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Replication bypass of *N*²-deoxyguanosine interstrand cross-links by human DNA polymerases η and ι

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Abstract

DNA-interstrand cross-links (ICLs) can be repaired by biochemical pathways requiring DNA polymerases that are capable of translesion DNA synthesis (TLS). The anticipated function of TLS polymerases in these pathways is to insert nucleotides opposite and beyond the linkage site. The outcome of these reactions can be either error-free or mutagenic. TLS-dependent repair of ICLs formed between the exocyclic nitrogens of deoxyguanosines (*N*²-dG) can result in low-frequency base substitutions, predominantly G to T transversions. Previously, we demonstrated *in vitro* that error-free bypass of a model acrolein-mediated *N*²-dG ICL can be accomplished by human polymerase (pol) κ , while Rev1 can contribute to this bypass by inserting dC opposite the cross-linked dG. The current study characterized two additional human DNA polymerases, pol η and pol ι , with respect to their potential contributions to either error-free or mutagenic bypass of these lesions. In the presence of individual dNTPs, pol η could insert dA, dG, and dT opposite the cross-linked dG, but incorporation of dC was not apparent. Further primer extension was observed only from the dC and dG 3'; termini and the amounts of products were low relative to the matched undamaged substrate. Analyses of bypass products beyond the adducted site revealed that dG was present opposite the cross-linked dG in the majority of extended primers and short deletions were frequently detected. When pol ι was tested for its ability to replicate past this ICL, the correct dC was preferentially incorporated, but no further extension was observed. Under the steady-state conditions, the efficiency of dC incorporation was reduced ~500-fold relative to the undamaged dG. Thus, in addition to pol κ -catalyzed error-free bypass of *N*²-dG ICLs, an alternative, albeit low-efficiency, mechanism may exist. In this pathway, either Rev1 or pol ι could insert dC opposite the lesion, while pol η could perform the subsequent extension.

Introduction

DNA interstrand cross-links (ICLs) are induced in cellular DNA by a number of endogenously produced chemical species and following exposure to various environmental and therapeutic agents. ICLs can interfere with replication, transcription, recombination, and other critical cellular processes, causing cell death or genomic instability. However, multiple proteins belonging to different pathways of DNA damage repair and tolerance assist mammalian cells in coping with ICLs. DNA polymerases represent one important class of proteins involved in ICL processing, being the key components of both recombination-dependent and recombination-independent ICL repair.^{1–6} The anticipated function for DNA polymerases in recombination-independent ICL repair is to fill a gap created by dual incision around the ICL on one of the two affected DNA strands. Although the initial steps of this gap-filling DNA synthesis may be conducted by major replicative DNA polymerases,

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such as pol δ , replication is likely to be blocked at an adducted deoxynucleotide present in the ICL repair intermediate. This event has been hypothesized to trigger a mechanism for recruitment of specialized translesion DNA synthesis (TLS) polymerases. Indeed, several eukaryotic TLS polymerases, including polymerase (pol) η , pol ζ , and Rev1, were shown to function in a pathway of ICL repair that is independent of recombination but involves a polymerase switch.¹⁻⁴

ICLs differ according to the identity of DNA atoms affected by modification,^{7,8} and this is an important determinant that can influence both the extent of the blockage to DNA synthesis and the mutagenic outcome of repair.⁹⁻¹¹ The linkages between the exocyclic nitrogens of deoxyguanosines (N^2 -dG) are common and in particular, they are induced in the 5'-CpG-3' sequence context by the metabolically activated anticancer agent Mitomycin C (MMC)¹² and as secondary modifications of N^2 -dG adducts, formed by acrolein, crotonaldehyde and other enal chemicals.¹³ The enal compounds are not only constituents of the environment,¹⁴ but are also generated *in vivo* as by-products of lipid peroxidation,^{15,16} and therefore may represent a significant source of naturally occurring ICLs.

The chemically stable, reduced derivatives of enal-mediated N^2 -dG ICLs (Figure 1A) have been used as tools in biological studies to address ICL repair, replication, and mutagenesis.^{10,17-21} When the fidelity of repair of these model lesions was assessed in mammalian cells, mutations, predominantly G to T transversions, occurred at frequencies of $\sim 3\%$.^{18,19}

In order to elucidate the specific functions of individual TLS polymerases in the bypass of N^2 -dG ICLs, biochemical assays were performed *in vitro* using purified proteins and DNA substrates that contained a site-specific reduced acrolein-mediated N^2 -dG ICL.^{10,19} The overall DNA structure was designed to mimic hypothetical intermediates in ICL repair, including the post-incision products in which nucleotides, either 3' or both 3' and 5' to the ICL have been removed (Figure 1B, ICL1 and ICL2, respectively). In agreement with the previously reported role of Rev1 in TLS-dependent repair of MMC-induced ICLs³ and with its recognized ability to insert the correct nucleotide, dC, opposite N^2 -dG monoadducts,²²⁻²⁴ *Saccharomyces cerevisiae* Rev1 efficiently incorporated dC opposite the cross-linked dG. The subsequent primer extension by Rev1 was not observed in the given sequence context, consistent with its known preference for dG over other template nucleotides.²⁵ Even though pol ζ has been implicated in conferring cellular resistance to MMC exposure^{6,26,27} and is involved in TLS-dependent repair of MMC-induced ICLs in higher eukaryotes,³ *S. cerevisiae* pol ζ failed to support DNA synthesis past acrolein-mediated N^2 -dG ICLs *in vitro*. This polymerase could not incorporate nucleotides opposite the lesion nor extend primers from the correct dC. However, limited extensions were observed from the mismatched primer termini. Human pol ν ¹⁰ and pol θ ²⁸ were incompetent in bypass of acrolein-mediated N^2 -dG ICLs. In contrast, human pol κ not only catalyzed accurate incorporation opposite the cross-linked dG, but also efficiently extended primers beyond the lesion. Consistent with a possible role for pol κ in replication bypass of N^2 -dG ICLs, both cell survival and chromosomal stability were compromised in pol κ -depleted human cells following MMC exposure.¹⁹

Thus, prior analyses have suggested that error-free bypass of N^2 -dG ICLs can be accomplished by pol κ , while Rev1 is likely to contribute to this bypass by inserting the correct nucleotide opposite the cross-linked dG. However, no DNA polymerase has yet been identified as a candidate for carrying out the mutagenic nucleotide insertions in the course of TLS-dependent repair of these lesions. In addition, alternative mechanisms of error-free bypass may exist and such a possibility should be considered. The current study was designed to address these questions by testing the abilities of two additional human DNA

polymerases, pol η and pol ι , to catalyze TLS past reduced acrolein-mediated N^2 -dG ICLs and evaluating the mutagenic outcome of these reactions.

