2152–2162 *Nucleic Acids Research, 2008, Vol. 36, No. 7* *Published online 14 February 2008*
doi:10.1093/nar/gkn058

Eukaryotic Y-family polymerases bypass a 3-methyl-2′-deoxyadenosine analog *in vitro* and methyl methanesulfonate-induced DNA damage *in vivo*

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Received November 9, 2007; Revised January 26, 2008; Accepted January 29, 2008

ABSTRACT

***N*3-methyl-adenine (3MeA) is the major cytotoxic lesion formed in DNA by S_N2 methylating agents. The lesion presumably blocks progression of cellular replicases because the *N*3-methyl group hinders interactions between the polymerase and the minor groove of DNA. However, this hypothesis has yet to be rigorously proven, as 3MeA is intrinsically unstable and is converted to an abasic site, which itself is a blocking lesion. To circumvent these problems, we have chemically synthesized a 3-deaza analog of 3MeA (3dMeA) as a stable phosphoramidite and have incorporated the analog into synthetic oligonucleotides that have been used *in vitro* as templates for DNA replication. As expected, the 3dMeA lesion blocked both human DNA polymerases α and δ. In contrast, human polymerases η, ι and κ, as well as *Saccharomyces cerevisiae* polη were able to bypass the lesion, albeit with varying efficiencies and accuracy. To confirm the physiological relevance of our findings, we show that in *S. cerevisiae* lacking Mag1-dependent 3MeA repair, polη (Rad30) contributes to the survival of cells exposed to methyl methanesulfonate (MMS) and in the absence of Mag1, Rad30 and Rev3, human polymerases η, ι and κ are capable of restoring MMS-resistance to the normally MMS-sensitive strain.**

INTRODUCTION

DNA is subject to a variety of chemical modifications that alter its structure. Such alterations can block basic cellular functions such as transcription and/or replication and can lead to cell death, mutagenesis and cancer in higher eukaryotes. One such modification is DNA methylation, which can be caused by endogenous chemicals, products of metabolism, environmental exposure or treatment with several cancer chemotherapeutics. Not surprisingly, cells have developed several evolutionarily conserved mechanisms for repairing or tolerating this type of DNA damage, including base excision repair (BER), nucleotide excision repair (NER), recombination and translesion DNA synthesis (TLS) (1).

Methylating agents primarily react with exocylic nitrogen or oxygen atoms on purines and pyrmidines, with the reaction mechanism (S_N1 or S_N2) determining the relative ratio of oxygen to nitrogen modifications (2). The major products in DNA exposed to S_N2 methylating agents are *N*7-methylguanine and *N*3-methyladenine (3MeA), while there is very little methylation of oxygen atoms on the bases or the sugar phosphate backbone. 3MeA accounts for ~20% of the base damage formed by S_N2 methylating agents (2) and is considered to be the major cytotoxic lesion produced by such chemicals, based on the fact that bacterial and viral DNA polymerases are blocked before adenine residues but not guanine, on templates treated with either S_N1 or S_N2 methylating agents (3).

3MeA is primarily removed by BER, although NER appears to provide an important back-up mechanism in the absence of BER in eukaryotes (4–7). Mouse embryonic fibroblasts (MEFs) lacking Aag, the DNA glycosylase that normally removes 3MeA from DNA, are sensitive to methyl methanesulfonate (MMS) and the compound methyl lexitropsin, which preferentially methylates *N*3 of adenine (8). Indeed, *Aag*−/− cells become arrested in S phase longer than their wild-type counterparts treated with either methylating agent, suggesting that the unrepaired 3MeA residues are a block to replication *in vivo*.

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However, it has been extremely difficult to prove that 3MeA blocks replication directly, as the half-life of 3MeA *in vitro* is estimated to be between 12 and 24 h (9), thereby precluding biochemical analysis. Furthermore, assuming 3MeA has a similar, or even faster decay *in vivo*, it seems likely that by the time the MMS-treated *Aag−/−* cells arrest in S phase, a significant portion of the 3MeA residues would be converted to replication-blocking abasic sites. The fact that the arrested cells eventually complete S phase (8) suggests that the replication-block is either removed by another repair mechanism, or that specialized DNA polymerases are able to bypass the damaged site.

Several eukaryotic DNA polymerases are capable of performing TLS. Perhaps the best-characterized eukaryotic TLS polymerases are polζ, a B-family polymerase (10,11), and polη, polι, polκ and Rev1, all of which are Y-family polymerases (12). Based upon structural studies, the Y-family polymerases appear to be good candidates to facilitate TLS of 3MeA, since unlike high-fidelity replicative polymerases, they do not make the same contacts with *N*3 of adenine in the minor groove of duplex DNA (13).

A major obstacle that has to date prevented the study of 3MeA TLS *in vitro* has been the inherent instability of the 3MeA lesion. To circumvent these problems, we have synthesized a stable 3-deaza analog of the nucleoside 3-methyl-2′-deoxyadenosine that can be incorporated into synthetic oligonucleotides as 3-deaza-3-methyladenine (3dMeA). Here, we show that human replicative polymerases polα and polδ are blocked by 3dMeA, while human and *Saccharomyces cerevisiae* Y-family polymerases are capable of bypassing the modified base *in vitro*. In agreement with our *in vitro* observations, we also demonstrate that human DNA polymerases η, ι and κ have the ability to restore MMS-resistance to a normally MMS-sensitive *mag1Δ rad30Δ rev3Δ* strain of *S. cerevisiae*.

MATERIALS AND METHODS

Oligonucleotides

Ethenoadenosine phosphoramidite was purchased from Glen Research (Sterling, VA, USA). All oligonucleotides used for *in vitro* replication and PCR assays, were synthesized by Lofstrand Labs Limited (Gaithersburg, MD, USA) and gel purified prior to use. Ethenoadenine and 3dMeA bases were incorporated into oligonucleotides using ultra-mild synthesis conditions.

Enzymes

Human polδ (14), GST-polι (15), His-polη (16) and *S. cerevisiae* polζ (GST-Rev3/Rev7) (17),were purified as previously described. Human polα was purchased from Chimerx (Milwaukee, WI, USA). Human polκ, *S. cerevisiae* polη and Rev1 protein were purchased from Enzymax (Lexington, KY, USA). Mouse Aag was purchased from Trevigen (Gaithersburg, MD, USA).

Synthesis of the 3-deaza-3-methyl-dA-phosphoramidite

A detailed protocol outlining the chemical synthesis of the 3-deaza-3-methyl-dA-phosphoramidite is available online as Supplementary Data.

