Unique misinsertion specificity of pol ι may decrease the mutagenic potential of deaminated cytosines

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DNA polymerase ι (pol ι) is a distributive error-prone enzyme that can incorporate nucleotides opposite a variety of DNA lesions. Further elongation is, however, either substantially inhibited or completely abolished. Here, we provide evidence that pol ι can facilitate the efficient bypass of uracil and its derivatives as well as oxidized cytosine and guanine residues. The fidelity of translesion replication depends upon the lesion encountered. Correct nucleotides were inserted preferentially opposite 7,8-dihydro-8-oxoguanine (8-oxoG) and 5-hydroxycytosine (5-OHC). However, when bypassing uracil, 5-hydroxyuracil (5-OHU) or 5,6-dihydrouracil (5,6-DHU), pol ι inserted T and G with a 4- to 26-fold preference over the Watson–Crick base, A. While the T:U, T:5-OHU and T:5,6-DHU mispairs were extended poorly, the G:U, G:5-OHU and G:5,6-DHU mispairs were extended with equal or greater efficiency than the correctly paired primer termini. Thus, pol ι -dependent misinsertion of G opposite uracil and its derivatives may actually provide a mechanism whereby mammalian cells can decrease the mutagenic potential of lesions formed via the deamination of cytosine.

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Introduction

Like other members of the recently described UmuC/DinB/Rev1/Rad30 (Y-family) of DNA polymerases (Ohmori *et al.*, 2001), human DNA polymerase ι (pol ι), encoded by the *POL1* (*RAD30B*) gene (McDonald *et al.*, 1999), is a highly distributive low-fidelity enzyme lacking intrinsic exonucleolytic proofreading activity (Johnson *et al.*, 2000a; Tissier *et al.*, 2000b, 2001; Zhang *et al.*, 2000; McDonald *et al.*, 2001). Still, its ability to misinsert G opposite template T with a 3- to 11-fold preference over the correct base, A, is unique amongst known human DNA polymerases (Johnson *et al.*, 2000a; Tissier *et al.*, 2000b; Zhang *et al.*, 2000; Vaisman *et al.*, 2001). Orthologs of *POL1* have been identified in *Mus musculus* (McDonald *et al.*, 1999) and *Drosophila melanogaster* (Ishikawa *et al.*, 2001). While the properties of the mouse enzyme are akin to those of the human enzyme (E.G. Frank, personal communication), the *Drosophila* enzyme mainly incorporates the normal Watson–Crick base A, opposite

template T (Ishikawa *et al.*, 2001). Thus, to date, the preferential incorporation of G opposite T appears to be restricted specifically to mammalian pol ι orthologs.

Pol ι is a paralog of pol η , defects in which result in the sunlight-sensitive skin cancer-prone XP-V phenotype (Johnson *et al.*, 1999; Masutani *et al.*, 1999) indicating that the primary function of pol η is to protect humans from the deleterious consequences of DNA damage. Presumably, pol ι has evolved similarly to provide a genome-protecting role. However, at first glance, the ability of pol ι to misinsert G opposite T at high frequency might appear counter-intuitive for the well being of mammalian cells, as it would generate an unacceptably high mutation rate that would most probably lead to cancer and possibly cell death. As a consequence, we have considered a number of scenarios whereby the unique enzymatic properties of pol ι might, in fact, be beneficial for mammalian cells. For example, the *in vitro* fidelity of human pol ι when extending a recessed primer terminus (Tissier *et al.*, 2000b), as well as its altered fidelity at the very end of a DNA template (Frank *et al.*, 2001; Poltoratsky *et al.*, 2001), support a possible role for pol ι in somatic hypermutation (Poltoratsky *et al.*, 2000).

The recent discovery of intrinsic deoxyribose lyase (dRpase) activity (Bebenek *et al.*, 2001) coupled with the fact that the catalytic activity of pol ι is stimulated at short gaps (Frank *et al.*, 2001) suggest that the enzyme may also participate in a specialized form of base excision repair (BER). For example, such a situation might occur when T is generated by deamination of 5-methylcytosine and the G from the resulting G:T mispair is excised aberrantly by a DNA glycosylase. Insertion of G opposite template T during a gap-filling reaction would actually be a ‘correct’ event, thereby preventing C:G to T:A transition mutations (Bebenek *et al.*, 2001).

It is relatively easy to envisage that such an hypothesis could be extended to unmethylated DNA, where pol ι might play a genome-protecting role by maintaining G opposite U that has arisen from spontaneous hydrolytic cytosine deamination (Figure 1A). This common type of lesion is a major biological problem especially for growing cells since cytosine deamination is >100 times faster in single-stranded DNA, which is generated during replication and transcription, than in double-stranded DNA (Lindahl, 1993; Lindahl and Wood, 1999). As a consequence, human cells are equipped with at least four DNA glycosylases (UNG, SMUG, TDG and MBD4) that are able to recognize U and initiate excision of the base from DNA (Lindahl and Wood, 1999; Pearl, 2000). Although such repair is efficient, some uracils nevertheless escape repair and are encountered by a replication fork. This is a problem, as most known polymerases studied to date incorporate A opposite U, thereby generating a C→T transition (Kunkel and Bebenek, 2000). The lone