Materials and Methods

Oligodeoxynucleotides

Unmodified oligodeoxynucleotides were synthesized by the Molecular Microbiology and Immunology Research Core Facility, Oregon Health & Science University (Portland, OR). ICL-containing oligodeoxynucleotides were a generous gift of Dr. Carmelo J. Rizzo (Vanderbilt University, Nashville, NT) and were synthesized as previously reported.¹⁹

Polymerases

Recombinant human pol η and pol ι were obtained from Enzymax Inc. (Lexington, KY). Both proteins were full-length and contained the calmodulin binding peptide at their N termini.

DNA Polymerase Bypass Assays

Prior to polymerase reactions, primer oligodeoxynucleotides were phosphorylated with T4 polynucleotide kinase (New England BioLabs) in the presence of [γ -³²P]-ATP (6000 Ci/mmol, PerkinElmer, Inc.), purified using P-6 Bio-Spin columns (Bio-Rad), and annealed to non-damaged (ND), ICL1, or ICL2 templates as described previously.²⁹

Polymerase bypass reactions were conducted at 37 °C in a buffer composed of 25 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 10% glycerol (v/v), 10 mM sodium chloride, 0.1 mg/ml bovine serum albumin, and 5 mM DTT. Primer extensions were carried out in the presence of 100 μ M each of the four dNTPs (Sigma). Single nucleotide incorporations were carried out in the presence of 20 μ M of an individual dNTP. Polymerase concentrations, primer/template concentrations, and incubation times varied and are given in the figure legends. Steady-state kinetic parameters for pol ι were measured according to a standard procedure.³⁰ The concentration of the primer/template DNA substrate was 10 nM. For ND substrate, the polymerase concentration was 0.5 nM, dCTP concentrations ranged from 1.95 μ M to 1 mM, and reactions were incubated for 8 min. For the ICL2 substrate, the polymerase concentration was 1 nM, dCTP concentrations ranged from 62.5 μ M to 4 mM, and reactions were incubated for 24 min. Polymerase reactions were terminated by the addition of an equal volume of stop solution consisting of 95% formamide (v/v), 20 mM EDTA, 0.2% bromophenol blue (w/v), and 0.2% xylene cyanol (w/v), followed by incubation at 90 °C for 3 min. DNA products were resolved through a 15% polyacrylamide gel in the presence of 8 M urea and visualized using a PhosphorImager screen (GE Healthcare). Quantitative analyses were performed using Image-Quant 5.2 software (Molecular Dynamics). The k_{cat} and K_m parameters with their error values were obtained from the best fit of the data to the Michaelis-Menten equation $v_{obs} = k_{cat}[E][dCTP]/(K_m + [dCTP])$ using Kaleida Graph 3.6 software (Synergy Software).

In order to characterize the mutagenic outcome of pol η -catalyzed bypass reactions, the ND and ICL2 template DNAs (5 pmols) were initially incubated with 20 units of terminal transferase (New England BioLabs) and 200 μ M 2',3'-dideoxy-ATP (Sigma) under conditions recommended by the enzyme supplier. As a result, 2',3'-dideoxy-AMP was added to the 3' end of the templates, creating a chain terminator for DNA synthesis in the downstream step of procedure. Following inactivation of terminal transferase at 75 °C for 20 min and purification from unincorporated 2',3'-dideoxy-ATP using P-6 Bio-Spin columns, the template DNAs were hybridized with the radioactively-labeled 76-mer primer oligodeoxynucleotide 5'-

GGGACCTGAACACGTACGGAATTCGATATCCTCGAGCCAGATCTGCGCCAGCTG GCCACCCTCGAGTCGGTACCAG-3'. Primers were extended in the reaction buffer described above using 10 nM primer/template DNA, 10 nM pol η , and 100 μ M each of the four dNTPs at 37 °C for 1 h. After separation through a 10% polyacrylamide gel in the presence of 8 M urea, the radioactively-labeled DNAs were visualized by autoradiography using X-OMAT Blue XB film (Kodak). The bands of interest were incised from the gel, and DNAs were eluted with a solution consisting of 500 mM ammonium acetate and 10 mM magnesium acetate. Following two sequential purifications through P-6 Bio-Spin columns, the bypass products were used as templates for polymerase chain reaction (PCR). Amplifications were performed with 2 $\times$ PCR master mix (Fermentas) and the combination of primers 5'-GGGACCTGAACACGTACGGAA-3' (forward) and 5'-AGCGTATTATGCAGCGATAGA-3' (reverse). PCR conditions were: 1) strand separation at 94 °C for 3 min; 2) 30 cycles of strand separation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and primer extension at 72 °C for 1 min; 3) final primer extension at 72 °C for 3 min. PCR products were ligated with pDrive vector (Qiagen) according to the manufacturer's protocol, and individual clones were obtained by selection on ampicillin (100 μ g/mL) LB agar coated with 50 mg/mL isopropyl- β -D-thiogalactopyranoside (Research Organics Inc.) and 20 mg/mL 5'-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Roche). Plasmids were isolated from a subset of the white colonies using a Bio-Spin plasmid purification kit (Qiagen) and sequenced by the Molecular Microbiology and Immunology Research Core Facility, Oregon Health & Science University using the forward PCR primer.

