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Implications for Damage Recognition during Dpo4-Mediated Mutagenic Bypass of m1G and m3C Lesions

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SUMMARY

DNA is susceptible to alkylation damage by a number of environmental agents that modify the Watson-Crick edge of the bases. Such lesions, if not repaired, may be bypassed by Y-family DNA polymerases. The bypass polymerase Dpo4 is strongly inhibited by 1-methylguanine (m1G) and 3-methylcytosine (m3C), with nucleotide incorporation opposite these lesions being predominantly mutagenic. Further, extension after insertion of both correct and incorrect bases, introduces additional base substitution and deletion errors. Crystal structures of the Dpo4 ternary extension complexes with correct and mismatched 3′-terminal primer bases opposite the lesions reveal that both m1G and m3C remain positioned within the DNA template/primer helix. However, both correct and incorrect pairing partners exhibit pronounced primer terminal nucleotide distortions, being primarily evicted from the DNA helix when opposite m1G or misaligned when pairing with m3C. Our studies provide insights into mechanisms related to hindered and mutagenic bypass of methylated lesions and models associated with damage recognition by repair demethylases.

INTRODUCTION

Alkylation damage to DNA is induced by naturally occurring endogenous agents (De Bont and van Larebeke, 2004; Sedgwick, 1997), as well as by many environmental carcinogens (Hecht, 1999; Singer and Grunberger, 1983) and by cancer chemotherapeutics (Allan and Travis, 2005; Park et al., 2010). Secondary cancers that arise as a consequence of chemotherapy with alkylating agents are not unusual and are associated with mutation accumulation, genome instability and defects in DNA repair (Allan and Travis, 2005). Among the diverse lesions produced by the Srs2 type alkylating agents are methylation at the N1-position of adenine (m1A) and guanine (m1G), and at the N3-positions of cytosine (m3C) and thymine (m3T) (Figure 1A) (Sedgwick, 2004; Shrivastav et al., 2010).

The addition of a methyl group to the endocyclic N-atoms that are normally involved in Watson-Crick base pairing is cytotoxic and blocks DNA replication in Escherichia coli in the absence of the AlkB repair protein (Delaney and Essigmann, 2004). This result suggests that the polymerase responsible for replicating genomic DNA, presumably the B-family Pol III, in E. coli, would be drastically impeded by the m1A, m1G, m3C, and m3T lesions. In vitro data indicate that the A-family gap-filling E. coli Pol I Klenow fragment is severely hindered by the m1A (Larson et al., 1985), m3C (Boiteux and Laval, 1982; Saffhill, 1984), and m3T (Huff and Topal, 1987) lesions. Activation of the SOS-response in E. coli induces production of Pol IV and Pol V, the low-fidelity Y-family translesion synthesis (TLS) polymerases (Yang and Woodgate, 2007), which increases bypass of the m1A, m3C, m1G, and m3T lesions in vivo by ~3–4-fold (Delaney and Essigmann, 2004). The resulting progeny contain a staggering fraction of mutations: ~70% in the case of the m3C- and m1G-modified templates and ~53% in the case of the m3T; only m1A is weakly mutagenic generating ~1% of errors (Delaney and Essigmann, 2004). In eukaryotes, as in E. coli, Y-family polymerases temporarily replace stalled high-fidelity polymerases with damage-bypass polymerases (McCulloch and Kunkel, 2008; Waters et al., 2009; Yang and Woodgate, 2007). Currently, structure-function studies that address TLS polymerase-catalyzed bypass of blocking m1A, m3C, m1G, and m3T lesions are lacking.

In contrast to high-fidelity B- and A-family polymerases that produce tight-fitting, solvent-excluding reaction-ready active sites on binding of a complementary dNTP (Johnson and Beese, 2004; Steitz and Yin, 2004; Swan et al., 2009a), Y-family polymerases have more spacious and solvent-accessible active sites (Biertumpfel et al., 2010; Silverstein et al., 2010; Yang and Woodgate, 2007). Furthermore, Y-family polymerases do not have an exonuclease domain. Additionally, they do not check the minor groove edge of the template/primer to proofread mismatches and they select for the correct dNTP guided predominantly by base pairing complementarity (Yang and Woodgate, 2007). These structural and functional features enable Y-family polymerases to bypass a variety of DNA lesions and at the same time cause a higher error rate on undamaged DNA templates (Broyde et al., 2008; Waters et al., 2009; Yang and Woodgate, 2007).
Bypass of Methylation Damage Lesions by Dpo4

