1.3\%$, and $5.7 \pm 1.1\%$, respectively. These values represent approximately 70%, 63%, and 80% of the C215, C216, and C217 repair observed in the three parental strains (Fig. 4, black columns), respectively. Similarly, the effect of knocking out *mutL* from the C215, C216, and C217 parental strains resulted in a decrease in repair to 68–76% that of wild-type. The percent repair efficiencies measured for the C215L, C216L, C217L, and C218L, *mutL*[−] strains (Fig. 4, hatched columns) were $67.4 \pm 1.0\%$, $52.1 \pm 1.1\%$, $64.8 \pm 2.6\%$, and $4.7 \pm 1.5\%$, respectively. These data shows that cells lacking MutS and MutL are less efficient at repairing O^6 mG by Ada or Ogt.

Most interesting, the *vsr*[−] strains (Fig. 4, dark gray columns) also displayed less efficient repair of O^6 mG as compared to their parent strains. Knocking out *vsr* from the C215 background conferred an approximately 20% decrease in repair, from $97.3 \pm 0.4\%$ (C215 parent) to $77.7 \pm 1.2\%$ (C215V *vsr* mutant). Absence of Vsr from the MTase single knockouts, however, lowered the levels of O^6 mG repair more significantly. Knocking out *vsr* from the C216 background dropped MTase repair by ~67% of that for the parent (from $68.4 \pm 2.9\%$ to $45.8 \pm 3.6\%$). The level of MTase repair in C217V ($69.5 \pm 3.5\%$) was ~73% of that for the parent ($94.9 \pm 0.7\%$). The repair efficiency for C218V ($7.1 \pm 1.2\%$), like that for C218S ($5.7 \pm 1.1\%$) and C218L ($4.7 \pm 1.5\%$), was statistically identical to that for the C218 parent ($6.3 \pm 1.0\%$), which lacks both the Ada and Ogt MTases.

4. Discussion

The efficiency to which an O^6 mG lesion is repaired in *E. coli* depends on the number of MTase molecules present in the cell and the affinities of those MTases for the lesion. During exponential growth, approximately 30 molecules of Ogt [15] and 1 molecule of Ada [38] are present. On this basis, we expected to observe a more dramatic decrease in repair in cells lacking Ogt (C216) as compared to the cells lacking Ada (C217). Data for the parental strains verify that the repair efficiency of the C216 strain is indeed lower than for the C217 strain. However, the affinity of O^6 mG for Ada is greater than that for Ogt [39], and therefore the difference in repair observed for the *ada* and *ogt* mutants reflects multiple parameters. We therefore decided to group the repair efficiencies according to the MTase background of the parent cell.

Interestingly, we observed data that were consistent with a small amount of O^6 mG repair in the C218 double mutant strain bearing no MTase activity. We rationalized that this low level of repair signal was most likely due to a population of genome construct containing guanine in place of O^6 mG. The synthetic route used to produce the 16-mer lesion-containing insert utilizes a deprotection step that was reported to convert small amounts of the lesion to guanine [40]. Mass spectrometry was conducted on the lesion-containing 16-mer and confirmed the presence of a species corresponding to ~5% guanine at the lesion site (data not shown). This result was consistent with the basal level of “repair” signal in the MTase deficient cells.

Strains lacking *dam* and *mutH* showed repair levels that were similar to their parental counterparts (Fig. 3), indicating that these MMR proteins do not play a role in affecting repair of O^6 mG:T mismatches to G:C base pairs. These results likely reflect the property of these proteins to act at d(GATC) sequences that are remote from the mismatch [19]; Dam and MutH would not be expected to be present at O^6 mG:T sites and hence they would not be expected to influence MTase repair of the lesion in our system. It was a formal possibility that MMR could also effectuate repair of O^6 mG:T mismatches to G:C base pairs by a mechanism whereby MMR occurred before MTase repair. We also view this possibility as unlikely. By this model, MMR would have to replace the mismatched thymine in the daughter strand with cytosine before MTase repair of O^6 mG to guanine could follow. As indicated above, O^6 mG is highly mutagenic as evidenced by the observation that thymine is placed opposite the lesion nearly 100% of the time [8]. Therefore, the absence of contribution from Dam or MutH to the repair of O^6 mG:T mismatches was expected. It should also be noted that the mutator phenotype, which is associated with *dam*⁻ and *mutH*⁻ strains [41], had no significant effect on MTase repair efficiency.