Fig. 1. Structures of modified bases and DNA sequence of oligonucleotide templates. (A) Uracil derivatives can arise from a variety of different cytosine deamination pathways. One of the stable products of cytosine oxidation is 5-OHC. (B) 8-oxoG is formed similarly *in vivo* by oxidation of guanine. (C) DNA templates with site-specific 8-oxoG, 5-OHC, 5-OHU, 5,6-DHU or uracil bases were hybridized to 32 P-labeled primers. Modified template bases (shown as X) were either adjacent to the 3' primer termini (using primer 1) to determine nucleotide incorporation opposite the lesion and total replicative bypass of damaged DNAs, or directly opposite the 3' primer terminus (using primer 2) to assess the efficiency of extension by polt. Primer 2 has either A, T, G or C as the 3'-terminal base. The undamaged controls utilized templates with G, C or T.

exception is human polt. In preliminary experiments, polt exhibited the same misincorporation spectrum opposite U as it did opposite T (Tissier *et al.*, 2001; K.Bebenek, A.Tissier, E.G.Frank, J.P.McDonald, R.Prasad, S.H.Wilson, R.Woodgate and T.A.Kunkel, unpublished data). As a consequence, polt-dependent misinsertion of G opposite U that has arisen from cytosine deamination would not be mutagenic. In a similar fashion, preferential dGMP misincorporation opposite uracil derivatives that are produced by cytosine deamination following exposure of DNA to ionizing radiation or oxidative agents (Teoule, 1987; Dizdaroglu *et al.*, 1993) would also be beneficial to a cell. These lesions include the poorly repaired 5-hydroxyuracil (5-OHU), which is formed by cytosine oxidation into an unstable cytosine glycol, followed by deamination and dehydration (Wagner *et al.*, 1992; Dizdaroglu *et al.*, 1993), and 5,6-dihydrouracil (5,6-DHU), a pyrimidine ring saturation product generated by ionizing radiation under anoxic conditions (Figure 1A) (Teoule, 1987). The present study was therefore designed to evaluate the efficiency and fidelity of polt-dependent translesion replication of several different uracil derivatives. For comparison, we also

used DNA containing 5-hydroxycytosine (5-OHC), another stable oxidative product of cytosine (Figure 1A) (Dizdaroglu *et al.*, 1993), and 7,8-dihydro-8-oxoguanine (8-oxoG), an extensively studied product of guanine oxidation (Figure 1B) (reviewed in Marnett, 2000).

Polt has previously been reported to have only a limited ability to bypass bulky DNA adducts (Johnson *et al.*, 2000a; Tissier *et al.*, 2000a; McDonald *et al.*, 2001; Zhang *et al.*, 2001). In the experiments described herein, we demonstrate that polt-catalyzed bypass of 8-oxoG and 5-OHC is, in fact, very efficient. The fidelity of 5-OHC lesion bypass is similar to that of undamaged C, with G preferentially inserted across from the lesion. In contrast, oxidation of G to 8-oxoG, alters polt's misincorporation spectra. Although C is still inserted preferentially opposite 8-oxoG, misinsertions of G also occur with very high frequency. In the case of replication through uracil and its derivatives, polt generally retains its unusual 'error-prone' properties by misincorporating G and T opposite U with 4- to 26-fold preference over the Watson-Crick base, A. While the misincorporated T is extended poorly, the misincorporated but non-mutagenic G is extended efficiently. Therefore, polt can catalyze efficient error-prone translesion replication of certain DNA lesions and, in selected cases, we suggest that its misincorporation specificity may actually lead to a decrease in damage-induced mutagenesis at these sites.

Results

Polt-dependent translesion replication of 8-oxoG, 5-OHC, uracil, 5-OHU and 5,6-DHU lesions *in vitro*

In order to examine the effects of various uracil derivatives as well as guanine and cytosine oxidative products on polt-dependent replication, 30mer oligonucleotides containing the respective site-specific adduct were used as substrates in 'standing-start' *in vitro* replication assays. As controls, we utilized undamaged templates containing either G, C or T immediately adjacent to the primer terminus. Figure 2A–D depicts time course reactions for primer elongation on these templates. Graphical representation of the time-dependent increase in total primer elongation (plots with filled symbols), as well as the increase in the replicative bypass of each lesion (plots with open symbols), reported as a percentage of total primer termini, is shown in Figure 2E–G. Consistent with its distributive nature, polt failed to reach the end of either the undamaged or damaged DNA template, and there were intense bands corresponding to every nucleotide in the sequence (Figure 2A–D). The initial primer utilization was very rapid and by 2 min almost all of the primers annealed to the undamaged DNA templates were elongated. Similar results were obtained with templates containing modified bases, where polt was clearly able to incorporate bases opposite the respective lesion, as well as promote complete lesion bypass.