E. coli AlkB is an iron(II)-and 2-oxoglutarate-dependent repair protein that directly reverses alkylation damage on the endocyclic N-atoms of DNA and RNA bases by oxidative demethylation (Falnes et al., 2002; Trewick et al., 2002). AlkB proficiently removes methyl groups from m1A and m3C adducts (Sedgwick et al., 2007; Shrivastav et al., 2010) predominantly in single-stranded DNA, but it is markedly less efficient on the more relatively minor m1G and m3T lesions (Delaney and Essigmann, 2004; Falnes et al., 2002; Trewick et al., 2002; Yu and Hunt, 2009). The human genome encodes at least nine AlkB family members, ABH1 through ABH8 (Aravind and Koonin, 2001; Sedgwick et al., 2007) and the fat mass- and obesity-associated FTO protein (Gerken et al., 2007). ABH2 is recognized as a primary demethylase that protects genomic DNA, because mice lacking ABH2 accumulate significant levels of m1A in their genome even in the absence of any exogenous methylating agent (Ringvoll et al., 2006). Consistently, in vitro, ABH2 works preferentially on double-stranded DNA and repairs m1A, m3C, and m3T (Aas et al., 2003; Duncan et al., 2002; Falnes, 2004; Ringvoll et al., 2006); m1G-containing DNA was not evaluated. ABH1 (Westbye et al., 2008), ABH3 (Aas et al., 2003; Duncan et al., 2002; Falnes, 2004; Ringvoll et al., 2006), and FTO (Gerken et al., 2007; Jia et al., 2008) are most efficient on ssDNA and ssRNA; however, their substrate specificities are different. The exact cellular roles of ABH1 and ABH3, as well as ABH4–7 remain elusive. Surprisingly, FTO is a key factor in energy homeostasis regulation (Fischer et al., 2009), and ABH8 is involved in methylation of tRNA and participates in regulation of the DNA-damage response pathway (Fu et al., 2010a, 2010b).

The crystal structures of AlkB with short ssDNA (Yu et al., 2006) and AlkB and ABH2 (Yang et al., 2008) with dsDNA, where both ss- and dsDNAs contained the m1A lesion, as well as ABH3 in the absence of DNA or RNA (Sundheim et al., 2006) and FTO with the m3C nucleobase (Han et al., 2010) revealed strikingly similar overall folds of the catalytic domains. These proteins flip damaged bases and insert them into the active site for repair (Han et al., 2010; Sedgwick et al., 2007; Sundheim et al., 2006; Yang et al., 2008; Yu et al., 2006). Thus far only ABH2 has been identified as a dsDNA repair protein; it interacts with both damaged and undamaged strands and employs an aromatic residue to intercalate into the duplex DNA and fill the gap resulting from the base flipping (Han et al., 2010; Yang et al., 2008). It is currently unclear how ABH2 identifies damaged bases opposite their cognate partners in the context of the vast amount of undamaged dsDNA. Moreover, no data are available on lesion recognition opposite incorrect base partners that were mistakenly incorporated by DNA polymerases during replication. The structures of the cognate m1A-T and m3C-G alignments within dsDNA duplexes have provided valuable information into a possible DNA scanning mechanism employed by ABH2 (Lu et al., 2010), but much remains to be elucidated.

The objectives of this work were to explore structural and biochemical features of Dpo4-catalyzed bypass of the m1G and m3C lesions. We demonstrate that this TLS polymerase, that is able to efficiently and in many cases accurately bypass a variety of DNA lesions ranging in size and chemical functionality (Bauer et al., 2007; Eoff et al., 2007; Ling et al., 2003; Rechkoblit et al., 2006, 2010; Zhang et al., 2009) and other Y-family polymerases (Lone et al., 2007; Nair et al., 2004; Swan et al., 2009b).
RESULTS

DNA Template/Primer Design for Structure Determination

To obtain the crystals of the Dpo4 extension ternary complexes with correct as well as with misinserted 3′ terminal primer bases opposite the m1G and m3C lesions (Figure 1A) we used 5′-CTAAC[X]C-3′ 19-mer templates, where [X] is m1G or m3C, and 2′,3′-dideoxy-Y terminated 13-mer primer strands, where Y is C, G, T, or A (Figure 1B). An incoming dGTP was added to pair with the template base C flanking the m1G or m3C on its 5′-side. The structures of the Dpo4 m1G-modified (designated m1G-C, m1G-G, m1G-T, m1G-A) and m3C-modified (m3C-C, m3C-A, m3C-G, m3C-T) ternary complexes were solved by the molecular replacement method. The crystal data, together with the data collection and refinement statistics are summarized in Table 1.