In vitro Aag excision assay

To measure DNA glycosylase activity on various substrates, 5′-[^{32}P] 29mer, 5′-GCT CGT CAG ACG **A**TT TAG AGT CTG CAG TG-3′ (with the adenine, ethenoadenine or 3dMeA underlined and in bold font), was annealed to its complementary strand. Double-stranded DNA of 0.4 pmol was treated with 3 U of mAag or mock treated for 1 h at 37°C. NaOH was added to a final concentration of 100 mM along with 10 mM Tris, 1 mM EDTA (final) and the samples were incubated at 37°C to cleave any resulting abasic sites. Samples were resolved on a 15% gel (8-M urea) and visualized with a Molecular Dynamics phosphorimager and ImageQuant software.

Replication assays

In vitro replication assays were performed using the 29mer oligonucleotide 5′-GCT CGT CAG ACG **A**TT TAG AGT CTG CAG TG-3′ as a template (with the location of the undamaged adenine, or 3dMeA underlined and in bold font). For most experiments described herein, this template was annealed to a [^{32}P]-labeled 16mer primer with the following sequence; 5′-CAC TGC AGA CTC TAA A -3′. For the extension assays reported in Table 3, the [^{32}P]-labeled primer was a 17mer with the sequence; 5′-CAC TGC AGA CTC TAA AX -3′, where X is either A, or T. Primer-template DNAs were prepared by annealing the 5′ [^{32}P]-labeled primer to the unlabeled template DNA at a molar ratio of 1:1.5. Standard 10-μl reactions contained 40 mM Tris–HCl at pH 8.0, 5 mM $MgCl_2$, 100 μM of each ultrapure dNTP (Amersham Pharmacia Biotech, NJ, USA), 10 mM DTT, 250 μg/ml BSA, 2.5% glycerol and 10 nM primer/template DNA. The concentration of polymerase added varied and is given in the legends to figures 3, 4, 5 and 7. After incubation at 37°C (or 30°C for yeast enzymes) for 5 min, reactions were terminated by the addition of 10 μl of 95% formamide/10 mM EDTA and the samples heated to 100°C for 5 min and briefly chilled on ice. Reaction mixtures (5 μl) were resolved on 15% polyacrylamide, 8M urea gels and analyzed with a Molecular Dynamics phosphorimager and ImageQuant software.

Steady-state reaction conditions

For steady-state kinetic reactions, each polymerase was assayed to determine the amount of enzyme and nucleotide that would result in <20% incorporation (18,19): 0.4 U/reaction for polα, 1.2 nM for human polη, 1.8 nM for polι, 1.5 nM for polκ and 1.4 nM for *S. cerevisiae* polη. All reactions were performed in 10 μl in the standard reaction buffer described earlier, except those involving polι, where the concentration of magnesium chloride was reduced from 5 to 0.25 mM. Reactions were initiated by the addition of the dNTP and lasted for 1.5–5 min for the

correct nucleotides and 5–10 min for incorrect nucleotides, depending on the polymerase. On unmodified templates, dNTP concentrations ranged from 0.01 to 100 μM for the correct dTTP and from 1 to 500 μM for the incorrect dNTPs. For Y-family polymerases on the 3dMeA containing template, dTTP concentrations ranged from 0.1 μM to 1 mM while incorrect dNTPs ranged from 10 μM to 1 mM (except for polι reactions where dATP ranged from 2 to 100 μM, while dGTP and dCTP ranged from 10 to 300 μM). For the data shown in Table 3, dCTP concentrations varied from 0.2 to 10 μM on the undamaged template and from 10 to 300 μM for 3dMeA-containing template. For polα with the 3dMeA-containing template, dATP and dTTP were varied from 0.1 to 1 mM. Replication products were separated on 15% polyacrylamide gels containing 8-M urea and visualized with a Molecular Dynamics phosphorimager and quantified with ImageQuant software.

The apparent V_{max} and K_m values for each enzyme and nucleotide were determined from a Hanes–Woolf plot by linear least-squares fit as described previously (18). The catalytic efficiency of nucleotide insertion was calculated as the ration of V_{max}/K_m and the frequency of misinsertion was calculated as $(V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$ as described previously (18) using SigmaPlot software (SPSS, Chicago, USA).

Generation of yeast strains and plasmids

All yeast strains were derived from the W303 background (20). MAG1 was disrupted by PCR amplification of the URA3 gene from pRS416 using primers with 40 nt of homology to upstream and downstream of MAG1 (MagUraF, 5′-ATG AAA CTA AAA AGG GAG TAT GAT GAG TTA ATA AAA GCA GCA GAG CAG ATT GTA CTG AGA GTG C-3′ and MagUraR, 5′-TTA GGA TTT CAC GAA ATT TTC TTC TGC CTT CAT CAT GGC AGC GGT ATT TTC TCC TTA CGC-3′) and transformed into C10-15a (W303 *RAD5 + mata)* (20). Positive disruptants were confirmed by PCR and MMS sensitivity. The *mag1Δ* haploid strain was mated to C10-10a, in order to obtain the *mag1Δ rad30Δ* double mutant (BPC1-4d) and a backcrossed mag1*Δ* (BPC1-2a) strain. BPC1-4d (*mag1Δ::URA3 rad30Δ:HIS3 matα*) was mated with C17-1A (*rev3Δ:HisG-URA3 mata*) to obtain *mag1Δ rev3Δ* (BPC2-8c), rad30*Δ* rev3*Δ* (BPC2-5a) double mutants and the *mag1Δ rad30Δ rev3Δ* (BPC2-13c) triple mutant. Since *MAG1* and *REV3* disruptions were both marked by the *URA3* gene, all strains genotypes were confirmed by PCR for these two genes by triplex PCR with the following reverse primer for *URA3* (URA3_44R; 5′-ACT AGG ATG AGT AGC AGC ACG-3′) and forward and reverse primers for either *MAG1* (MAG1_95upF; 5′-TGG CCA CTG CCC TCT GAT ATG-3′ and MAG1_298R; 5′-CTT GGC CAC TGA TCT GTT GAG-3′) or *REV3* (REV3_355upF; 5′-ACC ATT GTC CAA AGC TGT CGC-3′ and REV3_223R; 5′-ACG TGG CAC AAT ACT TGA TGC C-3′).

Plasmids expressing human and *S. cerevisiae* Y-family polymerases were constructed from pESC-LEU (Stratagene, La Jolla, CA, USA). *POLI* was cloned by digesting p6-1 (21) with NcoI, filling in the overhang with Klenow fragment, followed by digestion with AvaI and subsequent cloning into the SmaI site of pESC-LEU to generate pBP65. *POLH* was cloned as a NotI–BamHI fragment from pCDNA-XPV (22) into pESC-LEU digested with NotI and BglII to generate pPB66. *POLK* was cloned into pESC-LEU by first digesting pBP65 with NcoI, filling the ends with Klenow fragment to blunt end and subsequently digesting the vector with XmaI. An EcoRV–XmaI fragment from pHSE2 (a kind gift from Haruo Ohmori, University of Kyoto, Japan), encoding *POLK* was subsequently cloned into the vector to generate pBP98. *Saccharomyces cerevisiae RAD30* was cloned as an NcoI–PstI fragment from pJM231 into the similarly digested plasmid, pBP65, to generate pBP82.