Results

Replication Bypass of N^2 -Deoxyguanosine ICLs by pol η

Prior studies have demonstrated that when replicating DNAs containing N^2 -dG monoadducts, pol η manifests a wide spectrum of bypass abilities and fidelities. For example, human pol η preferentially inserts the correct nucleotide, dC, opposite relatively small N^2 -dG lesions, such as the ring-opened derivative of an acrolein dG adduct,²⁹ or methyl-, ethyl-, isobutyl-, benzyl-, and CH_2 -2-naphthyl-dGs.³¹ It also efficiently extends from the matched primer termini opposite such lesions. In contrast, this polymerase shows a very ineffective bypass and a high propensity for misincorporation when it replicates past bulky benzo[*a*]pyrene diol epoxide,³² CH_2 -9-anthracenyl, CH_2 -6-benzo[*a*]pyrenyl,³¹ or 2-amino-3-methylimidazo[4,5-*f*]quinoline³³ N^2 -dG adducts. Certain N^2 -dG lesions, such as the ring-closed analog of acrolein- N^2 -dG,²⁹ completely block pol η -catalyzed DNA synthesis *in vitro*, both at the insertion and extension step. Here, human pol η was characterized with respect to its ability to catalyze TLS past model acrolein-mediated N^2 -dG ICLs.

A ³²P-labeled 14-mer oligodeoxynucleotide primer (−10 primer, Figure 1C) complementary to the 3'-portion of the templates was hybridized with ND, ICL1 and ICL2 DNAs (Figure 1B), and primer extension reactions were performed in the presence of all four dNTPs. Full-length products were formed on both ICL-containing templates showing the ability of pol η to bypass these lesions (Figure 2A). However, DNA synthesis was strongly inhibited one nucleotide before the cross-linked dG (−1 site), opposite to it (0 site), and at the next nucleotide (+1 site). In addition, multiple pause sites were observed on the ICL1 primer/template at the initial steps of strand displacement synthesis. After 45 min of incubation, only ~ 3.2 % and 12.6 % of primers were extended beyond the +1 site on ICL1 and ICL2, respectively, versus ~ 91.0 % of primers being extended beyond the corresponding site on the ND template. It was also apparent that the patterns of bypass products on ICL-containing templates were different from those using the ND template; specifically, bands were detected with slightly faster gel mobilities relative to the full-length products. Thus, a subset of bypass products either resulted from incomplete primer extensions or contained deletions.

The identities of nucleotides that can be incorporated by pol η opposite N^2 -dG ICL were tested in the presence of individual dNTPs using the -1 primer (Figure 1C). On the ICL2 template, no primer extension was observed in the reaction supplemented with dCTP, while low but detectable levels of products were formed in the presence of all other dNTPs (Figure 2B and C). These initial observations suggested that pol η could be potentially responsible for the mutagenic bypass of N^2 -dG ICLs and in particular, cause G to T transversions, the major type of mutations generated as a consequence of repair of these lesions.^{18,19}

Since nucleotide incorporation by pol η opposite N^2 -dG ICL was found to be extremely error-prone, further experiments were designed to better characterize the extension step of pol η -catalyzed bypass. Reactions were performed in the presence of all four dNTPs using a series of 0 primers (Figure 1C) that contained either dC at the 3' terminus or one of the three mismatched nucleotides. When the correct dC was placed opposite the lesion, primer extension was inefficient but detectable (Figure 2D). Specifically, ~ 2.4 % of primers were converted to the full-length products versus ~ 52.8 % observed for the perfectly matched control sample. In addition, primers were extended from dG opposite the cross-linked dG with ~ 1.3 % of them reaching the end of the template.

Collectively, these data suggested that pol η -catalyzed TLS past N^2 -dG ICLs can result in deletions, G to C transversions, and possibly other types of base substitutions. To verify this assertion, the mutagenic outcome of an overall bypass reaction was assessed using the approach schematically shown in Figure 3. Briefly, primer extensions were carried out with a 76-mer primer annealed to either the ND or ICL2 template. The bypass products were gel-purified and subsequently amplified by PCR. The PCR products were inserted into a cloning vector and sequenced following isolation of individual clones from *E. coli* cells.

No mutations were found at the site corresponding to the cross-linked dG in twenty four clones analyzed for the ND template (Table 1). In contrast, this site was mutated in the majority of the clones that were analyzed following replication of the ICL2-containing template. The most common mutations were G to C transversions that were accompanied by deletion of the two 3' adjacent nucleotides in half of such mutated bypass products. Deletion of the target dG together with deletion of the two 3' adjacent nucleotides was another frequent type of mutation. Thus, at least a subset of the shortened products that were initially observed in the bypass reactions (Figure 2A) contained deletions and did not result from incomplete primer extensions. In addition, G to T transversions were detected in a small fraction of the clones that were analyzed. Two out of twenty four clones had no mutations at the target site. This observation indicates that occasionally, pol η can incorporate dC opposite the cross-linked dG and that preferential extension from the correctly paired primer termini can partially compensate for an extremely error-prone insertion step.