Structure of the m1G-C Extension Ternary Complex

The overall structure and conformation of the m1G-C extension ternary complex with correct primer C base opposite the lesion (Figure 1C) is similar (Cα root-mean-square deviation [rmsd] = 0.99 Å) to the type I unmodified ternary complex (Ling et al., 2001) (see Figure S1 available online). The Dpo4 polymerase embraces the template/primer DNA by the palm, finger, and thumb domains, that are present in all known DNA polymerases, and by the little finger or polymerase associated, PAD, domain that is unique to the Y-family (Johnson and Beese, 2004; Steitz Table 1. Data Collection and Refinement Statistics

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AU, asymmetric unit; rmsd, root-mean-square deviation. See also Figure S1.

Values in parentheses are for highest-resolution shell.

Table 1. Data Collection and Refinement Statistics

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and Yin, 2004; Swan et al., 2009a; Yang and Woodgate, 2007). The polymerase active site ions A and B, identified previously as Ca²⁺ under identical crystallization conditions (Rechkoblit et al., 2006), take analogous positions to the metal ions present in the ternary complexes of high-fidelity polymerases (Vaisman et al., 2005). The third ion, coordinated by the loop of the thumb domain, is found in the majority of the Dpo4 ternary complexes (Bauer et al., 2007; Eoff et al., 2007; Ling et al., 2001, 2003; Rechkoblit et al., 2006, 2010; Zhang et al., 2009); an ion at a similar position has been observed in other Y-family polymerases including human and yeast Rev1 (Swan et al., 2009b), human Pol κ (Lone et al., 2007), and Pol λ (Nair et al., 2004).

A nascent base pair at the (0) position of the Dpo4 active site pocket is formed by the C5 template base (5′ adjacent to m1G) and the incoming dGTP (Figure 2A). The m1G lesion at the (–1) position of the Dpo4 active site takes an anti conformation and stacks within the template/primer helix (Figures 2A and 2B). Such an arrangement of the m1G lesion is similar to that of a template G base at the same position of an unmodified Dpo4 complex (Protein Data Bank [PDB] ID 2AGQ) (Vaisman et al., 2005) (black lines in Figure 2B). We find that clashing of the methyl group at the N1 of m1G with the partner primer base C (that would occur as judged by the unmodified complex [Vaisman et al., 2005]) is avoided due to m1G retaining its spatial intrahelical Watson-Crick orientation with respect to an unmodified guanine, but placing the burden of distortion on the unmodified cytosine-containing opposing strand (black lines in Figure 2B). Thus, to accommodate the methyl group in the m1G-C complex, the base, sugar, and phosphate of C14 are pushed out of the DNA duplex toward the minor groove, as is evident from the simulated annealing Fo-Fc omit map shown in Figure 2B. The phosphate and sugar groups of C14 are well-defined in the electron density map shown in Figure 2B. However, the density for the base of C14 is partially disordered (base not shown in Figures 2A and 2B) indicating its conformational flexibility outside the double helix. Because the sugar of the C14 primer terminus and, thus, its putative 3′-OH is displaced and not properly positioned for an extension reaction to occur, the m1G-C complex is catalytically incompetent.
Mutagenic m1G·T, m1G·G, and m1G·A Extension Complexes
The m1G·T, m1G·G, and m1G·A ternary complexes represent an extension step from the misinserted T, G, or A primer bases opposite the mutagenic m1G lesion. The overall arrangements of these complexes and active site alignments (Figures S1 and S2) are similar to that of the m1G·C nonmutagenic complex (Figures 1C and 2A). Interestingly, as seen with the m1G·C, all mutagenic complexes show the m1G lesion to be in the normal anti conformation while maintaining base stacking with the adjacent template C5 and C7 bases (Figures 2C–2E; Figures S2A–S2C).

In the m1G·T complex, the position of the phosphate group of the partner primer base T14, that is well-defined in the electron density map (Figure 2C; Figure S2A), points to an inside-the-helix location for the T14 residue. However, despite the 1.89 Å resolution, the density for the sugar and base of T14 is poor; this suggests the possibility of alternate conformation(s) for T14. For the inside-the-helix location of the m1G·T pair (base and sugar of T are shown in light color due to the tentative nature of alignment in Figure 2C), the N2 of m1G could form a single hydrogen bond with the O2 of T14; this arrangement differs from the commonly found wobble G·T mismatch, where a T base is shifted toward the major groove and the N1 and O6 of G form two hydrogen bonds with the O2 and N3 of T, respectively (Hunter et al., 1987; Patel et al., 1982).

