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Replication Bypass of the Acrolein-Mediated Deoxyguanine DNA–Peptide Cross-links by DNA polymerases of the DinB Family

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Abstract

DNA–protein cross-links (adducts) are formed in cellular DNA under a variety of conditions, particularly following exposure to an α,β-unsaturated aldehyde, acrolein. DNA–protein cross-links are subject to repair or damage-tolerance processes. These adducts serve as substrates for proteolytic degradation, yielding DNA–peptide lesions that have been shown to be actively repaired by the nucleotide excision repair complex. Alternatively, DNA–peptide cross-links can be subjected to replication bypass. We present new evidence about the capabilities of DNA polymerases to synthesize DNA past such cross-links. DNAs were constructed with site-specific cross-links, in which either a tetrapeptide or dodecylpeptide was covalently attached at the N^2 position of guanine via an acrolein adduct, and replication bypass assays carried out with members of the DinB family of polymerases, human polymerase (pol)κ and *Escherichia coli* (*E. coli*) pol IV, and various *E. coli* polymerases that do not belong to the DinB family. Pol κ was able to catalyze both the incorporation and extension steps with an efficiency that was qualitatively indistinguishable from control (undamaged) substrates. Fidelity was comparable on all these substrates, suggesting that pol κ would have a role in the low mutation frequency associated with replication of these adducts in mammalian cells. When the *E. coli* orthologue of pol κ, damage-inducible DNA polymerase, pol IV, was analyzed on the same substrates, pause sites were detected opposite and three nucleotides beyond the site of the lesion, with incorporation opposite the lesion being accurate. In contrast, neither *E. coli* replicative polymerase, pol III, nor *E. coli* damage-inducible polymerases, pol II and pol V, could efficiently incorporate a nucleotide opposite the DNA–peptide cross-links. Consistent with a role for pol IV in tolerance of these lesions, the replication efficiency of DNAs containing DNA–peptide cross-links was greatly reduced in pol IV-deficient cells. Collectively, these data indicate an important role for the DinB family of polymerases in tolerance mechanisms of N^2-guanine linked DNA–peptide cross-links.

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Introduction

DNA–protein cross-links are formed in cells not only as a consequence of routine DNA metabolism, but also from exposure to a variety of chemical toxicants and metals (1). In particular, these lesions have been detected in DNA following treatment of cultured human cells with an α,β-unsaturated aldehyde, acrolein (2,3). Among aldehydes that can induce DNA–protein cross-links *in vitro*, acrolein is one of the most potent (4). It is cytotoxic and mutagenic (5–7), and has tumor initiating activity (8). Acrolein is ubiquitous in the environment, but more importantly, it is generated *in vivo*, mainly as a product of peroxidation of fatty acids (9–11). Thus, acrolein may represent a significant endogenous causative factor for induction of DNA–protein cross-links.

Being a strong bifunctional electrophile, acrolein reacts with DNA to modify nucleobases at various positions and in particular, at exocyclic nitrogen (N^2) of deoxyguanosine (dG[1]) (12–16). The major product of reaction between N^2-dG and acrolein is γ-hydroxypropano-deoxyguanosine (γ-HOPdG) (12), a mutagenic adduct (17) that has been detected in cellular DNA isolated from various tissues both following acrolein exposure and as endogenous lesion (10,18–20). The γ-HOPdG adduct undergoes spontaneous ring-opening and closing (Fig. 1A) with the equilibrium being shifted toward the ring-opened form in duplex DNA (21,22). The free aldehyde of the ring-opened γ-HOPdG can further react with amines in peptides and proteins to yield Schiff base-mediated linkages (23,24). Similar to the acrolein adduct, N^2-dG adducts of other biologically important α,β-unsaturated aldehydes, crotonaldehyde and *trans*-4-hydroxynonenal, have also been shown to form Schiff base-mediated DNA–peptide cross-links (23). Therefore, elucidation of a role for acrolein and other related compounds in living organisms cannot be complete without detailed understanding of cellular processing of DNA–protein cross-links.