Pol η -catalyzed replication also resulted in base substitutions at various positions other than the site of modification or the neighboring sites (Table 1). Such untargeted mutagenesis occurred on both the ND and ICL2 template with approximately equal frequencies. The cumulative frequency of these mutations was ~ 24×10^{-3} , which is close to the previously measured 32×10^{-3} frequency of base substitutions generated by human pol η during *in vitro* replication of an undamaged *lacZ* reporter sequence.³⁴

In summary, pol η showed a limited ability to replicate past model acrolein-mediated N^2 -dG ICLs, and the outcome of this reaction was extremely inaccurate. However, the types of pol η -induced mutations, mostly G to C transversions and deletions, differed from the mutation signature of N^2 -dG ICLs (predominantly G to T transversions).^{18,19} When the correct dC was provided, pol η could extend primers from opposite the cross-linked dG. The latter observation suggests that pol η may contribute to the non-mutagenic bypass of these lesions

by serving as an extender when TLS proceeds via a “two-step two-polymerase” mechanism.³⁵

Replication Bypass of *N*²-Deoxyguanosine ICLs by pol ι

Pol ι can utilize an unusual mechanism of replication, placing the adducted template base in the *syn* configuration and exposing the Hoogsteen edge of the base for interaction with an incoming nucleotide.^{36,37} This allows an efficient insertion of the correct dC opposite various minor groove lesions, including *N*²-methyl-dG, *N*²-ethyl-dG,³⁸ and *N*²-dG adducts induced by acrolein³⁹ and malondialdehyde.²⁴ Furthermore, pol ι can incorporate the correct nucleotide opposite a damaged dG even when the base is simultaneously modified at both N1 and *N*² sites.⁴⁰ However, pol ι tends to misinsert dT opposite *N*²-dG adducts, and as the adduct size increases, the efficiency and accuracy of incorporation dramatically decrease.³⁸

When human pol ι was tested for its ability to replicate past model acrolein-mediated *N*²-dG ICLs using the –10 primer, the processivity of this polymerase was so low that even under multiple hit conditions, all synthesis was terminated prior to the lesion (data not shown). However, when the –1 primer was used, pol ι could incorporate nucleotides opposite the cross-linked dG in ICL2, but no further extension was observed (Figure 4A). Single nucleotide incorporation data revealed that the correct nucleotide, dC, was predominantly inserted (Figure 4B). The primers were also extended in the presence of dTTP, but the amounts of products were negligible. The efficiency of dC incorporation was measured under the steady-state conditions. Relative to the undamaged dG, the efficiency of incorporation opposite to the cross-linked dG was reduced ~ 500-fold (Table 2). Confirming an inability of pol ι to extend after insertion of the correct nucleotide, no products were detected when the matched 0 primers were used on the ICL2 template (Figure 4C). Thus, acting on this small *N*²-dG ICL, pol ι could catalyze a relatively accurate nucleotide insertion, albeit with low-efficiency, but could not complete the bypass reaction.

Discussion

ICLs can be repaired by pathways that utilize the abilities of TLS polymerases to replicate DNA past damaged sites. Since the fidelities of the bypass reactions are typically well below the fidelity of normal replication, TLS-dependent ICL repair is a mutation-prone mechanism. However, it is anticipated based upon analogy with DNA unistrand lesions that the mutagenic outcome of cellular bypass of a particular ICL will depend on its chemical structure, the identities of DNA polymerases recruited to insert nucleotides opposite the ICL site, and the availability of DNA polymerases capable of extension from these either matched or mismatched primer termini.

In mammalian cells, TLS-dependent repair of the chemically stable derivatives of enal-mediated *N*²-dG ICLs resulted in point mutations at overall frequencies of ~ 3 %.^{18,19} This quite high accuracy of repair must be, at least in part, because of the remarkable ability of pol κ to replicate past such ICLs in an error-free manner.¹⁹ By efficiently inserting the correct dC opposite the ICL site, Rev1 is also likely to be involved in a non-mutagenic branch of repair. The current study has identified two additional DNA polymerases that may play roles in diminishing the mutagenesis associated with repair of these *N*²-dG ICLs. Specifically, pol ι could serve to insert dC, while pol η could extend from the correctly paired primer termini. Although the efficiencies of these reactions were low *in vitro*, they are likely to be higher in the presence of proliferating cell nuclear antigen and other cellular accessory factors. Thus, in addition to a pol κ-assisted pathway, the accurate bypass of enal-mediated *N*²-dG ICLs could be achieved via cooperation of pol η with either Rev1 or pol ι.

The results of this study together with the recently published data by Ho and coworkers¹¹ have further substantiated the idea that the ICL structure has a great impact on the fidelity of TLS by a DNA polymerase. In particular, pol ι faithfully incorporates dC opposite the minor groove acrolein-mediated N^2 -dG ICL, but is extremely promiscuous when inserting nucleotides opposite the major groove cisplatin or nitrogen mustard-like N7-dG ICLs.¹¹ Pol η appears incapable of recognizing the template dG, regardless of whether it was modified at the N^2 or N7 site. However, a correlation seems to exist between the degree of the DNA helix distortion imposed by a linkage and the fidelity of pol η -catalyzed extension from opposite the cross-linked site. Specifically, preferential extensions from the 3' dC were observed on templates containing the less distorting acrolein-mediated N^2 -dG ICL^{41–43} or the nitrogen mustard-like dimethylethylenediamine-linked N7-dG ICL¹¹ but not the cisplatin N7-dG ICL that introduces a high degree of helix distortion.⁴⁴

The products of pol η -catalyzed ICL2 bypass frequently contained dG opposite the target dG. This could be because dG was inserted directly opposite the cross-linked dG or alternatively, because the 5' adjacent dC (+1 site) was used as a template. In the latter case, bypass would proceed via a mechanism described by either the Streisinger slippage model⁴⁵ or the dNTP-stabilized misalignment model.⁴⁶ We hypothesize that such a mechanism could be operative in the majority of the bypass events and propose the following models to explain the formation of different types of mutated products. When incorporation of dG occurred from the -1 site, the primer terminus was likely repositioned opposite the cross-linked dG, and incorporation of the second dG followed. This would lead to a G to C transversion but not to deletion. In other cases, dG incorporation occurred from the -3 site, presumably due to formation of a looped-out structure on the template DNA strand. The outcome of such bypass would be deletion of the target dG together with the two 3' adjacent nucleotides. However, if the primer partially realigned with the template after dG incorporation but prior to the following insertion, the outcome would be a G to C transversion accompanied by deletions of the two 3' adjacent nucleotides.