Survival assays

MMS toxicity for each genotype was assessed on overnight cultures. Yeast were harvested and washed twice with PBS. MMS was diluted to 0.25% in PBS and aliquots of each strain were removed at selected time intervals, washed with PBS and diluted for plating on YPAD agar plates. Colonies were counted after 5 days at 30°C. For the complementation assays, strain BPC2-13A (*mag1Δ rad30Δ rev3Δ*) was transformed with pBP65 (expresses human polι), pBP66 (expresses human polη), pBP98 (expresses human polκ), pBP82 (expresses *S. cerevisiae* polη) or pESC-LEU. Yeast strains were cultured overnight in complete synthetic raffinose medium lacking L-leucine. One hour prior to MMS treatment, the cultures were harvested by centrifugation and transferred to synthetic galactose medium to induce the expression of polymerases. Cells were harvested and treated as described above, except dilutions of each culture were plated on synthetic galactose agar plates lacking L-leucine.

RESULTS

3dMeA is a stable analog of *N*3-methyladenine

3-Methyl adenosine is unstable *in vitro* with an estimated half-life of just 12–24 h (23). This short half-life has therefore limited biochemical or enzymatic studies on the lesion. To circumvent these problems, we have synthesized a 3-deaza-3-methyl-2′-deoxyadenosine analog of 3-methyl-2′-deoxyadenosine. The 3-deaza- analog has the same overall structure as the naturally occurring adduct (Figure 1A), but it lacks the positive charge associated with the *N*3 atom that normally destabilizes the glycosidic bond, and is therefore very stable. The analog can be synthesized as a phosphoramidite (Figure 1B) and can be incorporated into oligonucleotides by standard chemical DNA synthesis.

Since 3MeA is excised from DNA by the alkyladenine DNA glycosylase (Aag) (24), we determined if 3dMeA is also a substrate for Aag by treating either unmodified duplex DNA or DNA containing 3dMeA, or ethenoadenine (εA) with purified mouse Aag followed by hydroxide treatment. εA is a well-characterized substrate for Aag (25) and as noted in Figure 2, is completely excised from the substrate, as all of the εA oligonucleotide is cleaved

at the resulting abasic site, by hydroxide treatment (Figure 2). In contrast, the 3dMeA containing DNA shows relatively little cleaved substrate, and there is no detectable cleavage product in the unmodified control. This demonstrates that Aag can excise 3dMeA, but to a much lesser extent than εA and presumably the naturally occurring 3MeA.

Treatment with NaOH in the absence of Aag confirms that 3dMeA analog is indeed stable and that even boiling of the DNA to anneal the lesion containing strand to its complementary strand, did not result in abasic sites that could be subsequently hydrolyzed by treatment with NaOH. We suspect that the 3dMeA analog may not be removed as readily as naturally occurring 3MeA because of its stabilized glycosidic bond. Indeed, it has been proposed that the weakened glycosidic bond of several Aag substrates may facilitate excision by the glycosylase (26).

3dMeA is a strong kinetic block to replicative polymerases, but is bypassed by Y-family polymerases

To date, there has been no direct evidence of 3MeA blocking a replicative DNA polymerase. We therefore compared human polα and polδ to polη, polι, and polκ in the presence of the four standard deoxynucleotides to determine which enzymes were capable of replicating a template containing 3dMeA. Under standard reaction conditions and with an undamaged template, each polymerases utilizes ~10–20% of the primer (Figure 3A, left), however, virtually no extension of the primer annealed to the 3dMeA-containing template was observed in the presence of polα and polδ, indicating that the lesion is a strong kinetic block to replicative polymerases. By comparison, both incorporation and bypass of the lesion was observed in the presence of human polη, ι or κ (Figure 3). Steady-state kinetic analyses revealed that incorporation of T opposite the 3dMeA lesion only occurred with an efficiency of 0.15–3% of that opposite an undamaged A (Table 1). However, when one compares the catalytic activity (V_{max}/K_m) of the Y-family enzymes ability to incorporate opposite the 3dMeA lesion, it is 125- to 1200-fold more efficient than the incorporation by polα (Table 1) (full kinetic parameters are supplied as Supplementary Data).

Very recently, we discovered that the catalytic activity of polι *in vitro* is dramatically enhanced in the presence of low concentrations of Mg^{2+} or Mn^{2+} (27). Indeed, polι-dependent incorporation opposite the 3dMeA lesion increased significantly when comparing primer extension in 0.25 mM versus 5 mM $MgCl_2$ and lesion bypass was greatly stimulated in the presence of 0.25 mM $MnCl_2$ (Figure 4). Similar to studies with other B-family polymerases (28), low levels of Mn^{2+} also appeared to

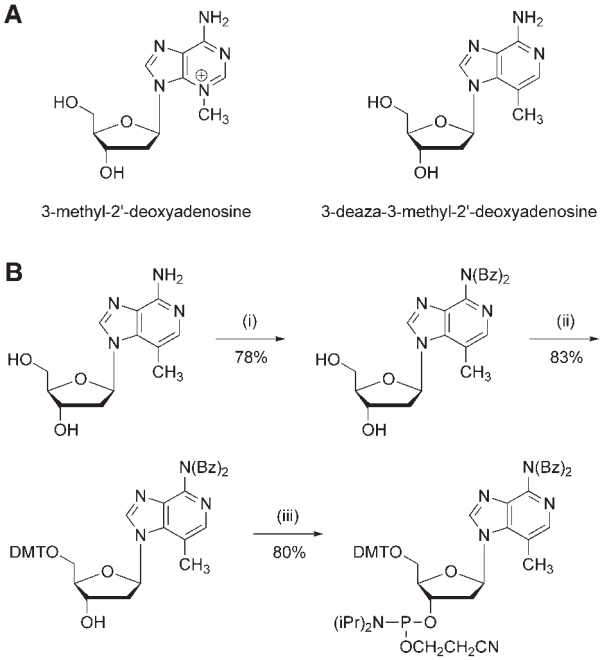

Figure 1. Synthesis of a synthetic 3-deaza-3-methyl-dA phosphoramidite. (**A**) Chemical structures of 3-methyl-2′-deoxyadenosine and 3-deaza-3-methyl-2′-deoxyadenosine. Replacement of the *N*3 with carbon removed the positive charge and helps stabilize the glycosidic bond. (**B**) Schematic of the synthesis of the 3-deaza-3-methyl-dA phosphoramidite (i) Di-benzoylation of 3-deaza-dA using benzoyl chloride in pyridine. (ii) Dimethoxytritylation using dimethoxytrityl chloride in pyridine. (iii) Phosphitylation using *N*,*N*-diisopropylamino-(2-cyanoethyl)phosphoramidic chloride and diisopropylethylamine in dichloromethane.