Although a subset of DNA–protein cross-links can undergo spontaneous reversal, others require active removal by DNA repair machinery or are processed through damage avoidance and tolerance pathways. Prokaryotic nucleotide excision repair (NER) has been demonstrated to function on DNA–protein adducts as large as 16,000 Da (25), while comparably sized cross-linked protein adducts were refractory to excision by the human NER complex (26–28). However, these data showing a lack of repair of intact proteins by human NER, do not rule out a role for this pathway in the ultimate processing of these lesions, since there is a literature precedent suggesting an initial proteolytic processing of these lesions prior to excision. In this regard, data obtained from the Zhitkovich's group (29) demonstrated that repair of formaldehyde-induced DNA–protein cross-links was significantly reduced in cells treated with lactacystin, a proteasome inhibitor. It was hypothesized that the cross-linked proteins were proteolytically degraded into substrates suitable for completion of the repair process. Consistent with these results, DNA–peptide cross-links serve as much better substrates relative to DNA–protein cross-links for both prokaryotic (25,30) and human NER (26–28). Baker *et al.* also demonstrated that incubation with the 26S proteasome inhibitor, MG132, resulted in a greater than 50% reduction in the intracellular repair of plasmids carrying site-specific DNA–protein cross-links (28). Collectively, these data indicate that biological processing of covalently linked protein adducts can proceed via a proteolytic degradative process, converting DNA–protein cross-links into DNA–peptide cross-links.

NER processing of DNA–peptide cross-links could be sufficiently delayed to make these lesions available for DNA replication bypass and potentially lead to mutations. Recently, we have assessed the mutagenic properties of Lys-Trp-Lys-Lys cross-links during

[1]***Abbreviations:*** dG, deoxyguanosine; γ-HOPdG, γ-hydroxypropano-deoxyguanosine; NER, nucleotide excision repair; pol, polymerase; *E. coli*, *Escherichia coli*.

extrachromosomal replication in mammalian cells. When this tetrapeptide was linked to the N^2position of dG via the acrolein-derived γ-HOPdG, lesion bypass was only moderately mutagenic (31). Previous investigations have suggested that the efficient and non-mutagenic replication bypass of bulky N^2-dG adducts can be performed by DNA polymerases of the DinB family, polymerase (pol) κ in mammalian cells and its bacterial orthologue, pol IV, in *Escherichia coli* (*E. coli*) (32–39). Here, we hypothesized that human pol κ as well as pol IV also function in replication bypass of N^2-dG–peptide cross-links. In order to test this hypothesis, we have created site-specifically modified oligodeoxynucleotides that contain either tetrapeptide (Lys-Trp-Lys-Lys) or dodecylpeptide (Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr) γ-HOPdG-mediated cross-links (Fig. 1) and performed replication assays *in vitro*. Additionally, the DNA–peptide cross-links were introduced into a single-stranded DNA vector (pMS2) (40) and the consequences of replication past these lesions were investigated in both the wild type and pol IV-deficient *E. coli* strains.

Materials and Methods

DNA polymerases

Human pol κ was purchased from Enzymax Inc, Lexington, KY. The polymerase is full length and tagged at its N-termini with the calmodulin-binding peptide. Subunits of the pol III replicase were purified as described before (41,42). Pol III core was reconstituted by mixing α,ε and θ subunits (1:1.5:3 molar ratio, respectively) and incubating for 30 min at 15 °C. Pol III core was resolved from unbound proteins by chromatography on MonoQ as described previously (43). The pol III replicase, containing two molecules of pol III core connected by the clamp loader, was reconstituted as follows. A mixture of γ and τ (2.5:1 molar ratio, respectively) was incubated for 90 min at 15 °C, followed by the addition of δ,δ',χ, and ψ (3-fold molar excess of each subunit over τ) followed by a further 1 hr incubation. The $\tau_2\gamma_1\delta\delta'\chi\psi$ complex was resolved from other species ($\tau_3\delta\delta'\chi\psi$, $\tau_1\gamma_2\delta\delta'\chi\psi$ and $\gamma_3\delta\delta'\chi\psi$) using a 1-ml MonoS column by elution with a 0.0–0.5 M NaCl gradient as described before (44). The $\tau_2\gamma_1\delta\delta'\chi\psi$ clamp loader was incubated with a 4-fold molar excess of pol III core, and pol III replicase was purified from excess pol III core by MonoQ anion exchange chromatography as reported previously (42). *E. coli* pol II (45), pol IV (46,47), pol V (48), and RecA (49) were purified as previously described.