The enal-mediated N^2 -dG ICLs are structurally related to those that are induced by the metabolically activated anticancer agent MMC. In both cases, the ICL occurs between exocyclic nitrogen (N^2) atoms of dG in 5'-CpG-3' sequences, connects DNA strands through the minor groove, and has only a minimal effect on the overall DNA structure.^{12,13,41–43,47,48} Not surprisingly, depletion of pol κ from GM639 human fibroblasts causes reduced cell survival and an increased number of chromosomal aberrations following MMC exposure.¹⁹ Depletion of pol η from HeLa cells has no apparent effect on cell survival in response to MMC exposure; however, MMC-induced chromatid gaps and breaks occur more frequently in both pol η -depleted HeLa cells and pol η -deficient human lymphoma cells relative to their respective controls.⁶ When repair of MMC-induced ICLs was examined following the intracellular reactivation of the plasmid-encoded luciferase reporter gene, the luciferase activity was reduced ~4-fold in the human pol η -deficient XP30RO cell line relative to the repair-proficient cell lines.¹ The capability for ICL repair of the XP30RO mutant was fully restored as a result of pol η expression from a vector DNA. Collectively, these data are consistent with the idea that TLS-dependent repair of N^2 -dG ICLs can proceed via pol κ - or pol η -assisted pathways. Given the fact that depletion of either of the two DNA polymerases leads to cellular defects in response to MMC exposure, both pathways are likely to be required to reach the maximal cellular capacity for repair of these lesions via a TLS-dependent mechanism.

Pol ι -deficient human lymphoma cells are not specifically sensitive to MMC exposure,⁶ and MMC-induced chromosomal abnormalities have not been elevated following pol ι depletion from GM639 cells.⁴⁹ These observations may suggest that pol ι has no role in TLS past MMC-induced ICLs. This is likely the case. Since pol ι poorly tolerates the bulk of DNA

adducts in the minor groove,³⁸ it is reasonable to expect that a MMC-induced ICL, in contrast to a relatively small acrolein-mediated ICL, completely blocks pol ι -catalyzed nucleotide insertion. However, it cannot be ruled out that pol ι function on MMC-induced ICLs is masked in the presence of Rev1 and pol κ but could become more apparent if one of these two polymerases is absent.

Although pol ζ is a critical component of the cellular defense system against MMC-induced damage,^{6,26,27} its role in TLS-dependent ICL repair of N^2 -dG ICLs has not been clearly defined. Deletion of the catalytic subunit of pol ζ from mouse embryonic fibroblasts or avian DT40 cells resulted in a significant reduction in the cellular capacity to repair MMC-induced ICLs in a TLS-dependent manner.³ Thus, pol ζ has been postulated to catalyze the replication bypass of these lesions. Surprisingly, *S. cerevisiae* pol ζ was completely blocked at the insertion step by an acrolein-mediated N^2 -dG ICL *in vitro* and showed a very limited ability for extension.¹⁹ The extension from the correctly paired primer termini was not observed. The apparent contradiction between the findings of cellular studies and the biochemical data calls for reconsideration of the previously postulated role for pol ζ in TLS-dependent ICL repair. We hypothesize that the major function of pol ζ in this process could be completion of gap-filling DNA synthesis after the primer is extended from opposite the ICL site by other DNA polymerases. Pol ζ may also be essential for repair of the second DNA strand that follows TLS-dependent restoration of the first DNA strand. In this hypothetical model, pol ζ would catalyze DNA synthesis in the context of mechanisms of recombination, consistent with recent data demonstrating that in human cells, pol ζ facilitates homologous recombination repair.⁵⁰ Finally, the major function of pol ζ in TLS-assisted ICL repair could be independent of its DNA-synthesizing activity. To the best of our knowledge, the latter possibility has not been experimentally tested.

With respect to ICL-induced mutagenesis, our data demonstrate that pol η and not pol ι has the potential to contribute to the mutagenic bypass of N^2 -dG ICLs. In particular, pol η could insert dG opposite our model cross-link and subsequently extend from such primer terminus. Relevant to this observation, G to C transversions have been detected following repair of the enal-mediated¹⁸ and MMC-induced ICLs,¹ though at low (1% or less) frequencies. Pol η could also insert dA opposite the cross-linked dG and extend beyond that site. Thus, this polymerase may be partially responsible for G to T transversions, the major type of mutations caused by N^2 -dG ICLs in mammalian cells.^{1,18,19} Although the corresponding mutated DNAs were minimally represented in the population of bypass products generated by pol η *in vitro*, they might be more readily formed in the presence of various cellular accessory factors. An additional possible scenario is that following incorporation of dA, pol η will dissociate from the DNA substrate, and another polymerase, for example pol ζ , will be recruited to complete replication bypass. The extension from dA opposite the model acrolein-mediated N^2 -dG ICL by *S. cerevisiae* pol ζ has been observed in our earlier study.¹⁹ Supporting a potential role for pol η in mutagenic bypass of N^2 -dG ICLs, TLS-dependent repair of a MMC-induced ICL was slightly less accurate in a pol η -deficient cell line relative to repair-proficient cell lines.¹ It is worth emphasizing that *in vitro*, misincorporation of dA opposite a N^2 -dG ICL has been detected only in the presence of pol η but not Rev1, pol ζ , pol κ ,¹⁹ pol ι (this study), pol ν ,¹⁰ or pol θ .²⁸

Based on the results of our study, several mechanisms can be envisioned regarding how pol η could contribute to the mutagenic bypass of N^2 -dG ICLs. Nevertheless, the data suggest that pol η is not the only polymerase responsible for these mutations and almost certainly, is not the major one. The reason for the latter assumption is a striking difference between the spectra of mutations induced by N^2 -dG ICLs in cells^{1,18,19} and the mutagenic outcome of pol η -catalyzed bypass of these lesions *in vitro*. Thus, we propose that pol η is rarely utilized *in vivo* to insert nucleotides opposite N^2 -dG ICLs. However, it may be recruited to

carry out the extension from the correctly paired dC, thereby contributing to the non-mutagenic branch of TLS-dependent ICL repair.