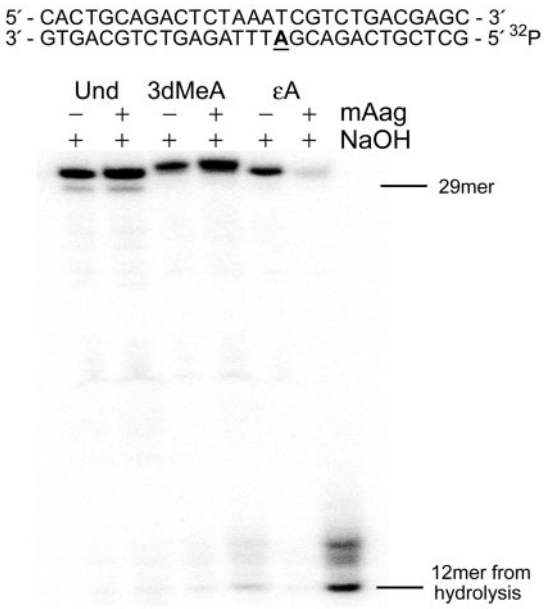

Figure 2. 3-deaza-3-methyl adenine is a stable analog of 3MeA. Mouse alkyladenine glycosylase (mAag) excises both 3-methyladenine (3MeA) and ethenodeoxyA (εA). 0.4 pmol of undamaged, 3dMeA- or εA-containing DNA was treated with 3 U of mAag, or mock treated for 1 h at 37°C. To hydrolyze the resulting abasic sites, NaOH was added to a final concentration of 100 mM along with 10 mM Tris, 1 mM EDTA (final) and the samples were incubated at 37°C. Samples were resolved on a 15% polyacrylamide gel containing 8-M urea. The nucleotide sequence of the 29mer duplex DNA is shown at the top of the panel and the position of the uncleaved ^{32}P-labeled 29mer oligonucleotide and 12mer product are shown on the right side of the gel.

stimulate human polδ's activity in the primer extension assays with the undamaged template, as well as enable a small amount of incorporation and extension beyond the 3dMeA lesion (Figure 4).

Next, we examined the single nucleotide insertion profile opposite the 3dMeA lesion promoted by polη, polι and polκ (Figure 5) and discovered that both polη and polι are error-prone, in that they readily misincorporate A opposite the 3dMeA lesion. Indeed, the ability of both polymerases to incorporate A opposite 3dMeA is consistent with the increase in A:T to T:A transversions observed *in vivo* in mice exposed to MMS (29). In contrast, polκ appears to be fairly accurate, as it primarily inserts T opposite 3dMeA. Analysis of the steady-state kinetics for each enzyme (Table 2) reflects the results shown in Figure 5. Polι and polη misinsert A opposite 3dMeA with a frequency of 0.46 and 0.48 relative to incorporation of the correct base T, respectively. In contrast, polκ is 10-fold more accurate than either polη or polι and misincorporates A opposite 3dMeA with a frequency of 0.04. Each polymerase appears to have higher than expected efficiency of inserting C, but this may simply occur as a consequence of the local sequence context, since the next 5′ template base is a G. While at first glance all three of the Y-family polymerases appear to be error-prone, they are, in fact, more accurate than polα, which actually misincorporates A opposite 3dMeA 4-fold better than T, in the steady-state assays (Table 2).

Finally, we examined the ability of polη, polι and polκ to extend from a base paired with 3dMeA. The primer terminus was either a 'correctly' paired T:3dMeA, or was an A:3dMeA mispair (Table 3). Both polη and polκ extended the correctly paired T:3dMeA primer terminus relatively well and did so with an efficiency of ~8–10% of that compared to a normal T:A base-pair (Table 3). In contrast, polι only extended the T:3dMeA primer with

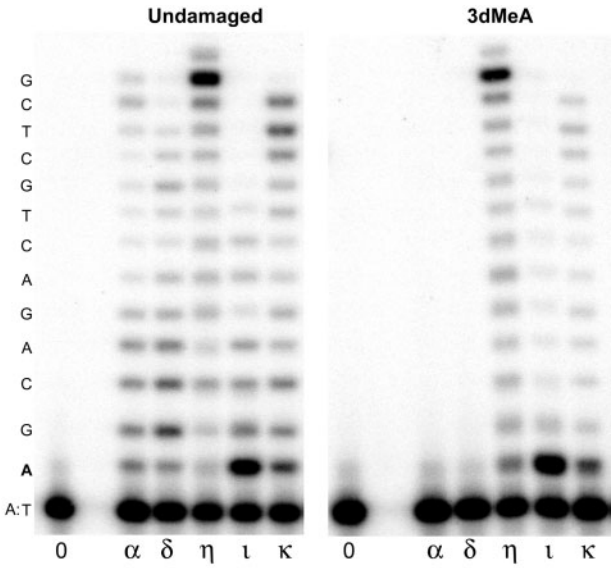

Figure 3. Ability of human DNA polymerases to bypass 3-deaza-3-methyl adenine *in vitro*. Standard reactions contained 100 μM all 4 dNTPs and lasted for 5 min at 37°C. Reactions contained 0.2 U polα, 5 nM polδ, 3 nM polη, 1.5 nM polι and 3 nM polκ. The nucleotide sequence of the template DNA is shown on the left-hand side of the gel. The 'A' in bold font is either undamaged (left-hand panel) or 3-deaza-3-methyl adenine (3dMeA; right-hand panel). As clearly seen, the 3dMeA lesion is a strong block to replication by human DNA polymerases α and δ, but can be bypassed by human polymerases η, ι and κ.

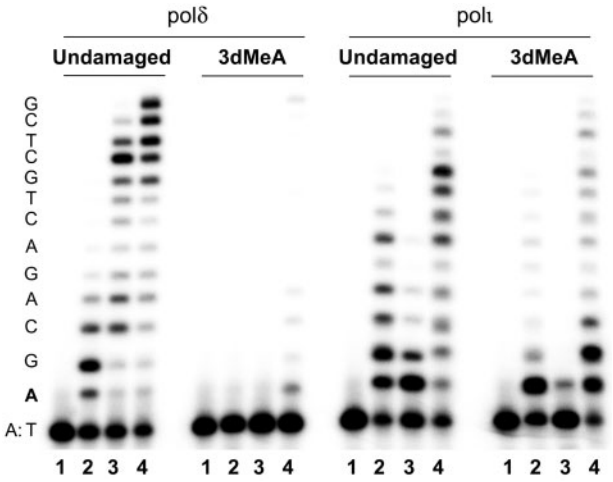

Figure 4. Ability of human DNA polymerases δ and ι to bypass 3-deaza-3-methyl adenine in the presence of low Mg^{2+}/Mn^{2+} *in vitro*. Standard reactions contained 100 μM all 4 dNTPs and lasted for 5 min at 37°C. Reactions contained 5 nM polδ and 4 nM polι. The nucleotide sequence of the template DNA is shown on the left-hand side of the gel. The 'A' in bold font is either undamaged (left-hand panel) or 3-deaza-3-methyl adenine (3dMeA; right-hand panel). Track 1, No dNTPs; Track 2, 0.25 mM $MgCl_2$; Track 3, 5 mM $MgCl_2$; Track 4, 0.25 mM $MnCl_2$. As clearly seen, the 3dMeA lesion is a strong block to replication by human DNA polymerases δ even in the presence of Mn. In contrast, polι-dependent incorporation opposite the lesion is stimulated by 0.25 mM $MgCl_2$ and significant bypass is observed in the presence of 0.25 mM $MnCl_2$.