Nucleotide incorporation opposite 8-oxoG was virtually as efficient as that observed opposite undamaged G (Figure 2A and E, plots with filled symbols); however, primer elongation past the damaged site decreased by ~20% (Figure 2E, plots with open symbols). In addition, a distinct pause site opposite the first template base downstream from 8-oxoG is evident (Figure 2A). This

Fig. 2. Time course experiments depicting polt-catalyzed primer extension on damaged and undamaged templates. Representative gels comparing polt-catalyzed primer extension on templates with undamaged G and 8-oxoG (A), with undamaged C and 5-OHC (B), with undamaged T (C) and with uracil, 5-OHU or 5,6-DHU (D). Template sequences are shown to the left of the gels. Damaged bases are indicated. The graphs (E–G) indicate the quantitation of the gels shown in (A–D) and plot the time course of primer elongation on damaged and undamaged templates as a percentage of total primer termini (filled symbols) and the extent of replication past the damaged site (open symbols). Primer elongation past the same site on undamaged templates (open symbols) is also shown for comparison. Primer elongation on templates with undamaged G (E), C (F) and U (G) is shown using open or filled circles. Primer elongation on templates with 8-oxoG (E), 5-OHC (F) and 5-OHU (G) is shown using open or filled triangles. Primer elongation on templates with 5,6-DHU (G) is shown using open or filled diamonds.

product consists of at least two bands with slightly different electrophoretic mobility. Because the 3'-terminal nucleotide has a significant influence on oligonucleotide mobility, the presence of doublets suggests that polt catalyzed a significant degree of misincorporation opposite either the 8-oxoG lesion or the base immediately after it, in this case at template A. Since previous data have shown that polt-dependent nucleotide incorporation opposite template A is rather accurate (Tissier *et al.*, 2000b), the existence of the doublet opposite template A suggests that at 8-oxoG there is extensive misincorporation followed by efficient elongation of both correctly paired and mispaired primers.

In contrast, 5-OHC appears to pose a greater block to polt-dependent bypass than 8-oxoG. On the 5-OHC-containing template, we observed a decrease in both nucleotide incorporation opposite the lesion and subsequent lesion bypass, compared with replication of the template with an undamaged C (Figure 2B and F). However, even in this case, replicative bypass reached ~60% in a 5 min assay (Figure 2F, plots with open symbols).

The relative distribution of replication products on templates containing U appears to be similar to that observed on undamaged templates with T in the same position (Figure 2C and D). In these cases, the efficient nucleotide incorporation opposite T or U is followed by less efficient primer extension. Previous reports have attributed the decreased efficiency of primer elongation past template T to the exceptionally high frequency of nucleotide misincorporation opposite this base (Tissier *et al.*, 2000b; Zhang *et al.*, 2000; Vaisman *et al.*, 2001). The data presented in Figure 3D suggest that polt's fidelity opposite template U is also very low. This also appears to be true for the templates containing 5-OHU and 5,6-DHU (Figure 2D and G). Indeed, the pattern of primer elongation on templates containing U, 5-OHU and 5,6-DHU is very similar. However, primer extension past 5,6-DHU appears to proceed more efficiently than that either past U or past 5-DHU (Figure 2G, plots with open symbols), even though the extent of nucleotide incorporation opposite 5,6-DHU, and therefore total primer elongation (Figure 2G, plots with filled symbols), is much lower than on the other modified templates.

Fig. 3. Specificity of polt-dependent nucleotide incorporation opposite unmodified and modified bases. Nucleotide misincorporation studies were performed for 10 min using 25 fmol of polt and a mixture of all four dNTPs or each dNTP individually present at 100 μ M. The template sequence is indicated on the left of the figure. (pr = primer).

Fidelity of pol β - and polt-dependent nucleotide incorporation opposite modified template bases

To determine the miscoding potential of DNA bases modified by deamination and oxidation, we repeated polt-dependent primer extension experiments in the presence of each nucleotide individually (Figure 3). In agreement with its known extremely low fidelity at template T, G or C (Johnson *et al.*, 2000a; Tissier *et al.*, 2000b; Zhang *et al.*, 2000), polt catalyzed significant levels of nucleotide misincorporation on all DNA templates used. In many cases, the resulting primer–template mismatches were elongated further by the incorporation of a second incorrect nucleotide. For example, primer elongation in the presence of dGTP using templates with U or 5-OHU immediately adjacent to the 3' primer terminus produced up to four distinct bands, with the last band containing three mismatched base pairs, and is consistent with polt's ability to extend mismatches promiscuously (Figure 3D) (Vaisman *et al.*, 2001).

Qualitatively, it appears that the pattern of misincorporation on the modified DNA templates is very similar to that observed with the corresponding undamaged DNA. (In these experiments, we assume that the correct undamaged 'control' for 5-OHU- and 5,6-DHU-containing templates, as for the uracil-containing DNA, would be a template with T rather than C, even though the oxidized uracil derivatives are formed as a result of cytosine deamination pathways.) One notable exception is the misinsertion pattern observed opposite 8-oxoG (Figure 3A). While G is the least incorporated nucleotide opposite the undamaged G, it appears to be incorporated at least as efficiently as the correct base, C, opposite 8-oxoG. Oxidation of guanine into 8-oxoG also changes the pattern of nucleotide incorporation by rat pol β , another low-fidelity DNA polymerase lacking intrinsic exonuclease activity (Figure 4A). However, in contrast to polt, pol β , like many other polymerases studied to date, misincorporates A opposite 8-oxoG (Figure 4A; Shibutani *et al.*, 1991). The nucleotide incorporation specificity of both polt and pol β opposite 5-OHC appears

to be very similar to that observed opposite undamaged C, which, in the case of pol β , is the predominant incorporation of the correct base G (Figure 4B). As expected, the misincorporation pattern of both polt and pol β opposite U, 5-OHU and 5,6-DHU resembled misincorporation specificities opposite T rather than the base from which they were derived, C (Figures 3C and D, and 4C and D). Thus, pol β mainly incorporates A opposite U, 5-OHU and 5,6-DHU, while polt incorporates G, T and A at comparable levels opposite these sites.