In the m1G·G complex, the partner residue G14 is displaced into the major groove (Figure 2D) and cannot support catalysis. Furthermore, in the m1G·A complex, the phosphate, sugar and base of the partner residue A14 are disordered (Figure 2E). Similar to G14, A14 is most likely displaced outside the helix, because modeling of A14 in either anti or syn conformation with intrahelical occupancy would result in collision with the m1G base (data not shown).

m3C Extension Complexes Opposite G, A, C, and T
The overall structures and conformations of the m3C-containing extension ternary complex are similar to each other, to m1G-modified complexes and to the type I unmodified ternary complex (Ling et al., 2001) (Figure S3). In contrast to the m1G-modified extension complexes (Figure 2), the smaller base of the m3C lesion allows its partner bases G, A, C, and T to enter the double helix (Figures 3A–3E; Figure S3). However, the electron densities for the base and sugar of the partner G14 and T14 residues are observed at a lower resolution than for the surrounding entities and, thus, may be indicative of the existence of alternate conformers (Figures 3B and 3E). For this reason, the intrahelical positioning of G14 (Figure 2B) and T14 (Figure 4E) are shown in light color due to the tentative nature of the alignments.

The “correct” m3C·G base pair with an intrahelical orientation of G is highly sheared, with m3C(anti) shifted into the major groove and its partner G14(anti) into the minor groove (Figures 3A and 3B). The base stacking interactions between m3C and the 3’-adjacent C7 template base, as well as between the G14 and G13 primer strand bases are disrupted; however, the base stacking between the 5’-adjacent C5 template base and the base of m3C is maintained. The sheared m3C·G pair is held together by hydrogen bond interactions, with the methyl group of the positively charged m3C potentially serving as a hydrogen bond donor to the O6 of G14 (3.0 Å separation; Figure 1B).

In the mutagenic m3C·A, m3C·C, and m3C·T complexes, the base of the m3C is positioned within the double helix and maintains stacking with the adjacent C5 and C7 template bases (Figures 3C–3E; Figures S4A–S4C). The C1’-C1’ distances between the m3C and its partner bases are in the range of 9.1 Å to 10.1 Å (Figures 3B–3E), less than the ~10.5 Å value for a normal Watson-Crick pair. In all these complexes, the CH3 group of the m3C lesion could serve as a donor for hydrogen bonding interactions and is positioned near the acceptor (Figures 3C–3E).

Examples of C-H···O/N acceptor hydrogen bonds have been observed in many protein-nucleic acid complexes (Braddock et al., 2002; Mandel-Gutfreund et al., 1998; Wahl and Sundaralingam, 1997). In these structures, the methyl group of T or the H5 atom of C serves as donors and oxygen atoms of amino acids serve as acceptors. In addition, in some structures, donor-acceptor arrangements have also been found between the methyl group of isoleucine and the O2 or N3 atoms of C (Braddock et al., 2002).

Efficiency and Fidelity of Dpo4 Translesion Synthesis
Here we show that Dpo4 is strongly inhibited and predominantly mutagenic during base insertion opposite the m1G and m3C lesions. Further extension after insertion of both correct and incorrect bases is inefficient and introduces additional base substitution and deletion errors.

Dpo4 readily elongates the 13-mer primer strand using the unmodified-G or C DNA templates, to produce predominantly full-length 19-mer extension products in the presence of all four dNTPs (Figure 4A, lanes 1–5 and 11–15, respectively). In contrast, primer elongation on the m1G- and m3C-modified templates is inefficient and produces only up to ~14% of fully extended products after 20 min reaction time with a higher concentration of Dpo4 (Figure 4A, lanes 6–10 and 16–20, respectively). Single base incorporation experiments (Figure 4B) indicate that Dpo4 generates ~90% mutations during the insertion step opposite m1G and ~60% opposite m3C, when calculated as the ratio of correctly inserted nucleotide over all inserted nucleotides. Surprisingly, the efficiencies of correct as well as incorrect base insertions opposite m1G and m3C lesions are smaller than the efficiencies of misincorporation opposite unmodified G or C. This is evident from a comparison of A, G, and T misincorporation opposite template G for 5 min under 10 nM Dpo4 with base insertion opposite m1G under the same conditions (Figure S5B); the same results are observed for misincorporation opposite template C and base insertion opposite m3C (Figure S5B). Therefore, we conclude that the efficiencies of C, A, G, and T incorporation opposite m1G and m3C are reduced by more than ~104-fold as compared to incorporation of the correct dNTPs opposite unmodified template bases (Rechkoblit et al., 2006; Zhang et al., 2009). We were not able to evaluate steady-state kinetic parameters for base insertions opposite the m1G and m3C lesions due to slow reaction rates that required high Dpo4 concentrations to reliably quantify the products; a high enzyme to substrate ratio is incompatible with the Michaelis-Menten kinetics (Creighton et al., 1995).