Table 1. Efficiency of insertion of T opposite undamaged A, or 3dMeA by various eukaryotic polymerases

Polymerase[a]	Template	V_{max}/K_m ($\mu M^{-1} min^{-1}$)	Template	V_{max}/K_m ($\mu M^{-1} min^{-1}$)	Efficiency of insertion[b]	Efficiency of insertion[c]
Hs α	A	3.4	3dMeA	0.0002	5.88×10^{-5}	1
Hs η	A	3.06	3dMeA	0.073	2.30×10^{-2}	365
Hs ι[d]	A	210	3dMeA	0.24	1.14×10^{-3}	1200
Hs κ	A	13.73	3dMeA	0.025	1.82×10^{-3}	125
Sc η	A	0.96	3dMeA	0.025	3.02×10^{-2}	125

[a]*Hs*, *Homo sapien*; *Sc*, *S. cerevisiae*.
[b]Insertion opposite 3dMeA relative to the insertion opposite an undamaged A.
[c]V_{max}/K_m opposite 3dMeA relative to the V_{max}/K_m opposite 3dMeA by *Hs* polα.
[d]In the presence of 0.25 mM $MgCl_2$.

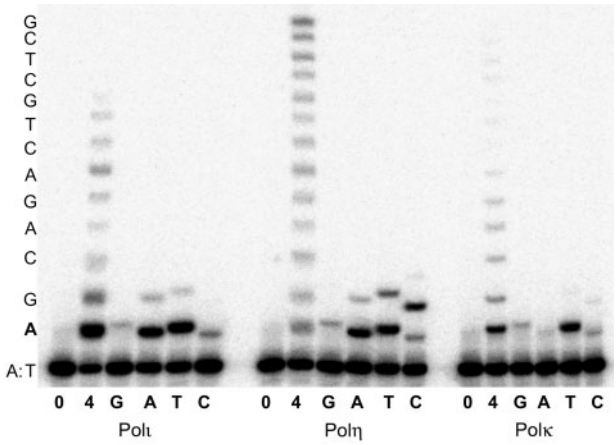

Figure 5. Ability of human DNA polymerases η, ι and κ to (mis)incorporate opposite 3-deaza-3-methyl adenine. Standard reactions contained 100 μM all 4 dNTPs (4), or each nucleotide separately (G, A, T, C) and lasted for 5 min at 37°C. Reactions contained 4 nM polη, 6 nM polι and 4 nM polκ. The nucleotide sequence of the template DNA is shown on the left-hand side of the gel. The 'A' in bold font indicates the location of the 3dMeA lesion.

Table 2. Fidelity of nucleotide insertion of opposite 3dMeA by various eukaryotic polymerases

Polymerase[a]	Incoming nucleotide	V_{max}/K_m ($\mu M^{-1}\,min^{-1}$)	f_{inc}
Hs α	A	0.0008	4
	T	0.0002	1
Hs η	G	0.017	2.3×10^{-1}
	A	0.035	4.8×10^{-1}
	T	0.073	1
	C	0.020	2.7×10^{-1}
Hs ι[b]	G	0.017	7.0×10^{-2}
	A	0.11	4.6×10^{-1}
	T	0.24	1
	C	0.018	7.5×10^{-2}
Hs κ	G	0.002	9.9×10^{-2}
	A	0.001	4.4×10^{-2}
	T	0.025	1
	C	0.003	1.3×10^{-1}
Sc η	G	0.002	6.2×10^{-2}
	A	0.004	1.6×10^{-1}
	T	0.025	1
	C	0.005	2.2×10^{-1}

[a]*Hs*, *Homo sapien*; *Sc*, *S. cerevisiae*.
[b]In the presence of 0.25 mM $MgCl_2$.

an efficiency of about 4% relative to an undamaged base-pair. Both polι and polκ extended the A:3dMeA mispair ~3- to 4-fold less efficiently than the T:3dMeA base-pair. In contrast, human polη actually extended the A:3dMeA mispair slightly better than the correctly paired T:3dMeA (Table 3).

Saccharomyces cerevisiae polη is important in tolerating MMS-induced damage in the absence of MAG1

Based on our *in vitro* findings with the human Y-family polymerases, we were eager to determine if Y-family polymerases play a role in tolerating 3MeA *in vivo*. We chose to use *S. cerevisiae* as a model because it has a limited number of DNA polymerases compared with higher eukaryotes. *Saccharomyces cerevisiae* polη is encoded by the *RAD30* gene, and it is reported that *rad30Δ* strains are somewhat sensitive to MMS (30,31). However, in the W303 background, a *RAD30* disruption is not sensitive to MMS (Figure 6A). Mag1 is the only DNA glycosylase that repairs 3MeA in *S. cerevisiae* and disruption of the *MAG1* gene makes yeast highly sensitive to methylating agents, such as MMS. In order to determine if *S. cerevisiae* polη is involved in tolerating lesions normally repaired by Mag1 (i.e. 3MeA), we generated a *rad30Δ mag1Δ* strain. Interestingly, the double mutant is more sensitive to MMS than the *mag1Δ* strain, suggesting that polη may help facilitate bypass of persisting 3MeA lesions *in vivo* (Figure 6A). Previous studies have shown that polζ is responsible for most MMS-induced mutagenesis (32,33), and deletion of *REV3* (encoding the catalytic subunit of polζ) further sensitizes *mag1Δ* strains to MMS (Figure 6B). Therefore, it is possible that both polη and polζ are important for survival after treatment with MMS in a *mag1Δ* background (Figure 6B). Indeed, the triple mutant is significantly more sensitive than either double mutant (Figure 6B). This suggests that polζ and polη act in independent repair pathways to tolerate unrepaired base damage caused by MMS. Similar observations and conclusions were recently drawn by Johnson *et al.* (34).