Bacterial strains

The *E.coli* strains ZK126 (W3110 D*lacU169 tna2*) and its pol IV-deficient derivative (ZK126 *dinB*::Kan) (50) used in this study were the generous gift of Dr. Steven Finkel, University of Southern California, Los Angeles.

DNA substrates

DNA oligodeoxynucleotides containing a site-specific γ-HOPdG (Fig. 1A) were synthesized and purified as previously described (51). This 30-mer had the following sequence: GCTAGTACTCGTC**G**ACAATTCCGTATCCAT, where the bolded **G** denotes the position of the γ-HOPdG. Procedures to convert γ-HOPdG into the permanently ring-opened adduct, reduced γ-HOPdG (Fig. 1B) were performed as previously described (31). The Lys-Trp-Lys-Lys tetrapeptide was synthesized by Sigma-Genosys. The Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr dodecylpeptide was purchased from Sigma-Genosys. Generation of the DNA–peptide cross-links (Fig. 1C) and purification of the DNA–peptide cross-link-containing oligodeoxynucleotides were carried out according to a published protocol (31). These 30-mer oligodeoxynucleotides were used as templates in replication reactions *in vitro* and also for preparation of site-specifically modified vectors for cellular studies. All other oligodeoxynucleotides were obtained from the Molecular Microbiology and Immunology Research Core Facility, Oregon Health & Science University.

Replication assays *in vitro*

Preparations of primer-template DNA substrates were performed as previously described (52). Polymerase bypass assays with human pol κ, and *E. coli* pol II, pol III, and pol IV were carried out using 5 nM primer-template DNA substrates in the presence of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10% (v/v) glycerol, 10 mM NaCl, 0.1 mg/ml bovine serum albumin, and 5 mM dithiothreitol at 37 °C. Polymerase bypass assays with *E. coli* pol V were carried out using 5 nM primer-template DNA substrates in the presence of 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 4% (v/v) glycerol, 10 mM sodium glutamate, 5 mM dithiothreitol, 100 μM EDTA, 2 μM a single-stranded 36-mer oligodeoxynucleotide, 12 μM RecA, and 1 mM adenosine 5'[γ-thio]triphosphate. Prior to pol V-catalyzed reactions, single-stranded oligodeoxynucleotide and RecA were preincubated in reaction buffer in the presence of adenosine 5'[γ-thio]triphosphate at 37 °C for 3 min and then added to the primer-template DNA substrates. The reactions were initiated by addition of dNTPs and pol V and conducted at 37 °C. Protein concentrations, dNTPs concentrations, and incubation times are given in the figure legends. Polymerase reactions were terminated by the addition of an equal volume of a solution of 95% (v/v) formamide, 20 mM EDTA, 0.2% (w/v) bromphenol blue, and 0.2% (w/v) xylene cyanol. Products were resolved through a 15% (w/v) denaturing polyacrylamide gel in the presence of 8 M urea and visualized using a PhosphorImager screen (GE Healthcare).

Replication of DNAs containing site-specific DNA–peptide cross-links in *E. coli*

The pMS2 phagemid shuttle vector (40) was a gift from Dr. M. Moriya, State University of New York, Stony Brook. Single-stranded, site-specifically modified pMS2 vectors were constructed as in previous studies (17,31,40). Transformations of *E. coli* cells with these vectors and analyses of the progeny DNAs by differential hybridization were performed as previously described (17,31). Individual DNAs were isolated using Qiagen plasmid minipreparation kits and tested by ScaI restriction endonuclease according to manufacturer's recommendations.