Strains lacking MutS and MutL displayed a decrease in MTase repair. These results were interesting as MutS can bind to O^6 mG base pairs [10] and can theoretically shield the lesion from MTase repair. Indeed, it could be postulated that MutS complexes should compete with MTases proteins for access to O^6 mG base pairs. However, our data suggest otherwise. Strains bearing MutS and MutL proteins displayed a higher efficiency of O^6 mG repair by MTases, suggesting these proteins cooperate with Ada and Ogt.

The mechanism by which the presence of MutS and MutL stimulates MTase repair of O^6 mG would likely involve (i) the identification of the lesion by MutS/MutL followed by (ii) the dissociation of the complex from the mismatch to present and facilitate and (iii) downstream repair events (Fig. 5). MutL has been shown to assist MutS in mismatch presentation by stimulating the formation of an α -shaped DNA loop containing the mismatch [42], and the observation that the *mutL*⁻ and *mutS*⁻ mutants displayed similar decreases in MTase repair is consistent with previous observations that mutating either gene results in an identical

defect in mismatch recognition [43]. The observation that *dam*⁻ and *mutH*⁻ mutants do not show decreased levels of MTase repair makes it highly unlikely that the effects seen from knocking out *mutS* and *mutL* are the result of creating a mutator phenotype. Taken together, it therefore appears that a novel role of MutS and MutL mismatch repair proteins in assisting *O*⁶mG repair by MTases has been uncovered.

The *vsr*⁻ strains, in addition to *mutS*⁻ and *mutL*⁻ strains, show a significant decrease in MTase repair (Fig. 4, dark gray columns). We rationalize that Vsr dependent stimulation of one or both MTases (Fig. 5) increases the likelihood that (after mismatch recognition by MutS and MutL) Vsr, and not MutH, initiates repair of G:T mismatches that result from MTase repair of *O*⁶mG:T base pairs. Subsequently, VSPR, and not MMR, corrects the mismatch to the native G:C pairing with high efficiency.

This model is attractive from a number of perspectives. First, VSPR is the most energetically advantageous mechanism by which to process *O*⁶mG:T mismatches to G:C base pairs. As described above, VSPR requires fewer protein components and characteristically replaces a much shorter segment of the error-containing DNA strand as compared to MMR. Second, it may be that the cell depends on VSPR for correction of G:T mispairs. Given that *O*⁶mG:T mismatches arise through replication past the lesion-containing DNA strand, MMR would be targeted to the DNA strand opposite the lesion and reiteratively regenerate a MMR substrate. Eventually, if futile attempts to repair the mismatch do not kill the cell, the DNA becomes fully methylated at the d(GATC) sites by Dam, thereby inactivating MMR. At this point, correct repair of *O*⁶mG:T mismatches depends on MTase repair followed by VSPR. Therefore, stimulation of MTase repair by VSPR proteins may promote cooperative repair, which ultimately becomes necessary. Third, it is possible that cooperation between the VSPR and MTase systems could have assisted cellular survival and influenced the evolution of *E. coli*. If these bacteria have been continually challenged with the task of correcting post-replicative *O*⁶mG:T mismatches, it is plausible that cooperation between the MTase and VSPR proteins developed over time. In doing so, the organism would have gained a survival advantage by networking repair pathways to correct a cytotoxic lesion with high efficiency. Finally, the model presented here, based upon observations made on viruses replicating in living cells, could be studied in parallel from a biochemical perspective to provide further mechanistic insight into cooperation among DNA repair pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Repair of O^6 mG base pairs to counteract their mutagenic and toxic properties

Fig. 2. Overview of experimental methods

Fig. 3. Percent O^6 mG repair in parental, dam^- , and $mutH^-$ strains. Percent repair is determined from the (number of dark blue plaques/total plaques) $\times 100$; see Section 2 for details

Fig. 4. Percent O^6 mG repair in parental, $mutS^-$, $mutL^-$, and vsr^- strains. Percent repair is determined from the (number of dark blue plaques/total plaques) $\times$ 100; see Section 2 for details

Fig. 5. Hypothetical mechanism for stimulation of MTase repair by VSPR proteins