Primer extension from the correctly paired unmodified G-C and C-G termini is robust; only single bands corresponding to
partially extended 15-, 16-, 17- and 18-mers are evident (Figure 4C, lanes 1–2 and 21–22; Figure S6, top). In contrast, with correct m1G
C and m3C
G termini (Figure 4C, lanes 9–10 and 29–30), as well as with mismatched damaged termini (Figure 4C, lanes 11–14 and 25–28, 31–32), extension efficiency is low; furthermore, it is less than that from the mismatched A and G opposite the template G (Figure 4C, lanes 3–6) and from the mismatched C, A, and T opposite the template C (Figure 4C, lanes 17–20 and 23–24). These results indicate that the extension from m1G
C and m3C
G, as well as from the damaged mismatches, is at least \( \leq 10^3 \)-fold less efficient than from the unmodified m1G
C or m3C
G template, indicating mutagenic extension (Figure 4C: lanes 9–10 versus 1–2, lanes 29–30 versus 21–22, and lanes 25–26 versus 17–18; Figure S6, bottom). This reflects substitution errors that arose during extension from the correctly paired and mismatched damaged termini and, thus, indicates that m1G and m3C lesions can also cause semi-targeted substitution mutations. In addition, a predominant 18-mer product is detected during extension from m3C
C termini (Figure 4C, lanes 25–26) suggesting a semi-targeted deletion mutation.

**DISCUSSION**

Dpo4-Catalyzed Insertion Opposite m1G and m3C

The structures of the Dpo4 complexes reveal that during extension, both the m1G and the m3C lesions stay within the DNA template/primer helix and present their Watson-Crick edges (sterically compromised by the bulky methyl group) for base pairing. This causes either eviction of m1G’s correct partner C from

**Figure 3. Pairing Alignments of the m3C Lesion with Correct G and Misinserted A, C, or T 3'-Terminal Primer Bases at the (–1) Position of Extension Ternary Complexes**

(A) Structure of the active site of the m3C-G complex. m3C(anti) at the (–1) position is opposite the 3'-terminal G14(anti) base of the primer strand. Simulated annealing Fo-Fc omit map contoured at 3σ level and colored in blue (2.80 Å resolution) is shown for m3C and G14 residues, and at 2σ level colored in gray, is shown for G14.

(B) m3C opposite correct G14 of the primer strand. The electron density for the base of G14 is not well defined, thus the base of G14 is shown in light coloring due to an uncertainty regarding its precise orientation.

(C) m3C(anti) opposite A14(anti) of the primer strand of the m3C-A complex.

(D) m3C(anti) opposite C14(anti) of the primer strand of the m3C-C complex.

(E) m3C(anti) opposite T14(anti) of the m3C-T complex. Given the poor density for the sugar and base of T14, there is uncertainty regarding the precise orientation of this residue, with the intrahelical alignment shown in light coloring. Simulated annealing Fo-Fc omit maps contoured at 3σ level and colored in blue are shown for damaged pairs in the m3C-A, m3C-C, and m3C-T complexes at 2.70 Å, 2.50 Å, and 2.80 Å resolution, respectively; in addition, omit map at 2σ level and colored in gray is shown for T14. Water molecules are shown as small red spheres. The overall structural superposition of the m3C-G, m3C-A, m3C-C, m3C-T and unmodified Dpo4 complexes is shown in Figure S3. See also Figure S4.
the double helix into the minor groove (Figure 2B), or displacement of m3C’s correct partner G into the minor groove (Figure 3B); the incorrect partner bases are also primarily evicted opposite m1G or misaligned opposite m3C. During the insertion step, these positions of m1G and m3C would hinder entrance of a dNTP into the Dpo4 active site, consistent with the relative to the m1G terminal primer residue, which follows the T, is crosslinked to the protein and flips out the double helix.

**Dpo4’s Catalytic Feasibility for Extension Past m1G and m3C Lesions**

In the m1G-modified Dpo4 complexes, further extension from the primer bases C, G, and A in their extrahelical positions (Figures 2A, 2B, 2D, and 2E) would not be possible. However, extension from m1G-C, m1G-G, and m1G-A termini still occurs, although with very low efficiency, and results in correct, as well as mutagenic extension products (Figure 4C). To explain this we hypothesize that for correct extension these primer residues would enter the helix and position their 3′-OH groups for elongation, and thereby dislocate the m1G from its internal helical position (these rare events are not captured in the crystal structures). The observed mutagenic extension could arise due to pairing of the template base 5′ to the m1G with the 3′-terminal primer base, so as to skip m1G; a −1 frameshift, similar to the one observed with an abasic lesion (Ling et al., 2004), could potentially form. Alternatively, semi-targeted misalignment events are possible as proposed earlier for the bulky 2-aminofluorene-dG lesion (Rechkoblit et al., 2010).