Results

Preparation of DNA–Peptide Cross-links

Although there are multiple potential sites of adduction within both DNA and protein, we have chosen to model DNA–peptide cross-links via acrolein-modified DNA at N^2-dG. Acrolein reacts with DNA primarily at N^2-dG, yielding γ-HOPdG, a lesion that undergoes spontaneous ring-opening and closing (Fig. 1A) (21). In the presence of $NaBH_4$, γ-HOPdG can be converted to its reduced derivative (Fig. 1B) that serves in our assays as a model for the permanently ring-opened form of the adduct. The free aldehyde of the natural, unreduced γ-HOPdG can react with primary and secondary amines in peptides and proteins (23,24). When DNAs containing these lesions are incubated in the presence of peptides that contain both alpha and epsilon amino groups, the relative pKa of the alpha amino group dominates the course of the cross-linking reaction, forming a carbinolamine linkage between the N-terminal alpha amino group and the aldehyde of the γ-HOPdG adduct. The initial carbinolamine linkage can dehydrate to a Schiff base, that can be reductively trapped in the presence of a mild reducing agent such as $NaCNBH_3$ to form an irreversible cross-link (Fig. 1C) (23). Thus, DNAs incubated as described above have stable peptide cross-links that are directly amenable to replication bypass analyses. These linkages through the alpha amino group are common intermediates for a variety of DNA glycosylase/abasic site lyases (53) and thus, our DNA–peptide cross-links model biologically relevant lesions.

The 30-mer oligodeoxynucleotides containing DNA–peptide cross-links were prepared as previously described (31) and tested for purity using denaturing (8M urea) polyacrylamide gel. No bands were detected that would suggest either the presence of DNAs without cross-link or decomposition of oligodeoxynucleotides (data not shown).

Bypass of DNA–peptide cross-links by human pol κ

Our previous studies had indicated that both the γ-HOPdG adduct and its reduced derivative posed a severe block to DNA synthesis by eukaryotic replicative polymerases, pol δ and pol ε (17,54). Based on these observations, we considered it unlikely that either of them would be able to replicate past bulky γ-HOPdG-mediated DNA–peptide cross-links. In contrast to replicative polymerases, human pol κ could efficiently incorporate the correct nucleotide, C, opposite the ring-opened, reduced γ-HOPdG (54,55). Further, pol κ will efficiently extend a 0 primer if the correct C has been placed opposite either γ-HOPdG or its reduced derivative (54,55). In the current study, we compared the ability of pol κ to carry out translesion synthesis past tetra- and dodecylpeptides linked to DNA via the ring-opened γ-HOPdG adduct versus control (unadducted) DNAs (Fig 1C).

Using a - 4 primer, pol κ showed a significant processivity on nondamaged DNA, with a large percentage of primers being extended to near full length (Fig. 2, upper panel, lanes 2–5). Surprisingly, neither of the peptide-modified γ-HOPdG substrates blocked the progression of pol κ (Fig. 2, upper panel, lanes 6–13). Although there was a minor pause site, two nucleotides prior to the adducted site, there was no evidence of even a minor replication pause at the site of the lesion, and primers could be extended to full length comparable with the control DNAs.

Since pol κ incorporated a nucleotide opposite both lesions, the relative fidelity of the reactions was examined by carrying out single nucleotide incorporation assays using a −1 primer (Fig 2, lower panel). These assays revealed that in addition to these DNA–peptide cross-links not posing a block, pol κ utilized dCTP with a very strong preference over any other of the dNTPs (Fig. 2, lower panel). Taken together, these data indicate that pol κ has the capacity to accurately bypass the complex N^2-dG acrolein-derived peptide adducts.