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Abbreviations

ICL	DNA interstrand cross-link
TLS	translesion DNA synthesis
pol	DNA polymerase
MMC	mitomycin C
ND	non-damaged
PCR	polymerase chain reaction

References

1. Zheng H, Wang X, Warren AJ, Legerski RJ, Nairn RS, Hamilton JW, Li L. Nucleotide excision repair- and polymerase η - mediated error-prone removal of mitomycin C interstrand cross-links. *Mol. Cell. Biol.* 2003; 23:754–761. [PubMed: 12509472]
2. Sarkar S, Davies AA, Ulrich HD, McHugh PJ. DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase ζ . *EMBO J.* 2006; 25:1285–1294. [PubMed: 16482220]
3. Shen X, Jun S, O'Neal LE, Sonoda E, Bemark M, Sale JE, Li L. REV3 and REV1 play major roles in recombination-independent repair of DNA interstrand cross-links mediated by monoubiquitinated proliferating cell nuclear antigen (PCNA). *J. Biol. Chem.* 2006; 281:13869–13872. [PubMed: 16571727]
4. Räschle M, Knipscheer P, Enoiu M, Angelov T, Sun J, Griffith JD, Ellenberger TE, Schärer OD, Walter JC. Mechanism of replication-coupled DNA interstrand crosslink repair. *Cell.* 2008; 134:969–980. [PubMed: 18805090]
5. Moldovan GL, Madhavan MV, Mirchandani KD, McCaffrey RM, Vinciguerra P, D'Andrea AD. DNA polymerase POLN participates in crosslink repair and homologous recombination. *Mol. Cell. Biol.* 2009; 30:1088–1096. [PubMed: 19995904]
6. Hicks JK, Chute CL, Paulsen MT, Ragland RL, Howlett NG, Gueranger Q, Glover TW, Canman CE. Differential roles for DNA polymerases η , ζ , and REV1 in lesion bypass of intrastrand versus interstrand DNA cross-links. *Mol. Cell. Biol.* 2010; 30:1217–1230. [PubMed: 20028736]
7. Noll DM, Mason TM, Miller PS. Formation and repair of interstrand cross-links in DNA. *Chem. Rev.* 2006; 106:277–301. [PubMed: 16464006]
8. Guainazzi A, Schärer OD. Using synthetic DNA interstrand crosslinks to elucidate repair pathways and identify new therapeutic targets for cancer chemotherapy. *Cell. Mol. Life Sci.* 2010; 67:3683–3697. [PubMed: 20730555]
9. Smeaton MB, Hlavin EM, Noronha AM, Murphy SP, Wilds CJ, Miller PS. Effect of cross-link structure on DNA interstrand cross-link repair synthesis. *Chem. Res. Toxicol.* 2009; 22:1285–1297. [PubMed: 19580249]
10. Yamanaka K, Minko IG, Takata K, Kolbanovskiy A, Kozekov ID, Wood RD, Rizzo CJ, Lloyd RS. Novel enzymatic function of DNA polymerase ν in translesion DNA synthesis past major groove

- DNA-peptide and DNA-DNA cross-links. *Chem. Res. Toxicol.* 2010; 23:689–695. [PubMed: 20102227]
11. Ho TV, Guainazzi A, Derkunt SB, Enoiu M, Schärer OD. Structure-dependent bypass of DNA interstrand crosslinks by translesion synthesis polymerases. *Nucleic Acids Res.* 2011; 39:7455–7464. [PubMed: 21666254]
 12. Tomasz M. Mitomycin C: small, fast and deadly (but very selective). *Chem. Biol.* 1995; 2:575–579. [PubMed: 9383461]
 13. Minko IG, Kozekov ID, Harris TM, Rizzo CJ, Lloyd RS, Stone MP. Chemistry and biology of DNA containing 1,*N*²-deoxyguanosine adducts of the α,β -unsaturated aldehydes acrolein, crotonaldehyde, and 4-hydroxynonenal. *Chem. Res. Toxicol.* 2009; 22:759–778. [PubMed: 19397281]
 14. Stevens JF, Maier CS. Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol. Nutr. Food Res.* 2008; 52:7–25. [PubMed: 18203133]
 15. Chung FL, Nath RG, Nagao M, Nishikawa A, Zhou GD, Randerath K. Endogenous formation and significance of 1,*N*²-propanodeoxyguanosine adducts. *Mutat. Res.* 1999; 424:71–81. [PubMed: 10064851]
 16. Nair U, Bartsch H, Nair J. Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: a review of published adduct types and levels in humans. *Free Radic. Biol. Med.* 2007; 43:1109–1120. [PubMed: 17854706]
 17. Mu D, Bessho T, Nechev LV, Chen DJ, Harris TM, Hearst JE, Sancar A. DNA interstrand cross-links induce futile repair synthesis in mammalian cell extracts. *Mol. Cell. Biol.* 2000; 20:2446–2454. [PubMed: 10713168]
 18. Liu X, Lao Y, Yang IY, Hecht SS, Moriya M. Replication-coupled repair of crotonaldehyde/ acetaldehyde-induced guanine-guanine interstrand cross-links and their mutagenicity. *Biochemistry.* 2006; 45:12898–12905. [PubMed: 17042508]
 19. Minko IG, Harbut MB, Kozekov ID, Kozekova A, Jakobs PM, Olson SB, Moses RE, Harris TM, Rizzo CJ, Lloyd RS. Role for DNA polymerase κ in the processing of *N*²*N*²-guanine interstrand cross-links. *J. Biol. Chem.* 2008; 283:17075–17082. [PubMed: 18434313]
 20. Kumari A, Minko IG, Harbut MB, Finkel SE, Goodman MF, Lloyd RS. Replication bypass of interstrand cross-link intermediates by *Escherichia coli* DNA polymerase IV. *J. Biol. Chem.* 2008; 283:27433–27437. [PubMed: 18697749]
 21. Ben-Yehoyada M, Wang LC, Kozekov ID, Rizzo CJ, Gottesman ME, Gautier J. Checkpoint signaling from a single DNA interstrand crosslink. *Mol. Cell.* 2009; 35:704–715. [PubMed: 19748363]
 22. Washington MT, Minko IG, Johnson RE, Haracska L, Harris TM, Lloyd RS, Prakash S, Prakash L. Efficient and error-free replication past a minor-groove *N*²-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase ζ . *Mol. Cell. Biol.* 2004; 24:6900–6906. [PubMed: 15282292]
 23. Choi JY, Guengerich FP. Kinetic analysis of translesion synthesis opposite bulky *N*²- and *O*⁶-alkylguanine DNA adducts by human DNA polymerase REV1. *J. Biol. Chem.* 2008; 283:23645–23655. [PubMed: 18591245]
 24. Maddukuri L, Eoff RL, Choi JY, Rizzo CJ, Guengerich FP, Marnett LJ. *In vitro* bypass of the major malondialdehyde- and base propenal-derived DNA adduct by human Y-family DNA polymerases κ , ι , and Rev1. *Biochemistry.* 2010; 49:8415–8424. [PubMed: 20726503]
 25. Zhang Y, Wu X, Rechkoblit O, Geacintov NE, Taylor JS, Wang Z. Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. *Nucleic Acids Res.* 2002; 30:1630–1638. [PubMed: 11917024]
 26. Wu HI, Brown JA, Dorie MJ, Lazzeroni L, Brown JM. Genome-wide identification of genes conferring resistance to the anticancer agents cisplatin, oxaliplatin, and mitomycin C. *Cancer Res.* 2004; 64:3940–3948. [PubMed: 15173006]
 27. Sonoda E, Okada T, Zhao GY, Tateishi S, Araki K, Yamaizumi M, Yagi T, Verkaik NS, van Gent DC, Takata M, Takeda S. Multiple roles of Rev3, the catalytic subunit of pol ζ in maintaining genome stability in vertebrates. *EMBO J.* 2003; 22:3188–3197. [PubMed: 12805232]
 28. Minko IG, Wood RD, Lloyd RS. Unpublished results.