Interestingly, extension from a T base opposite m1G occurs faster than from the m1G-C pair (Figure 4C). The observed intra-helical position of the partner T base (at lower α level) appears to be feasible for extension (Figure 2C). Even though the 3′-carbon of the sugar of the primer terminal T14 in m1G-T (the 3′-OH is absent) is relocated by ~1.6 Å toward the major groove compared to the 3′-C position of C in the unmodified G-C complex (Figure S7), the distance between the 3′-carbon of T14 and the α-phosphate of dGTP is ~3.8 Å, similar to the analogous distance in the unmodified complex with a 2′, 3′-dideoxy primer terminus (~4.2 Å, PDB ID 2AQG) (Vaisman et al., 2005). However, the catalytic cation A, which coordinates the 3′-OH group and activates it for reaction (Steitz and Yin, 2004) is repositioned by ~2.2 Å relative to its location in the unmodified complex (Figure S7). The A-site metal ion has often been found to be displaced in Dpo4 complexes that contain inhibiting mismatches or lesions (Rechkoblit et al., 2010; Vaisman et al., 2005; Yang and Woodgate, 2007). It has been suggested that the correct geometry of ion binding is critical for the chemical bond formation step and only a suitable substrate allows the ion to be properly positioned (Vaisman et al., 2005; Yang and Woodgate, 2007). Thus, repositioning of the A ion is consistent with inhibition of extension from T14 with respect to an unmodified complex.

In the m3C-modified complexes the 3′-primer termini of the G, A, C, and T partner bases relocate by no more than ~1.0 Å relative to the position observed in the unmodified G-C complex (Figures 5A and 5B). Hence, the distances from the 3′-carbon of the sugar to the α-phosphate of dGTP are in a productive 3.8 to 4.2 Å range. However, for all four complexes, the catalytic cation A repositions by ~2.5 Å relative to its location in the unmodified complex (Figure 5). Thus, efficient catalysis is not promoted, allowing time for misalignment events to occur.

**m3C and G Form a Pair with CH3 of m3C Acting as a Hydrogen Bond Donor**

Our observed m3C-G pair with a 10.0 Å C1′-C1′ distance (Figure 3B) features a novel hydrogen bond where the methyl group of m3C serves as a donor. Consistently, the CH3 group forms hydrogen bonds with acceptor atoms of A, C, and possibly T bases (Figures 3C-3E). By contrast, a different positioning of m3C opposite G was reported for the m3C-G alignment in duplex DNA using a protein-linked protein-DNA system (Lu et al., 2010). In this structure, both m3C and opposing G are shifted toward the major and minor grooves respectively, but are not hydrogen-bonded to each other, and exhibit a long 14.5 Å C1′-C1′ separation. This distinctly different alignment most likely reflects contributions resulting from the disruption of two base pairs that are immediately adjacent to the m3C-G in this structure; the T base 3′ to the m3C skips its partner A and forms a noncanonical pair with the next A, whereas the C, which follows the T, is crosslinked to the protein and flips out of the double helix.

**Proposed Model for ABH2 Recognition of Alkylated DNA Lesions**

The lesion search mechanism employed by the repair demethylase ABH2 is unclear and is of great interest in the field. To elucidate this process, structures of cognate m1A-T and m3C-G alignments within DNA duplexes have been investigated (Lu et al., 2010) and m1A has been shown to adopt a syn conformation presenting its Hoogsteen edge to form a base pair with T; the latter shifted from its position in a normal A(anti)-T Watson-Crick pair (C1′-C1′ distance of 10.5 Å) to compensate for the shorter 9 Å distance between C1′ of m1A(syn) and C1′ of T.

We find that a common theme in our observed correct m1G(anti)-C and m3C(anti)-G pairs, as well as the m1A(syn)-T pair (Lu et al., 2010), is the displacement of the partner C, G and T bases from their respective normal positions in duplex DNA. Thus, it is tempting to propose that ABH2 employs interactions with the complementary undamaged strand of the DNA duplex while scanning DNA for lesions (ABH2 is known to prefer dsDNA substrates (Aas et al., 2003; Duncan et al., 2002; Falnes, 2004; Ringvoll et al., 2006)). Such a lesion recognition strategy has been proposed for the glycosylase AlkD (Rubinson et al., 2010) and the nucleotide excision repair protein Rad4/XPC (Min and Pavletich, 2007).