Replication of DNAs containing DNA–peptide cross-links by *E. coli* DNA polymerases

Whereas the blocking effect of γ-HOPdG and reduced γ-HOPdG has been shown for eukaryotic replicative polymerases (17,54), the replicative polymerase in *E. coli* cells, pol III, has not been previously tested with any of these adducts. Here, the primer extensions by pol III were conducted with – 4 primers that were annealed with DNA templates containing either γ-HOPdG, reduced γ-HOPdG, or γ-HOPdG-derived DNA–peptide cross-links (Fig. 3). The form of the multi-subunit pol III replicase employed here is the dimeric replicase that is utilized for chromosome replication; it contains two molecules of pol III core that attach to two τ subunits of the clamp loading assembly. The observed results reveal that while the pol III replicase could efficiently catalyze DNA synthesis utilizing the nondamaged substrate (Fig. 3, lanes 2 and 3), no bypass was detected in reactions containing any of the adducted templates; all primer extensions were terminated one nucleotide prior to the lesion even at the highest enzyme concentrations (Fig. 4, lanes 6, 9, 12, and 15). Previously, it has been shown that the exonuclease-deficient Klenow fragment of pol I, which is another major DNA polymerase in *E. coli*, catalyzed very inefficient and extremely error-prone DNA synthesis past the γ-HOPdG adduct (56,57). Collectively, these data suggest that similar to mammalian cells, DNA synthesis past γ-HOPdG-derived lesions in bacteria is likely to utilize specialized bypass polymerases.

Given that human pol κ could readily bypass these lesions, it was hypothesized that *E. coli* pol IV, which is an orthologue of mammalian pol κ (58), might be capable of catalyzing translesion synthesis past these adducts. As anticipated, pol IV extended a - 4 primer annealed with an undamaged template to full length product (Fig. 4, upper panel, lanes 2–5). In contrast, extension of the - 4 primer annealed to the DNA template containing γ-HOPdG was significantly blocked one nucleotide prior to the lesion and modestly opposite the lesion (Fig. 4, upper panel, lanes 6–9). However, given a sufficient number of enzyme-DNA encounters, extension could proceed to full length. Similar reactions, but using the reduced γ-HOPdG

showed only a modest pause site one nucleotide prior to the lesion (Fig. 3, upper panel, lanes 10–13). Thus, the permanently ring-opened analog of the lesion affords efficient bypass.

In contrast to these data, replication of the templates containing the γ-HOPdG adducts with tetra- and dodecylpeptide revealed different patterns of inhibition. For the DNA modified with tetrapeptide, the dominant pause site occurred following incorporation of a nucleotide opposite the lesion (Fig. 4, upper panel, lanes 14–17). However, using increasing enzyme concentrations, blockage opposite the lesion could be overcome, only to encounter replication blockage one and three nucleotides beyond the lesion (Fig. 4, upper panel, lanes 16, 17). The block at the third position beyond the lesion was very poorly extended, suggesting that there are structural impediments to the passage of the bulky adducted duplex DNA through the backside of pol IV. Comparable analyses using the dodecylpeptide-containing substrate revealed a less significant blockage opposite the lesion, but an equivalent pause site at the third position beyond the lesion (Fig. 4, upper panel, lanes 18–21). These data confirm that the presence of N^2-dG-linked peptide inhibits pol IV-catalyzed processive synthesis several nucleotides past the site of the lesion. However, since the magnitude and site of the pause was not affected by the increased bulk of the larger peptide, these data suggest that the impeding interactions are localized to the first few amino acids of the adducted peptides.

Single nucleotide incorporation analyses revealed that pol IV preferentially incorporated the correct nucleotide, C, opposite all of these lesions; however, misincorporations of A and T opposite the γ-HOPdG and reduced γ-HOPdG adducts were also observed (Fig. 4, lower panel).

In addition to pol IV, *E. coli* possesses two other DNA damage-induced polymerases, pol II and pol V (59). Here, the abilities of pol II and pol V to synthesize DNA past γ-HOPdG, reduced γ-HOPdG, and γ-HOPdG-derived DNA–peptide cross-links have been tested by conducting primer extension experiments similar to those described above. The data revealed that pol II was strongly inhibited by all these lesions one nucleotide prior to the site of modification (Fig. 5). The blockage was less severe on the DNA substrate containing the ring-opened, reduced γ-HOPdG adduct with a significant percentage of primers being extended to the end of the template (Fig. 5, lanes 8 and 9). In contrast, almost no full-length products were detected in reactions using substrates that contained either γ-HOPdG (Fig. 5, lanes 5 and 6) or DNA–peptide cross-links (Fig. 5, lanes 11, 12, 14, and 15). When pol V was examined with the same set of modified DNAs (Fig. 6), patterns of inhibition of DNA synthesis appeared to be very similar to that observed for pol II, with the only exception that in pol V-catalyzed reactions, no bypass products were detected on substrates adducted with DNA–peptide cross-links (Fig. 6, lanes 8 and 10). Thus, among *E. coli* DNA polymerases, pol IV was uniquely able to tolerate N^2-dG linked DNA–peptide cross-links *in vitro*.