29. Minko IG, Washington MT, Kanuri M, Prakash L, Prakash S, Lloyd RS. Translesion synthesis past acrolein-derived DNA adduct, γ -hydroxypropanodeoxyguanosine, by yeast and human DNA polymerase η . *J. Biol. Chem.* 2003; 278:784–790. [PubMed: 12401796]
30. Creighton S, Bloom LB, Goodman MF. Gel fidelity assay measuring nucleotide misinsertion, exonucleolytic proofreading, and lesion bypass efficiencies. *Methods Enzymol.* 1995; 262:232–256. [PubMed: 8594351]
31. Choi JY, Guengerich FP. Adduct size limits efficient and error-free bypass across bulky N^2 -guanine DNA lesions by human DNA polymerase η . *J. Mol. Biol.* 2005; 352:72–90. [PubMed: 16061253]
32. Rechkoblit O, Zhang Y, Guo D, Wang Z, Amin S, Krzeminsky J, Louneva N, Geacintov NE. Trans-lesion synthesis past bulky benzo[*a*]pyrene diol epoxide N^2 -dG and N^6 -dA lesions catalyzed by DNA bypass polymerases. *J. Biol. Chem.* 2002; 277:30488–30494. [PubMed: 12063247]
33. Choi JY, Stover JS, Angel KC, Chowdhury G, Rizzo CJ, Guengerich FP. Biochemical basis of genotoxicity of heterocyclic arylamine food mutagens: Human DNA polymerase η selectively produces a two-base deletion in copying the N^2 -guanyl adduct of 2-amino-3-methylimidazo[4,5-*f*]quinoline but not the C8 adduct at the NarI G3 site. *J. Biol. Chem.* 2006; 281:25297–25306. [PubMed: 16835218]
34. Matsuda T, Bebenek K, Masutani C, Rogozin IB, Hanaoka F, Kunkel TA. Error rate and specificity of human and murine DNA polymerase η . *J. Mol. Biol.* 2001; 312:335–346. [PubMed: 11554790]
35. Prakash S, Johnson RE, Prakash L. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* 2005; 74:317–353. [PubMed: 15952890]
36. Nair DT, Johnson RE, Prakash S, Prakash L, Aggarwal AK. Replication by human DNA polymerase ι occurs by Hoogsteen base-pairing. *Nature.* 2004; 430:377–380. [PubMed: 15254543]
37. Johnson RE, Prakash L, Prakash S. Biochemical evidence for the requirement of Hoogsteen base pairing for replication by human DNA polymerase ι . *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:10466–10471. [PubMed: 16014707]
38. Choi JY, Guengerich FP. Kinetic evidence for inefficient and error-prone bypass across bulky N^2 -guanine DNA adducts by human DNA polymerase ι . *J. Biol. Chem.* 2006; 281:12315–12324. [PubMed: 16527824]
39. Washington MT, Minko IG, Johnson RE, Wolfle WT, Harris TM, Lloyd RS, Prakash S, Prakash L. Efficient and error-free replication past a minor-groove DNA adduct by the sequential action of human DNA polymerases ι and κ . *Mol. Cell. Biol.* 2004; 24:5687–5693. [PubMed: 15199127]
40. Wolfle WT, Johnson RE, Minko IG, Lloyd RS, Prakash S, Prakash L. Human DNA polymerase ι promotes replication through a ring-closed minor-groove adduct that adopts a *syn* conformation in DNA. *Mol. Cell. Biol.* 2005; 25:8748–8754. [PubMed: 16166652]
41. Dooley PA, Tsarouhtsis D, Korbel GA, Nechev LV, Shearer J, Zegar IS, Harris CM, Stone MP, Harris TM. Structural studies of an oligodeoxynucleotide containing a trimethylene interstrand cross-link in a 5'-(CpG) motif: model of a malondialdehyde cross-link. *J. Am. Chem. Soc.* 2001; 123:1730–1739. [PubMed: 11456774]
42. Dooley PA, Zhang M, Korbel GA, Nechev LV, Harris CM, Stone MP, Harris TM. NMR determination of the conformation of a trimethylene interstrand cross-link in an oligodeoxynucleotide duplex containing a 5'-d(GpC) motif. *J. Am. Chem. Soc.* 2003; 125:62–72. [PubMed: 12515507]
43. Cho YJ, Kim HY, Huang H, Slutsky A, Minko IG, Wang H, Nechev LV, Kozekov ID, Kozekova A, Tamura P, Jacob J, Voehler M, Harris TM, Lloyd RS, Rizzo CJ, Stone MP. Spectroscopic characterization of interstrand carbinolamine cross-links formed in the 5'-CpG-3' sequence by the acrolein-derived γ -OH-1, N^2 -propano-2'-deoxyguanosine DNA adduct. *J. Am. Chem. Soc.* 2005; 127:17686–17696. [PubMed: 16351098]
44. Huang H, Zhu L, Reid BR, Drobny GP, Hopkins PB. Solution structure of a cisplatin-induced DNA interstrand cross-link. *Science.* 1995; 270:1842–1845. [PubMed: 8525382]