**Incorrect Partner Bases Opposite m3C Could Complicate Recognition by ABH2**

Generally, DNA glycosylases, that flip alkylated bases, exhibit enhanced excision activity for lesions in distorted mispaired arrangements (O’Brien and Ellenberger, 2004). We observe that the positions of the bases in the correct m3C-G pair deviate the most from ones in the unmodified C-G pair compared to the mutagenic m3C-A, m3C-C, and m3C-T alignments (Figure 5B). This leads us to speculate that ABH2 could detect m3C in the
Figure 4. Efficiency and Fidelity of Base Incorporation and Extension of Primers Bound to Unmodified-G, m1G-, Unmodified-C, and m3C-Templates by Dpo4

(A) Time course of extension of 32P 5’-end-labeled 13-mer primers bound to 19-mer templates in the presence of all four dNTPs. The 3’ end of the 13-mer primer was paired with the base on the 3’ side of the unmodified-G, m1G-, unmodified-C, or m3C template. The reactions were conducted with 10 nM Dpo4 on the
Structure
Bypass of Methylation Damage Lesions by Dpo4

Figure 5. Superposition of the m3C-G, m3C-A, m3C-C, m3C-T and Unmodified Dpo4 Complexes
(A) View of 5′-…(G5-C3-T3-A5-G3) template and 3′-Y14-G13 (Y = G, A, C, or T) primer segments plus dGTP and equivalent entities of the unmodified complex (PDB ID 2AOQ) (Vaisman et al., 2005). See labels for color coding. Structures are superimposed by Cs atoms of the active site forming palm and finger domains of Dpo4. Asp7, Asp105, Glu106, and Tyr12 are shown in sticks for the m3C-G (pink) and unmodified (silver) complexes. In all four m3C-modified complexes the catalytic cation A, shown as a color-coded sphere, repositions by ~2.5 Å relative to its location in the unmodified complex (silver sphere).
(B) View of m3C-containing and unmodified base pairs looking down the helix axis.
correct m3C-G pair more efficiently than in the incorrect base pairs resulting in longer persistence of this lesion opposite mismatches. If true, this would suggest a mechanism whereby a mutagenic insertion opposite m3C may have another chance of being removed by the exonuclease domain of a high-fidelity polymerase (or perhaps by another DNA repair protein) before making such a mutation permanent by repair of m3C-A, C, or T to C-A, C, or T mismatches by ABH2, thus increasing the overall fidelity of replication. This would be especially important if mismatch repair was lost, as seen in various tumor cells (Karamurzin and Rutgers, 2009; Poulogiannis et al., 2010). This paradigm would be analogous to the GO system by which the E. coli DNA glycosylase MutM and its human homolog OGG1 will only excise 8-oxoguanine if the opposite base is cytosine, whereas another repair glycosylase (E. coli MutY and human MYH) will remove mismatched adenine opposite 8-oxoguanine (Fromme et al., 2004).

Bypass of m1G and m3C by Human Y-Family Polymerases
Our results provide opportunities for proposing hypotheses concerning processing of these alkylated lesions by human bypass polymerases. We propose that Pol ι (Lone et al., 2007) and Pol η (Biertumpfel et al., 2010), which, like Dpo4, employ Watson-Crick base pairing between the templating base and dNTP, are most likely to be inefficient during insertion opposite m1G and m3C. In contrast, Pol 1 that rotates adenine (Nair et al., 2004) and guanine (Nair et al., 2005) templating bases into the syn conformation to employ their Hoogsteen edges for dNTP binding, might be able to bypass the m1G lesion. However, structural (Jain et al., 2009) and kinetic (Choi et al., 2009) data suggest that Pol 1 engages the Watson-Crick edge of template pyrimidines for base pairing with dNTP, thus presenting a challenge for base insertion opposite the m3C. In addition, Rev1 polymerase, which displays template G outside the DNA helix and uses an arginine residue to pair with incoming dCTP (Swan et al., 2009b), might be efficient in bypass of m1G. Future experimental investigations, both structural and biochemical, are needed to further elucidate the functions of the human polymerases in processing these lesions.