To determine which TLS polymerases in *S. cerevisiae* are capable of bypassing unrepaired 3MeA, we assayed the ability of *S. cerevisiae* polη, polζ, Rev1 and polζ in conjunction with Rev1 to bypass 3dMeA *in vitro* (Figure 7). Similar to human polη, *S. cerevisiae* polη bypasses the 3dMeA lesion reasonably efficiently. In contrast, polζ exhibits a much weaker ability to bypass the lesion (Figure 7, left-hand panel). Rev1, a dCMP transferase that is necessary for the function of

Table 3. Kinetics of single nucleotide extension[a] from matched/mismatch primer termini paired with 3-deaza-3-methyl adenine (3dMeA) by Y-family DNA polymerases

Polymerase[b]	Primer: Template	V_{max} (%ext/min)	K_m (μM)	V_{max}/K_m ($\mu M^{-1}\,min^{-1}$)	f_{ext}
Hs η	T:A	1.1	0.6	1.8	1
	T:3dMeA	1.1	7.7	0.14	8.0×10^{-2}
	A:3dMeA	0.77	4.9	0.16	9.0×10^{-2}
Sc η	T:A	2.0	1.0	2	1
	T:3dMeA	4.0	14.4	0.27	1.35×10^{-1}
	A:3dMeA	1.14	18	0.06	3.0×10^{-2}
Hs ι[c]	T:A	7.8	1.1	7.1	1
	T:3dMeA	1.8	6.4	0.28	4.0×10^{-2}
	A:3dMeA	0.8	14	0.06	8.5×10^{-3}
Hs κ	T:A	1.0	1.4	0.7	1
	T:3dMeA	0.63	9.4	0.07	1.0×10^{-1}
	A:3dMeA	0.36	17.6	0.02	3.0×10^{-2}

[a]Incorporation of C opposite undamaged G.
[b]*Hs*, *Homo sapien*; *Sc*, *S. cerevisiae*.
[c]In the presence of 0.25 mM $MgCl_2$.

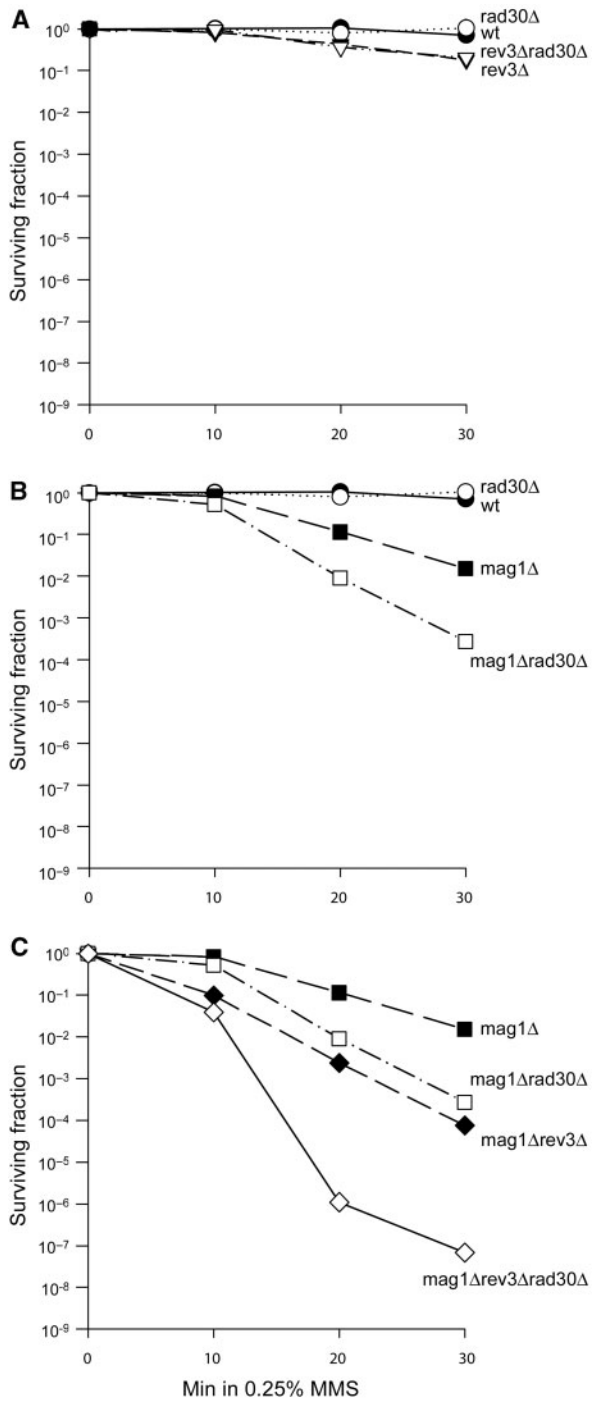

Figure 6. Survival of *S. cerevisiae* exposed to MMS. Exponentially growing strains of *S. cerevisiae* were exposed to 0.25% MMS for 10, 20 or 30 min, washed and subsequently plated on YPAD for 5 days at 30°C. (**A**) Disruption of *REV3* makes *S. cerevisiae* mildly sensitive to MMS, but disruption of *RAD30* has no observable effect on MMS sensitivity. (**B**) Disruption of *MAG1* sensitizes *S. cerevisiae* to MMS, and disruption of *RAD30* sensitizes the strain to MMS, indicating that the Rad30 encoded polη helps protect *S. cerevisiae* from lesions that are normally repaired by Mag1. (**C**) Disruption of *REV3* also sensitizes a *mag1Δ* strain to MMS, and the disruption of both *RAD30* and *REV3* synergistically enhances the lethality of MMS, indicating that polη and polζ may operate in separate pathways to repair lesions normally removed from the genome by Mag1. Three independent isolates were tested for each strain and standard deviations, which were all below 1 log of survival have been omitted for clarity.

polζ *in vivo*, also has minimal activity on either the undamaged A-template, or 3dMeA-containing template, as well as having little, to no stimulatory effect, on polζ's ability to bypass the 3dMeA lesion. Rev1 is clearly catalytically active under our assay conditions, as the enzyme is able to insert C opposite an undamaged template G, as well as further stimulate polζ activity (Figure 7, right-hand panel). Our *in vitro* data, combined with the MMS sensitivities of the *mag1Δ rad30Δ* and the *mag1Δ rad30Δ rev3Δ* strains therefore supports the idea that polη replicates past unrepaired 3MeA lesions in the absence of Mag1.

Human Y-family polymerases rescue the MMS sensitivity of *mag1Δ rad30Δ rev3Δ* strains of *S. cerevisiae*

The enhanced MMS sensitivity of the *mag1Δ rad30Δ rev3Δ* strain gave us an opportunity to test the ability of human polη, polι and polκ to bypass alkylation damage *in vivo*. When each human polymerase (as well as *S.cerevisiae* polη, as a control), was expressed from a galactose inducible promoter in the triple mutant, we discovered that all of the human polymerases rescued the MMS-sensitivity of the *mag1Δ rad30Δ rev3Δ* strain, albeit to varying degrees (Figure 8). Quite remarkably, expression of human polκ confers MMS resistance on the *mag1Δ rad30Δ rev3Δ* strain to the same extent as overproducing *S. cerevisiae* polη. Both human polη and polι also confer MMS-resistance, but to a lesser degree than human polκ or *S. cerevisiae* polη.