Steady-state kinetics of polt-catalyzed nucleotide insertion opposite modified and undamaged bases

To determine more accurately the specificity of polt-dependent nucleotide (mis)incorporation opposite each modified base, we utilized a steady-state kinetic assay (Boosalis *et al.*, 1987; Creighton *et al.*, 1995) (Table I). Surprisingly, the efficiency of dCMP incorporation opposite 8-oxoG was 6.5 times lower than that observed opposite undamaged G. This contrasts with the apparently efficient primer elongation observed with the 8-oxoG-modified DNA in the presence of all four nucleotides (Figure 2A and E; plots with filled symbols). The explanation for this discrepancy comes partly from the comparison of the $V_{\max}/K_m$ values for dGMP incorporation (Table I). The efficiency of dGTP incorporation opposite 8-oxoG is actually 14-fold higher than that observed opposite an unmodified G and only twice as low as the efficiency of dCTP incorporation opposite 8-oxoG. Still, the combined $V_{\max}/K_m$ for dCTP and dGTP incorporation opposite 8-oxoG (0.59 ± 0.28) is only ~23% of that opposite undamaged C (3.83 ± 0.02) (Table I). The seeming inconsistencies between the kinetic data (Table I) and results of primer extension experiments (Figure 2A and E) are explained in part by the higher enzyme concentration used in the primer extension studies, but also by the fact that the decreased efficiency of dCTP incorporation opposite 8-oxoG compared with an undamaged G is determined to a larger extent by an increase in the K_m value (3.5-fold) rather than by the

Table I. Frequency of misincorporation and relative efficiency of nucleotide incorporation on undamaged and damaged DNA templates

Target template base	Incoming dNTP	$V_{\max}/K_m^a$	Relative $V_{\max}/K_m^b$	f_{inc}
Undamaged G	C	3.83 ± 0.40	—	1
	G	0.02 ± 0.01	—	0.005
	T	0.07 ± 0.01	—	0.018
	A	0.045 ± 0.006	—	0.012
8-oxoG	C	0.59 ± 0.11	0.15	1
	G	0.28 ± 0.08	14	0.47
	T	0.037 ± 0.006	0.53	0.063
	A	0.020 ± 0.001	0.44	0.034
Undamaged C	G	3.71 ± 0.75	—	1
	C	0.15 ± 0.04	—	0.04
	A	0.07 ± 0.01	—	0.019
	T	0.13 ± 0.02	—	0.035
5-OHC	G	1.59 ± 0.50	0.43	1
	C	0.09 ± 0.02	0.60	0.057
	A	0.05 ± 0.01	0.71	0.031
	T	0.19 ± 0.04	1.46	0.12
U	G	1.32 ± 0.08	0.36	1
	C	0.022 ± 0.005	0.15	0.017
	A	0.31 ± 0.05	4.43	0.23
	T	4.7 ± 0.6	36.2	3.56
5-OHU	G	0.49 ± 0.02	0.13	1
	C	0.021 ± 0.008	0.14	0.043
	A	0.10 ± 0.04	1.43	0.2
	T	2.4 ± 0.7	18.5	4.9
5,6-DHU	G	0.18 ± 0.03	0.049	1
	C	0.016 ± 0.003	0.11	0.089
	A	0.018 ± 0.005	0.26	0.10
	T	0.10 ± 0.02	0.77	0.56

^aStanding-start kinetic assays were performed for 1 min and the dNTP concentrations ranged from 0.5 to 500 μM . Data for $V_{\max}/K_m$ are means ($\pm$ standard error) from 3–4 experiments. Units for $V_{\max}/K_m$ are the percentage of primer elongation product/min/ μmol of nucleotide.

^bSince uracil species are considered to be derivatives of cytosine, the efficiency of nucleotide incorporation opposite these bases was determined relative to incorporation opposite C and the frequency of misincorporation was determined relative to dGTP incorporation.

Table II. Frequency of misincorporation opposite template T and uracil derivatives relative to the correct incorporation of dAMP

Target template base	Incoming dNTP	f_{inc}
T	A	1
	T	4.5
	G	9.7
	C	0.2
U	A	1
	T	15.2
	G	4.3
	C	0.07
5-OHU	A	1
	T	26
	G	4.9
	C	0.2
5,6-DHU	A	1
	T	5.6
	G	10
	C	0.9

derivatives with greater efficiency than A (Figure 3D; Table II). If the mispair is elongated successfully, the incorrect base will be fixed as a mutation. To test the

Fig. 5. Extension of primers with various 3'-terminal bases opposite 8-oxoG and uracil derivatives. Primer extension was assayed after a 10 min reaction containing 25 fmol of polt and a mixture of all four dNTPs (A and B) or each dNTP individually (C) present at 100 μM . The extension of an 8-oxoG containing template is shown in (A), extension on templates containing uracil, 5-OHU and 5,6-DHU are shown on (B) and (C). The template sequence is indicated on the left of each panel. The primer-template structure for DNA containing uracil derivatives is shown below the gel.