Replication of plasmid DNA containing DNA–peptide cross-links in *E. coli*

Having observed replication bypass of the γ-HOPdG-derived DNA–peptide cross-links by pol IV *in vitro*, we hypothesized that this polymerase may function in such a process intracellularly. To test this hypothesis, 30-mer DNA oligodeoxynucleotides adducted with either tetrapeptide or dodecylpeptide or containing no damage, were individually inserted into a single-stranded pMS2 vector, and these vectors were used to transform the wild type and pol IV-deficient *E. coli* cells. Following overnight selection on ampicillin-containing plates, colonies were counted, and the relative efficiency of transformation was calculated as the ratio of the number of clones originating from the adducted vectors to the number of clones originating from the nondamaged control. The average relative efficiencies with their standard deviations obtained from three independent experiments are given in Table 1, third column. These data suggested that DNA–peptide cross-links represented more severe block for replication in pol IV-deficient mutant than in wild-type cells. However, based on our previous experience (31), it was possible that a substantial percentage of the progeny DNAs originated from ligated pMS2 vectors did

not contain the insert sequences, and therefore, they should be excluded from the analyses. To evaluate the number of progeny DNAs having specific insert sequences, the following approach was utilized. A subpopulation of clones were individually grown in the 96-well plates, transferred onto Whatman 541 paper, and subjected to differential hybridization, utilizing the 5′-radioactively labeled oligodeoxynucleotide probe G. This probe was designed to be complementary to the insert sequence, assuming no mutations were introduced. Additional 20-mer oligodeoxynucleotide probes, L and R were designed to be complementary to the vector sequences located 60 and 52 nucleotides from the site of insertion. Hybridization with these probes allowed for the exclusion of the progeny DNAs with deletions involving the region of interest. Individual DNAs that hybridized with a mixture of L and R probes, but did not hybridize with G probe, were isolated and analyzed further. Specifically, they were tested by cleavage with ScaI endonuclease. Since both the original pMS2 vector and the inserted DNAs contain ScaI recognition sites, cleavage of progeny DNAs containing the insert sequences can be easily identified by the generation of DNA fragments, ~1.9 and ~3.2 kb in length. Thus, the combination of differential hybridization and analyses of isolated plasmids by cleavage with ScaI facilitated the determination of the percentage of the progeny DNAs having inserted sequences (Table 1, fifth column). Subsequently, these values were used to re-evaluate the relative efficiencies of transformation, taking into account only the transformants in which vectors with inserts, but not other products of ligation and/or intracellular DNA rearrangements, were replicated. These data indicated that relative to wild type cells, the pol IV-deficient mutant was very inefficient in replicating the adducted vectors, thus, confirming the intracellular role for pol IV in bypass of γ-HOPdG-derived DNA–peptide cross-links.