In some regard, it is really quite amazing that the human polymerases are able to confer MMS-resistance in the heterologous yeast survival assay, given the myriad of protein interactions that are believed to be required for the activity of the polymerases *in vivo* (35). Clearly, most of these protein–protein interactions must be conserved throughout evolution for the human polymerases to be able to function in *S. cerevisiae*. However, it is unlikely that these protein–protein interactions occur with the same efficiency in the heterologous system, and as a result, it is possible that the ability of human polι to restore MMS-resistance in *S. cerevisiae* is compromised by weakened protein–protein interactions with *S. cerevisiae*'s TLS accessory proteins. The same cannot be said of human polη's inability to restore MMS-resistance to the same extent as *S. cerevisiae* polη, as human polη has previously been shown to fully complement the UV-sensitivity attributed to a polη-deficiency in a *rad52Δ rad30Δ S. cerevisiae* strain (36).

DISCUSSION

We have described a novel procedure for the synthesis of a phosphoramidite that is a stable 3-deaza analog of 3-methyl-2′-deoxyadenosine (Figure 1). By using this analog in replication assays, we provide the most direct evidence currently available that 3MeA is a significant block to two of the three main replicases in eukaryotes, namely polα and polδ. Furthermore, we demonstrate that three human Y-family polymerases (polη, polι and polκ) are capable of insertion opposite the 3dMeA lesion, as

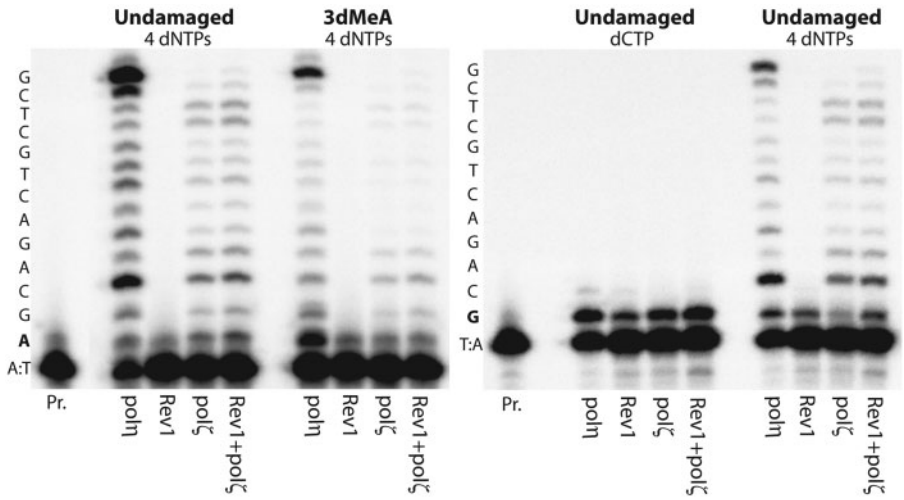

Figure 7. Ability of *S. cerevisiae* DNA polymerases to bypass 3-deaza-3-methyl adenine *in vitro*. Standard reactions contained 100 μM all 4 dNTPs and lasted for 20 min at 30°C. Reactions contained 4 nM polη, 6 nM polζ and 15 nM Rev1. The nucleotide sequence of the DNA templates is shown on the left-hand side of each gel. Left-hand panels: the 'A' in bold font is either undamaged adenine or 3-deaza-3-methyl adenine. As can be seen, polη bypasses the 3dMeA lesion much more efficiently than polζ. Rev1 has negligible activity on either template, and did not appreciably stimulate the activity of polζ on the 3dMeA template. Right-hand panels: both Rev1 and polζ are catalytically active, as they are able to incorporate dCMP opposite undamaged G. Rev1 also stimulates polζ in the presence of dCTP alone as well as in the presence of all 4 dNTPs.

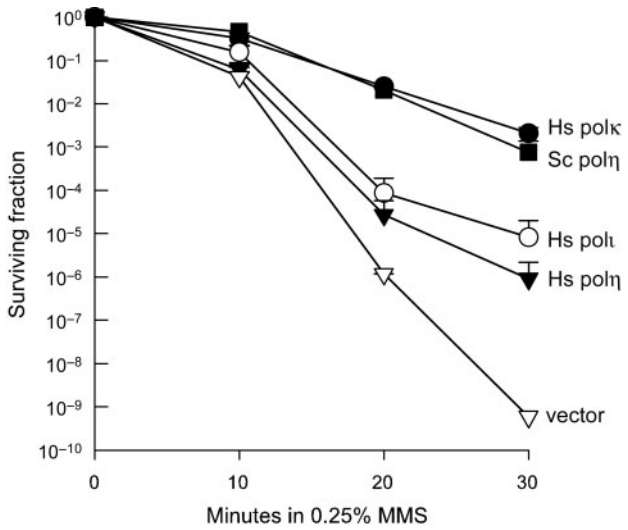

Figure 8. Human Y-family polymerases can restore MMS-resistance to a normally MMS-sensitive *rad30Δ rev3Δ mag1Δ* strain of *S. cerevisiae*. Exponentially growing strains of *S. cerevisiae* harboring plasmids expressing human polymerases η, ι and κ or *S. cerevisiae* polη under the control of a galactose inducible promoter were induced in complete synthetic galactose media without leucine. Media was removed, and cells were washed and exposed to 0.25% MMS for 10, 20 or 30 min, washed and subsequently plated on complete synthetic galactose plates lacking leucine for 5 days at 30°C. Three independent isolates were tested for each strain and standard deviations, which were all below 1 log of survival, have been omitted for clarity. As clearly seen, human polη and ι can restore MMS-resistance to the normally MMS-sensitive strain, but the greatest effect was observed with human polκ, which was as efficient as native *S. cerevisiae* polη in restoring MMS-resistance.

well as extension beyond the modified base (Figures 2–4), with polκ being the most accurate and polη the most efficient *in vitro* (Tables 1 and 2). Similarly, *S. cerevisiae* polη bypassed the 3dMeA lesion with the greatest efficiency of the Y-family polymerase assayed (Table 1), whilst polζ showed little ability to traverse the lesion *in vitro* (Figure 6A). Human polη, *S. cerevisiae* polη and human polκ, all extended bases incorporated opposite 3dMeA with an efficiency of 8–14% relative to an undamaged primer terminus (Table 3). Human polη did not discriminate between a correctly paired, or mispaired 3dMeA primer terminus, while human polκ and *S. cerevisiae* polη both preferred to extend the correctly paired T:3dMeA primer terminus 3- to 4-fold better then the A:3dMeA mispair. Polι extended the T:3dMeA base-pair poorly, but like human polκ and *S. cerevisiae* polη preferred the correctly paired primer terminus over the mispair.