45. Streisinger G, Okada Y, Emrich J, Newton J, Tsugita A, Terzaghi E, Inouye M. Frameshift mutations and the genetic code. *Cold Spring Harb. Symp. Quant. Biol.* 1966; 31:77–84. [PubMed: 5237214]
46. Tippin B, Kobayashi S, Bertram JG, Goodman MF. To slip or skip, visualizing frameshift mutation dynamics for error-prone DNA polymerases. *J. Biol. Chem.* 2004; 279:45360–45368. [PubMed: 15339923]
47. Norman D, Live D, Sastry M, Lipman R, Hingerty BE, Tomasz M, Broyde S, Patel DJ. NMR and computational characterization of mitomycin cross-linked to adjacent deoxyguanosines in the minor groove of the d(T-A-C-G-T-A).d(T-A-C-G-T-A) duplex. *Biochemistry.* 1990; 29:2861–2875. [PubMed: 2346750]
48. Rink SM, Lipman R, Alley SC, Hopkins PB, Tomasz M. Bending of DNA by the mitomycin C-induced, GpG intrastrand cross-link. *Chem. Res. Toxicol.* 1996; 9:382–389. [PubMed: 8839039]
49. Jakobs PM, Moses RE. Personal communication.
50. Sharma S, Hicks JK, Chute CL, Brennan JR, Ahn JY, Glover TW, Canman CE. REV1 and polymerase ζ facilitate homologous recombination repair. *Nucleic Acids Res.* 2012; 40:682–691. [PubMed: 21926160]

Figure 1. Structures of DNA substrates. (A) Structure of reduced enal-mediated N^2 -deoxyguanine crosslinks ($R = H$ or CH_3 in an acrolein- or crotonaldehyde-mediated cross-link, respectively). (B) Schematic of the crosslinked (ICL1 and ICL2) DNA substrates used in replication bypass analyses. (C) The oligodeoxynucleotide primers used in replication bypass analyses. The underlined deoxyguanosine is associated with the crosslink in ICL-containing templates. The asterisk indicates the position of the radioactive label.

Figure 2.

Replication bypass of ICLs by human pol η . (A) Primer extensions by pol η (2 nM) were conducted for the indicated period of time. (B) Single nucleotide incorporations by pol η (1 nM) were carried out for 30 min. (C) The percent of primers extended in reactions shown in B. (D) Primer extensions by pol η (1 nM) were carried out for 30 min.

Figure 3.
 Experimental approach to assess the mutagenic outcome of a pol η-catalyzed bypass reaction.

Figure 4.

Replication bypass of ICLs by human pol ι . (A) Primer extensions by pol ι (2 nM) were conducted for the indicated period of time. (B) Single nucleotide incorporations by pol ι (1 nM) were carried out for 30 min. (C) Primer extensions by pol ι were carried out for 20 min.

Table 1

The mutagenic outcome of pol η -catalyzed DNA synthesis on the non-damaged and ICL2-containing DNA templates^a

Template DNA	Products of pol η -catalyzed DNA synthesis		Summary
	Sequences	Number	
Nondamaged	CGATGCTATCGT	18	Number of sequences analyzed: 24
	<u>T</u> GATGCTATCGT	1	
	CGA <u>A</u> GCTATCGT	1	
	CGAT <u>T</u> CTATCGT	1	Mutations at dG corresponding to the cross-linked dG: 0
	CGATGCT <u>G</u> TCGT	2	
	CGATGCTATC <u>A</u> T	1	
ICL2	CGATGCTATCGT	2	Number of sequences analyzed: 24
	CGATGCTAT <u>G</u> GT	2	
	<u>T</u> GATG <u>A</u> TAT <u>G</u> GT	1	
	C <u>A</u> ATGCTAT <u>G</u> GT	2	Mutations at dG associated with the cross-link: 22
	CGA <u>A</u> GCTAT <u>G</u> GT	1	
	CGATGC <u>A</u> AT <u>G</u> GT	1	
	CGATGCT- <u>G</u> GT	7	Additional mutations: 7
	CGATGCT--GT	5	
	<u>T</u> GATGCT--GT	1	
	CGATGCTAT <u>A</u> GT	2	

^aSites of misincorporations or deletions are underlined. Sites opposite the cross-linked dG or the corresponding unmodified dG are in bold.

Table 2Steady-state kinetic parameters for dCMP insertion by pol α

DNA substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	Relative efficiency
ND	0.65 ± 0.01	31 ± 4	21×10^{-3}	1
ICL2	0.028 ± 0.001	729 ± 52	0.038×10^{-3}	0.002