ability of polt to extend lesion-containing mispairs, we performed primer elongation studies using primers each with a different 3' terminus opposite the modified base. Under these conditions, the G:8-oxoG mispair is elongated by one nucleotide as efficiently as the correct C:8-oxoG base pair (Figure 5A). However, further elongation is inhibited substantially. Therefore, efficient misincorporation of dGMP opposite 8-oxoG and its subsequent elongation would lead to a replication pause one base after the 8-oxoG lesion. As a consequence, one would expect a doublet band opposite A, the first template base downstream of the 8-oxoG lesion, which would represent extension from both correctly paired and mispaired bases inserted opposite 8-oxoG. Indeed, such a doublet is clearly visible in the presence of all four dNTPs (Figure 2A).

Extension of primers with 3' termini opposite uracil, 5-OHU and 5,6-DHU reveals that all four terminal bases appear to be elongated relatively easily (Figure 5B). In the presence of all four dNTPs, primers ending with T appear to be extended with the lowest efficiency, while primers with a 3'-terminal G are elongated almost as efficiently as primers ending in A. To determine the accuracy of elongation, primer extension reactions were performed using a single dNTP substrate present at a relatively high concentration (100 μM) (Figure 5C; misincorporation patterns were similar on templates containing all three uracil derivatives and, as a consequence, only the results with template U are shown). Under these conditions, extension of primers with T and C incorporated opposite uracil, 5-OHU and 5,6-DHU is accurate, with misincorporation bands barely detectable in all instances. Polt also extends primers with a 3' A or G by the preferential insertion of the correct base T opposite template A. However, it is clear that when extending primers with A and G opposite uracil, 5-OHU and 5,6-DHU, polt also makes multiple errors, as we also observed misincorporations of A, C or G opposite the next template A and bases further downstream (Figure 5C).

Table III. Steady-state kinetic analysis of primer extension by the incorporation of dTMP opposite template A and the overall efficiency of polt-dependent bypass of uracil, 5-OHU and 5,6-DHU

Primer–template	$V_{\max}/K_m^a$	f_{ext}	$f_{\text{inc}} \pm f_{\text{ext}}^b$
A:U	300 $\pm$ 60	1	1
T:U	0.18 $\pm$ 0.01	6.0×10^{-4}	8.8×10^{-3}
G:U	63 $\pm$ 9	0.21	0.87
A:5-OHU	135 $\pm$ 33	1	1
T:5-OHU	0.048 $\pm$ 0.001	3.6×10^{-4}	9.2×10^{-3}
G:5-OHU	23 $\pm$ 3	0.17	0.83
A:5,6-DHU	29 $\pm$ 3	1	1
T:5,6-DHU	0.034 $\pm$ 0.006	1.2×10^{-3}	6.6×10^{-3}
G:5,6-DHU	7 $\pm$ 1	0.24	2.4

^aStanding-start kinetic assays were performed for 1 min using 10 fmol of polt and 100 fmol of primer–template. dTTP concentrations ranged from 0.004 to 4 μ M when A or G was the 3'-terminal primer base and from 30 to 500 μ M when the 3'-terminal base was T. Data for $V_{\max}/K_m$ are means ($\pm$ standard error) from 3–5 experiments. Units for $V_{\max}/K_m$ are the percentage of primer elongation product/min/ μ mol of nucleotide.

^b f_{inc} values used for calculation of overall efficiency of translesion replication are taken from Table I.

We have characterized the efficiency of the primer extension reactions further using steady-state gel kinetic analysis. In doing so, we focused on determining the kinetic parameters for the reactions that apparently represent the best misincorporation and/or extension at the modified bases. Therefore, we determined the catalytic efficiencies ($V_{\max}/K_m$ values) for extension of primers with a 3'-terminal A, T and G opposite uracil, 5-OHU and 5,6-DHU by the correct incorporation of T opposite the next template base, A (Table III). According to our kinetic measurements, Watson–Crick A:U, A:5-OHU and A:5,6-DHU base pairs were extended with the greatest efficiency. However, extension of primers with a 3'-terminal G opposite uracil or its derivatives is also quite efficient, occurring with an efficiency of ~20% relative to the efficiency of extension of Watson–Crick base pairs (Table III). Such mispair-extension efficiencies are remarkably high for most polymerases, and are at the high end of the range seen with other low-fidelity enzymes, such as HIV reverse transcriptase (Mendelman *et al.*, 1990), or those proficient at mispair extension such as *Saccharomyces cerevisiae* pol ζ (Johnson *et al.*, 2000a; Lawrence *et al.*, 2000). Indeed, the values reported here are close to the maximum that we recently reported for polt-dependent extension of mispairs at undamaged template sites (Vaisman *et al.*, 2001).