Mutagenic properties of γ-HOPdG-mediated DNA–peptide cross-links in *E. coli*

Earlier reports (17,56,57) indicated that translesion synthesis past the γ-HOPdG adduct was essentially error-free in *E. coli*. Although these studies assayed bacterial strains with various genetic backgrounds and utilized different vector systems, mutations (G to T transversions) were consistently observed at less than 1 % frequencies or not detected at all. In this report, we examined the potential the γ-HOPdG-mediated DNA–peptide cross-links to cause mutations during extrachromosomal replication in *E. coli*. As described above, following intracellular replication of site-specifically modified pMS2 vectors, individual clones were tested by differential hybridization using a probe that hybridized only when nonmutagenic replication bypass had occurred. Using this method, the progeny DNAs containing no mutations were readily identified. In order to verify the accuracy of hybridization procedure, approximately 10 % of such clones were analyzed by DNA sequencing using oligodeoxynucleotide R as a primer. Among these DNAs, no clone had any sequence alteration. However, a few clones were identified that did not hybridize with probe G, but according to the ScaI cleavage analyses, they contained inserts. Targeted mutations were detected in these clones by DNA sequencing indicating that in both the wild type and pol IV-deficient cells the γ-HOPdG-mediated DNA–peptide cross-links were only marginally miscoding. Specifically, two mutations were observed in the wild type cells: one G to A transition (caused by the tetrapeptide cross-link) and one G to T transversion (caused by the dodecylpeptide cross-link). In pol IV-deficient mutant, the only mutation found was G to C transversion (caused by the tetrapeptide cross-link).

Discussion

Previous investigations have provided insights into the mechanisms by which DNA–protein cross-links are processed and removed (1). Although there have been inconsistent reports in the literature on the role of nucleotide excision repair in the removal of these adducts, these apparent contradictions may potentially be explained by differences in both the damage-inducing agents and the assays used to assess repair capacity. With the advent of technologies

to create site-specific DNA–protein and DNA–peptide adducts, a much clearer picture has emerged for the role of NER in processing of these lesions. Specifically, data in both bacterial and mammalian systems indicate that relative to DNA–protein cross-links, DNA–peptide cross-links are preferable substrates for the initiation of repair by NER proteins (25–28,30). These data are highly consistent with a role for proteolytic degradation of cross-linked proteins occurring prior to repair, since proteasomal inhibitors significantly reduce repair (29).

Since proteolytic processing of DNA–protein cross-links appears to be integral to the ultimate repair, these data raise the possibility that replication forks could encounter these intermediates prior to nucleotide excision repair. Therefore, it is likely that replicative polymerases, such as pol δ and pol ε in mammalian cells and pol III in bacteria, would be blocked. In such a scenario, specialized translesion polymerases could be recruited to these sites. Germane to this hypothesis, the study presented herein shows that pol κ was able to catalyze high fidelity efficient bypass of DNA–peptide cross-links, where the linkage of the peptide to DNA was via an acrolein-mediated bridge to N^2-dG. These biochemical data suggest that pol κ contributes to the non-mutagenic bypass events during intracellular replication past N^2-dG DNA–peptide cross-links. Thus, the relatively accurate replication past the γ-HOPdG-mediated tetrapeptide cross-link that previously was observed in mammalian cells (31) maybe be provided, at least partially, by pol κ. Pol κ has been shown to catalyze high fidelity bypass of benzo[*a*]pyrene and other bulky N^2-dG adducts (32–38). Recently, we have extended the substrate repertoire of pol κ to include replication bypass of DNA interstrand cross-links (60). In this study, DNAs were utilized containing site-specifically engineered N^2-N^2-dG interstrand cross-links that model DNA repair intermediates, hypothesized to be in a repair pathway not requiring homologous recombination. We demonstrated that pol could catalyze bypass of cross-linked substrates in which nucleotides 3' to the cross-link had been removed. Data were also obtained which demonstrated that loss or even reduction in cellular levels of pol κ, resulted in increased cytotoxicity and chromosomal damage when cells were exposed to mitomycin C, an N^2-N^2-dG cross-link-inducing agent.

Collectively, these data reveal a consistent pattern of pol κ being an efficient and high fidelity enzyme in the bypass of extremely bulky N^2-dG adducts. Relative to these and other catalytic bypass substrates, it is of interest that the co-crystal structure has been solved for the catalytic core of pol κ with a primer-template complex and an incoming nucleotide (61). This structure clearly shows pol κ completely encircling the duplex DNA, using an N-terminal clasp. It was hypothesized that this structure would enhance the thermodynamic stability of the core complex, thus facilitating replication bypass. Given this tight-fitting encircling of the DNA, we hypothesized that there must be considerable flexibility in the hinge domains to allow such bulky lesions to pass through the core of the complex. Thus, it is of considerable interest to determine the structures of additional complexes to reveal the conformational changes that must take place to permit these error-free reactions.