EXPERIMENTAL PROCEDURES
m1G- and m3C-Modified DNA Templates
m1G- and m3C-modified 19-mer oligonucleotides (Figure 1B) were synthesized by automated solid-phase synthesis, deprotected with concentrated ammonium hydroxide, purified by anion exchange high-performance liquid chromatography (HPLC), and characterized by matrix-assisted laser desorption/ionization essentially as described previously (Delaney and Essigmann, 2004); the m1G and m3C oligonucleotides were deprotected at 37°C for 4 hr (m1G) or 12 hr (m3C) before lyophilization and HPLC purification.

unmodified G- and C-templates and with 50 nM Dpo4 on the m1G- and m3C-templates; the concentration of template/primer DNAs was 10 nM. See Figure S5A for the experiments conducted on the damaged templates with 10 nM Dpo4.
(B) dCTP, dATP, dGTP, or dTTP single nucleotide insertion. The green triangles represent insertion of the correct nucleotide; the magenta triangles represent mutagenic insertion opposite the unmodified G template strand. The reactions were conducted for 5 min with 10 nM Dpo4 on the unmodified G- and C-templates and for 20 min with 50 nM Dpo4 on the m1G- and m3C-templates. See Figure S5B for the experiments conducted for 5 min with 10 nM Dpo4 on the damaged templates. The percentages of C, A, G, and T incorporation into the primer strand on the unmodified-G template were 93%, 6.3%, 1.9%, and 4.9%, respectively, and on the unmodified-C template were 2.5%, 5.5%, 95%, and 5.8%, respectively. Note that two G bases are correctly incorporated opposite template C and 5′-adjacent C template bases. The percentages of C, A, G, and T incorporation into the primer strand on the m1G template were 2.2%, 5.6%, 9.8%, and 2.5%, respectively, and on the m3C template were 5.2%, 11%, 12%, and 1.8%, respectively.
(C) Efficiency of extension from C, A, G, and T bases opposite the unmodified-G, m1G, unmodified-C and m3C by Dpo4 (see labels). Additional 15-, 16-, 17-, and 18-mer bands that migrate with different mobilities than the correctly elongated bands arising from the extension from matched unmodified G-C and C-G termini, are detected, thus indicating mutagenic extension. An overexposed image of the gel is used to show mutagenic primer extension events. The green triangles represent the correctly extended products; the magenta triangles represent mutagenic extension. 20-mer products indicate (+1) nontemplate directed addition of dNTP. Profiles were determined by PhosphorImager analysis with images exposed for varying times as indicated.

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Crytalization

The crystals of the Dpo4 extension ternary complexes containing m1G- or m3C-modified 19-mer templates and 13-mer primers terminated with 2',3'-dideoxy-C, 2',3'-dideoxy-A, 2',3'-dideoxy-G, or 2',3'-dideoxy-T (Figure 1B) opposite m1G- or m3C- lesions were grown in the presence of dGTP and flash-frozen in liquid nitrogen for X-ray data collection under conditions described previously (Rechkoblit et al., 2006). Several rounds of micro seeding were employed to produce the diffraction quality crystals.

We were not able to obtain crystals of the insertion ternary complexes with dCTP, dATP, dGTP, or dTTP opposite the m1G or m3C and a primer terminated with 2',3'-dideoxy-G opposite a C7 base, 3' to the lesion site.

Structure Determination and Refinement

X-ray diffraction data were collected at the NE-CAT 24-ID-C beam line at the Advanced Photon Source (Argonne National Laboratory, Chicago). The data were processed and scaled using the HKL2000 suite. The structures of the extension complexes were solved by the molecular replacement method, using our published oxoG-modified extension ternary Dpo4-DNA-dGTP structure (Rechkoblit et al., 2009) as a search model. The model building was manually finished in TURBO-FRODO (http://www.afmb.univ-mrs.fr/-TURBO- ) based on the electron density maps calculated in REFMAC (http://www.ccp4.ac.uk/html/refmac5.html), and the resulting models were refined in REFMAC. The crystal data, together with the data collection and refinement statistics for all structures are summarized in Table 1. The simulated annealing omit maps were calculated in CNS (http://cns-online.org/v1.21/) with the m1G, m3C and partner bases omitted from the models before they were heated to 2000 K and then slowly cooled.

Primer Elongation Assays

Primer elongation assays were conducted as described by our group in the literature (Rechkoblit et al., 2006).

ACCESSION NUMBERS

Coordinates and structure-factor amplitudes for the m1G-C, m1G-T, m1G-G, m1G-A, m3C-G, m3C-A, m3C-C, and m3C-T complexes have been deposited in the Protein Data Bank with accession codes 3RAX, 3RB0, 3RB3, 3RB4, 3RB6, 3RBD, and 3RBE, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.str.2011.03.020.

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