Even though T is misincorporated 5- to 25-fold better than A opposite uracil and its derivatives, the distortion generated by the T:U primer–template terminus strongly inhibits subsequent elongation ($f_{\text{ext}} = 1 \times 10^{-3}$ – 6×10^{-4}). This phenomenon also resembles polt-dependent misinsertion and bypass at a *cis*–*syn* thymine–thymine dimer, where T is inserted with the greatest efficiency, yet is poorly extended (Tissier *et al.*, 2000a). To evaluate the overall outcome of translesion synthesis on templates with uracil and its derivatives, we have determined the relative frequency of formation and extension from the A, G or T base pairs with damaged bases as the product of the efficiency of nucleotide insertion and the efficiency of

subsequent extension (Table III). Insertion of A opposite template U, 5-OHU or 5,6-DHU and its subsequent extension by the incorporation of dTMP opposite the downstream template A was chosen as the standard, with an overall frequency value of 1. During replication past uracil derivatives, dATP and dGTP are favored over dTTP by two orders of magnitude due to an $\sim 10^3$ - to 10^4 -fold preference in their extension. These kinetic data help explain the results of our primer extension studies, which showed more efficient elongation past 5,6-DHU compared with that past U and 5-OHU (Figure 2, plots with open circles). Indeed, the most easily extended G:5,6-DHU base pair (Table II) is formed 2–10 times more frequently than T:5,6-DHU or A:5,6-DHU (Table I), while the most frequently formed T:5-OHU and T:U pairs (Table I) are extended inefficiently (Table II). The overall slight preference for dATP during bypass of U and 5-OHU occurs at the extension step, while the 2.4-fold bias for dGTP on 5,6-DHU-containing templates is determined by the initial misincorporation step. Our data suggest, therefore, that translesion replication on templates containing deaminated cytosine derivatives is least mutagenic for 5,6-DHU-containing DNA. However, even on U- and 5-OHU-containing templates for which the formation of the wobble but 'correct' G:U base pair occurs less frequently, the overall efficiency of dGMP incorporation and extension of the resulting base pair is similar to the efficiency of the formation and extension of Watson–Crick A:U and A:5,6-DHU base pairs. This is in dramatic contrast to other DNA polymerases, even those considered as being low fidelity, such as pol β , which still inserts A opposite U much more frequently than it misinserts G opposite U (Figure 4).

Discussion

Exposure of cells to both endogenous and exogenous oxidizing agents produces >100 different DNA oxidative products including oxidized purines and pyrimidines, some of which are unstable and give rise to additional DNA lesions (Laval, 1996). Among them are 8-oxoG, a major stable purine product produced by guanine oxidation, and 5-OHC and 5-OHU, both stable products of cytosine oxidation (Laval, 1996). In contrast, spontaneous and damage-induced cytosine deamination results in the formation of uracil and 5,6-DHU in DNA (Lindahl, 1993; Laval, 1996). If not readily removed, these lesions pose a serious threat to genome integrity. In the present study, we determined the efficiency and fidelity of polt-dependent translesion synthesis past 8-oxoG, 5-OHC, 5-OHU, 5,6-DHU and uracil.

Polt-dependent bypass of DNA lesions in vitro

Previous *in vitro* experiments using polt and a variety of damaged DNAs (Johnson *et al.*, 2000a; Tissier *et al.*, 2000a; McDonald *et al.*, 2001; Zhang *et al.*, 2001) indicate that even when polt is able to incorporate nucleotides opposite the lesion efficiently, further primer elongation is either completely abolished (as in the case with an abasic site; Johnson *et al.*, 2000a; McDonald *et al.*, 2001; Zhang *et al.*, 2001) or considerably inhibited [as in the case with *N*-2-acetylaminofluorine- and UV-induced *cis*–*syn* thymine–thymine dimers and TT (6–4) photoproducts;

Tissier *et al.*, 2000a; McDonald *et al.*, 2001]. Zhang *et al.* (2001) recently reported that 8-oxoG also significantly blocks polt-catalyzed primer elongation. However, in contrast, we show here that polt in fact catalyzes efficient bypass of 8-oxoG and, in the process, it misincorporates dGMP with extremely high frequency ($f_{\text{inc}} = 0.47$), while dAMP and dTMP are incorporated ~ 10 -fold less efficiently ($f_{\text{inc}} = 3.4 \times 10^{-2}$ for dAMP and 6.3×10^{-2} for dTMP). This misincorporation specificity differs from that reported for polt in an independent study, where A was the most frequently misinserted base opposite 8-oxoG (Zhang *et al.*, 2001). These contradictory results possibly may be attributed to the different sequence context in the vicinity of the lesion. Indeed, sequence context differences in the fidelity of nucleotide incorporation opposite 5-OHC and 5-OHU have been reported for the exonuclease-deficient Klenow polymerase (Purmal *et al.*, 1994). Our data on specificity of polt-dependent misincorporation opposite 8-oxoG are also very different from those reported for other DNA polymerases, where A is incorporated predominantly opposite 8-oxoG (Shibutani *et al.*, 1991). The fact that polt not only misincorporates G opposite 8-oxoG with high frequency but also extends the mispair efficiently (Figure 5A) suggests that if polt-dependent bypass of an 8-oxoG lesion would occur *in vivo*, it would lead to a high level of G→C transversions in mammalian cells. This mutation would be particularly harmful for the cell since, at the present time, there is no known glycosylase that excises G from a G:8-oxoG mismatch. In contrast, the evolutionarily conserved MutY protein efficiently removes most adenine residues misincorporated opposite 8-oxoG (Tsai-Wu *et al.*, 1991; McGoldrick *et al.*, 1995). *In vivo* studies show that the most common 8-oxoG-induced mutations are G→T transversions (Moriya, 1993; Le Page *et al.*, 1995), which presumably arise because the misincorporated A escapes correction by the MutY protein. Consistent with such a hypothesis, the level of G→T transversions increases in knockout mice defective in the removal of 8-oxoG (Klungland *et al.*, 1999; Minowa *et al.*, 2000), implying that while polt can bypass an 8-oxoG lesion efficiently *in vitro*, it is probably not involved in the replicative bypass of 8-oxoG *in vivo*.