Our kinetic data on the ability of human pols η, ι and κ to misinsert bases opposite a 3dMeA lesion *in vitro* does not agree well with a recent report in which 3MeA was modeled into the active site of the respective enzymes (34). Based upon molecular modeling it was hypothesized that polκ and polη should be able to insert a base opposite the 3MeA lesion equally as well as opposite an undamaged base. However, while significantly better than human polα, human polκ and polη inserted a base opposite 3dMeA with an efficiency of ~0.2–2% of that opposite an undamaged base, suggesting some steric hindrance of the 3dMeA lesion in the active site of the respective Y-family enzymes. Similarly, it was also hypothesized that polι should be able to extend a T:3MeA base pair efficiently (34), but in our hands, this only occurred with an efficiency of about 4% of that of an undamaged base pair.

To examine the role of Y-family polymerases in tolerating 3MeA *in vivo*, we utilized strains of *S. cerevisiae* that carried a Rad30 (polη) deletion. At least in the wild-type W303 and CL1265-7C backgrounds (data for CL1265-7C not shown), the absence of polη did not appear to render the strain sensitive to MMS (Figure 6A).

However, in the S288C background, a mild sensitivity has been previously reported (37). Since a large number of genes are known to be important for tolerating MMS in yeast (31), it is possible that subtle genetic differences between the W303 and the S288C backgrounds might account for the discrepancy between our observations and the data reported by others. Thus, despite the fact that *S. cerevisiae* polη bypasses the 3dMeA lesion *in vitro* (Figure 7), it does not appear to play a primary role in protecting wild-type cells from the cytotoxic effects of alkylation damage *in vivo*. Presumably such observations can be explained by the fact that 3MeA is not only intrinsically labile, but is efficiently removed from the genome by the Mag1 glycosylase (38). Interestingly, *S. cerevisiae* strains lacking both Mag1 and polη are significantly more sensitive to the cytotoxic effects of MMS than a wild-type strain (Figure 6B). We believe that such observations reveal an important role for polη in the bypass of *persisting* 3MeA lesions *in vivo*. It is also possible that the increased MMS-sensitivity may be partially due to a requirement for polη-dependent bypass of abasic sites. However, previous studies indicate that polη has limited ability to traverse an abasic site *in vitro* (39) and as a consequence, it is believed that polη plays only a minor role in the bypass of abasic site *in vivo* (40).

While polζ showed little ability to bypass the 3dMeA lesion *in vitro*, a *rev3Δ* strain nevertheless exhibited mild MMS-sensitivity *in vivo* (Figure 6A) (41). However, it should be noted that the strain is proficient for Mag1 and it is conceivable that the MMS sensitivity is actually due to an inability to bypass abasic lesions generated through the actions of the Mag1 glycoslyase, rather than defects in the bypass of 3MeA (32,33). The idea that polη and polζ potentially act in two separate pathways to facilitate bypass of 3MeA and abasic sites, respectively, is supported by the fact that the *mag1Δ rad30Δ rev3Δ* triple mutant is considerably more MMS-sensitive than either the *mag1Δ rad30Δ* or *mag1Δ rev3Δ* strains (Figure 6C).

The enhanced MMS sensitivity of the *mag1Δ rad30Δ rev3Δ* triple mutant allowed us to assay the role of human polη, polι and polκ in the tolerance of alkylation damage *in vivo*. While both expression of polη and polι increased MMS-resistance, expression of polκ resulted in MMS-resistance that was only rivaled by overexpression of endogenous *S. cerevisiae* polη (Figure 8). We believe that our data reflects how these enzymes might participate in the tolerance of alkylation damage in higher eukaryotes. Indeed, polκ appears to be important for survival after MMS exposure in both polκ-deficient MEFs and in polκ-deficient DT40 chicken lymphoblasts (42), and in both cases, it is assumed that the BER pathway in these cell lines remains fully functional. Furthermore, the role for polκ/polIV-like polymerases in tolerating cellular alkylation damage appears to be well conserved throughout evolution, as it has recently been reported that *Escherichia coli dinB* (polIV)-deficient strains are considerably more sensitive to MMS damage than wild-type strains (43).

The role of polη in tolerating MMS-induced lesions *in vivo* appears less clear. Polη appears to be important for budding yeast to tolerate MMS-induced damage, but only in the absence of BER (Figure 6A), and a similar situation may arise in human cells. Individuals with the variant form of *Xeroderma Pigmentosum* (XP-V) lack functional polη, and are susceptible to sunlight-induced skin cancer and while cells from these individuals are mildly sensitive to ultraviolet light, they are not sensitive to methylating agents, such as MMS (44).

The role of polι in the TLS of alkylation damage in mammals remains enigmatic. The *Poli* gene in the 129-derived inbred strain of mice has a stop codon in the second exon, effectively making the mice homozygous *Poli*(−/−) (45). Mice and embryonic stem cell lines derived from 129-derived strains are widely used in the study of DNA repair and mutagenesis, and appear to have no obvious sensitivity to methylating agents. However, it is possible that several 'knockout' mice generated using 129-derived embryonic stem cells could be 'double knockouts' for both *Poli* and the target gene of interest (46,47). Of direct importance to our current study, is the fact that two separate groups generated *Aag*(−/−) mice and cell lines from 129-derived embryonic stem cells (29,48). Interestingly, there were differences between the two studies in the sensitivity of the mice and MEFS to various alkylating agents. Elder *et al.*, found that the *Aag*(−/−) primary fibroblasts exhibited mild sensitivity to MMS, but not bischroroethyl nitrosourea or mitomycin C, while Engelward and colleagues, who generated homozygous Aag(−/−) cells directly from a 129-derived embryonic stem line, observed that the MEFS were hypersensitive to MMS, bischloroethyl nitrosourea and mitomycin C. Essentially, the cells used by Elder *et al.* (29), were *Aag*(−/−) while those used by Engelward *et al.* (48), were likely to have been *Aag*(−/−), *Poli*(−/−). A subsequent study by Sobol and colleagues (49) found that cells independently derived from *Aag*(−/−) were not sensitive to MMS at all. Thus, subtle genotypic strain differences could readily account for the various phenotypes. As a consequence, it will be interesting to assay the MMS sensitivity of congenic C57Bl6-derived mice lacking *Aag* and *Poli*, to determine if polι plays a role in protecting mammalian cells from alkylation-induced DNA damage.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

This work was supported by funds from the NICHD/NIH Intramural Research Program. We thank Alexandra Vaisman for help preparing the figures and Haruo Ohmori for kindly providing the polκ plasmid, pHSE2. Funding to pay the Open Access publication charges for this article was provided by the NICHD/NIH Intramural Research Program.

Conflict of interest statement. None declared.

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