Similarly to 8-oxoG, 5-OHC was also bypassed readily by polt, although in this case bypass of the lesion was more accurate and did not result in any significant change in misinsertion specificity compared with the undamaged C template. In contrast, another product of cytosine oxidation, 5-OHU, is characterized by a very different nucleotide incorporation pattern. Unlike other DNA polymerases, polt preferentially incorporated T opposite 5-OHU as well as opposite uracil. Polt-dependent nucleotide incorporation opposite these bases proceeds with a relative efficiency of T>G>A>>C (Table I). Nevertheless, the preferential insertion of dTMP opposite uracil and 5-OHU is opposed by the preferential extension from A and G paired with uracil and 5-OHU (Table III). Comparison of the overall efficiencies of translesion replication shows a slight preference for the incorporation and extension of dAMP opposite both lesions. Since cytosine is the precursor of uracil and 5-OHU, if the resulting A:U Watson–Crick base pair is extended further, it will lead to a C→T transition. Therefore, polt-catalyzed replication bypass of U and 5-OHU will still be

‘error-prone’. However, the mutation frequency will be significantly lower than for other DNA polymerases, which predominantly incorporate A opposite U and 5-OHU by several orders of magnitude over the misincorporation of G (Boiteux and Laval, 1982; Duncan and Weiss, 1982; Cabral-Neto *et al.*, 1993; Purmal *et al.*, 1994).

In contrast to U and 5-OHU, 5,6-DHU mainly pairs with dGMP in polt’s active site. The relative stability of the G:5,6-DHU pair is confirmed further by the efficient extension of primers with a 3’ G opposite 5,6-DHU. In this case, the non-mutagenic base therefore provides the best fit, even though its incorporation leads to the formation of a wobble base pair. The insertion of G opposite 5,6-DHU and the extension of the resulting base pair is, in fact, favored over A and T by ~ 2.4 - and 360-fold, respectively. Therefore, these data suggest that exceptionally error-prone polt appears to be the least mutagenic among DNA polymerases capable of replicating damaged DNA containing deaminated cytosines. Polt’s involvement in the apparently non-mutagenic bypass of specific lesions seems logical and clearly parallels the observation that pol η , which like polt is a low-fidelity enzyme, plays a major role in the error-free bypass of UV-induced *cis-syn* thymine–thymine dimers (Masutani *et al.*, 1999; Johnson *et al.*, 2000b).

Does polt bypass uracil, 5-OHU or 5,6-DHU *in vivo*?

DNA lesions vary significantly in the extent with which they inhibit DNA replication and direct nucleotide misincorporation. Previous *in vitro* replication data have shown that uracil, 5-OHU or 5,6-DHU result in no more than a transient replication pause for most DNA polymerases [for example, see Figure 4 for rat pol β , Purmal *et al.* (1994) for 5-OHU and Liu and Doetsch (1998) for 5,6-DHU with *Escherichia coli* DNA polymerases], suggesting that these lesions are unlikely to be lethal or cytotoxic. However, published data also revealed that uracil and its derivatives are potentially mutagenic DNA lesions (Marnett, 2000). Both uracil and 5,6-DHU pair mainly with dAMP (Boiteux and Laval, 1982; Duncan and Weiss, 1982; Cabral-Neto *et al.*, 1993; Liu and Doetsch, 1998). In contrast, the outcome of 5-OHU translesion replication catalyzed by the exonuclease-deficient Klenow fragment of *E.coli* polymerase I is dependent upon DNA sequence context (Purmal *et al.*, 1994). In one sequence context, mutagenic dAMP was incorporated opposite 5-OHU, while in a second sequence context, the Klenow polymerase predominantly incorporated dCMP opposite 5-OHU (Purmal *et al.*, 1994). In either case, incorporation of A or C opposite a residue that once paired with G is, of course, highly mutagenic.

Intuitively, it would make sense that to reduce the mutagenic potential of these lesions, cells might employ a polymerase, such as polt, that would incorporate G opposite the deaminated cytosine much more frequently than other polymerases. However, this is not an easy task. Polt is a distributive enzyme (Tissier *et al.*, 2000b) and is unlikely to compete with replicative polymerases for a 3’ primer terminus, unless a significant pausing event occurs at the lesion *in vivo*. The other problem is that the cell has to discriminate between a cytosine base that has undergone deamination to uracil, and a dUMP moiety that has been

incorporated into nascent DNA erroneously. While incorporation of G opposite U (that was once a C) would be error-free, incorporation of G opposite dUMP would be highly error-prone. Interestingly, vertebrates seem to have evolved a system of distinguishing between the two uracil moieties. Recent studies suggest that dUMP is removed primarily by uracil DNA glycosylase (encoded by *UNG2*) (Nilsen *et al.*, 2000), whereas deaminated cytosines are removed by the SMUG1 glycosylase (Nilsen *et al.*, 2001). Is it possible that the targeting of polt and the discrimination between U/deaminated C and dUMP is achieved via a common signaling pathway involving SMUG1? If it is, it may be no mere coincidence that both *SMUG1* and *POLI* appear to have similar evolutionary distributions (McDonald *et al.*, 1999; Nilsen *et al.*, 2001). At the present time, the number of identified *POLI* orthologs is limited but, like *SMUG1*, no readily identifiable ortholog is found in the completely sequenced *Caenorhabditis elegans*, *Arabidopsis thaliana* or *S.cerevisiae* genomes, suggesting that both *SMUG1* and *POLI* may encode proteins with specialized functions in higher eukaryotes. Clearly, much remains to be learned about how polt (or any of the recently discovered DNA polymerases) is regulated and specifically targeted for its *in vivo* functions. Whether polt can replicate uracil, 5-OHU or 5,6-DHU *in vivo* will probably only be known when a knockout *Poli* mouse is available and it is crossed with mice defective in *Ung2* and *Smug1*.

Materials and methods

Construction of damaged DNA templates

All primer-templates were constructed from synthetic oligonucleotides and were synthesized by Loftstrand Laboratories (Gaithersburg, MD) using standard techniques and gel purified prior to use. The 8-oxoG, 5-OHC, 5-OHU and 5,6-DHU phosphoramidite bases were all purchased from Glen Research (Sterling, VA). The sequences of templates containing site-specific lesions are listed in Figure 1C. Primers (16 and 17mers) were 5'-end-labeled using T4 polynucleotide kinase and [γ - 32 P]ATP and annealed to undamaged or damaged 30mer templates at a 1:1.5 molar ratio. Hybridization was achieved by heating the mixture of required oligonucleotides in an annealing buffer [50 mM Tris-HCl pH 8, 5 mM MgCl₂, 50 μ g/ml bovine serum albumin (BSA), 1.42 mM 2-mercaptoethanol] for 10 min at 100°C followed by slow cooling to room temperature over a period of ~2 h. Annealing efficiencies were >95%, as evidenced by the different electrophoretic mobility of the 32 P-labeled primers on non-denaturing polyacrylamide gels before and after hybridization to the template (data not shown).

Primer extension assays

GST-tagged polt was purified as described previously (Tissier *et al.*, 2000b). Recombinant human pol β was generously provided by Dr S.Wilson (NIEHS). Primer extension assays were performed essentially as described previously (Tissier *et al.*, 2000b; Vaisman *et al.*, 2001). A 100 fmol concentration of primer-template (expressed as primer termini) was incubated with 25 fmol of polt at 37°C in 10 μ l reactions containing 100 μ M of either all four dNTPs or each dNTP individually, 40 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 250 μ g/ml BSA and 2.5% glycerol. Reaction times are indicated in the respective figure legends. Primer extension reactions containing pol β were performed using 100 fmol of DNA templates and 25 fmol of enzyme. Reactions were incubated at 37°C for 10 min in 10 μ l reactions containing 100 μ M of either all four dNTPs or each dNTP individually, 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 2 mM DTT, 20 mM NaCl, 200 μ g/ml BSA and 2.5% glycerol. All reactions were terminated by the addition of an equal volume of formamide loading dye solution (90% formamide, 500 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Before loading onto the gel, the reactions were denatured by heating at 100°C for 5 min and immediately transferred to ice for 5 min.

Products were resolved by denaturing polyacrylamide gel electrophoresis (8 M urea, 20% acrylamide, 4 h at 2000 V) and then visualized and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. Total primer elongation was calculated as the sum of all elongation products and expressed as a percentage of total primer termini (elongated and unelongated). The extent of translesion replication on damaged templates was calculated as the sum of elongation products past the DNA lesion and is expressed as a percentage of total primer termini.

Steady-state polymerization kinetics

Steady-state kinetic parameters K_m and V_{max} for dNTP incorporation were measured in standing-start reactions as described previously (Creighton *et al.*, 1995; Tissier *et al.*, 2000b; Vaisman *et al.*, 2001). DNA substrates (100 fmol) were replicated for 1 min at 37°C in 10 μ l reaction mixtures containing 10 fmol of polt and variable concentrations of dNTPs. Fewer than 20% of the primers were extended under the steady-state conditions, ensuring single hit conditions. The velocity of dNTP incorporation opposite the template target site was determined as a percentage of primer elongation divided by the reaction time. The relationship between v and dNTP concentration conformed to a Michaelis-Menten equation, as indicated by linearity in a Hanes-Woolf plot of $[dNTP]/v$ versus $[dNTP]$. V_{max} and K_m were determined from a Hanes-Woolf plot by linear least squares fit. The efficiency of nucleotide incorporation by each polymerase was calculated as V_{max}/K_m . To facilitate the comparison of different DNA lesions, the relative insertion efficiency f_{rel} is also reported: $f_{rel} = (V_{max}/K_m)_{damaged}/(V_{max}/K_m)_{undamaged}$. The fidelity of dNTP incorporation (f_{inc}) was determined as the ratio of incorrect to correct incorporation efficiencies, where $f_{inc} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$.

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