Our data also demonstrated that the *E. coli* pol III, pol II, and pol V were all incapable of replicating past DNA–peptide cross-links as assayed herein, but that pol IV was modestly efficient at the incorporation and extension steps and that incorporation of the correct nucleotide opposite the lesions was highly preferred. However, unexpectedly, processive synthesis of pol IV was strongly inhibited beyond the site of the lesions. Such blocks were not observed with the γ-HOPdG, reduced γ-HOPdG (this study) or furfuryl N^2-dG adducts (34). These data suggest that the ability of pol IV to continue processive synthesis is blocked via a physical obstruction of the peptide cross-link with a portion of pol IV in a location where the newly synthesized DNA exits the polymerase. Such downstream blockages have been previously observed with the HIV reverse transcriptase replicating N^2-dG styrene oxide-modified DNAs (62,63). This blockage could be attributed directly to the interaction of α-helix H and I, tracking in the minor groove of the newly synthesized duplex DNA, such that site-

directed mutagenesis of selected amino acid residues in the tracking face of α-helix H modulated the downstream pause sites (62,64). We hypothesize that similar "reading heads" of pol IV may monitor the newly synthesized duplex DNA. Insights into this mechanism await crystal and co-crystal structures of pol IV.

Although DNA synthesis past the γ-HOPdG-mediated DNA–peptide cross-links by pol IV *in vitro* was partially inhibited relative to the non-damaged DNA, our cellular studies clearly implicate pol IV as a major contributor to the replication bypass of these lesions *in vivo*. In its absence, we observed ~ 5- and 20-fold reduction in recovery of plasmids when the dodecylpeptide or tetrapeptide, respectively, was site-specifically linked to the single-stranded vector DNAs, and these DNAs replicated in *E. coli* cells. Surprisingly, such cross-links appeared to be only marginally miscoding in both the wild-type and pol IV-deficient strains. In wild type *E. coli*, mutations were observed at overall frequencies of ~ 1 %, which is significantly less than the frequency of mutations caused by the identical tetrapeptide cross-link in mammalian cells, in which ~ 8 % of bypass events resulted in targeted mutations (31). Interestingly, the γ-HOPdG adduct is also less miscoding in *E. coli* relative to mammalian system (17,56,57). Overall, these data give strong support for the role of DinB family polymerases in the bypass of very bulky N^2-dG adducts.

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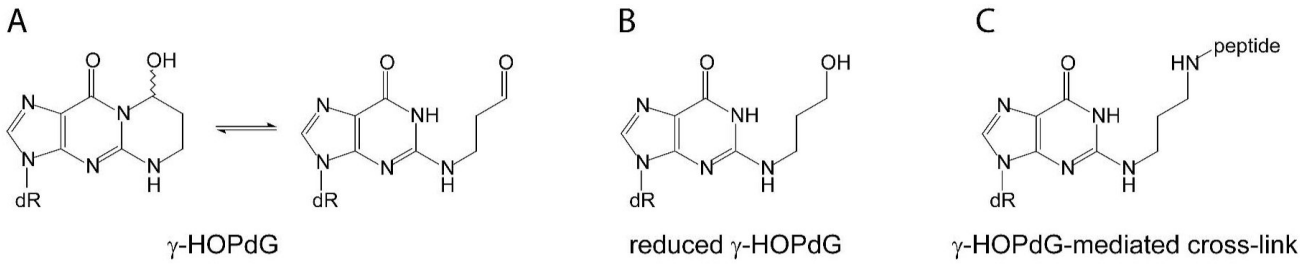

Fig. 1.

Structure of DNA adducts used in the current study. A. γ-hydroxypropano deoxyguanosine shown as the equilibrium between the ring-closed and ring-opened forms; B. reduced γ-hydroxypropano deoxyguanosine; C. reduced γ-hydroxypropano deoxyguanosine-mediated peptide cross-link.

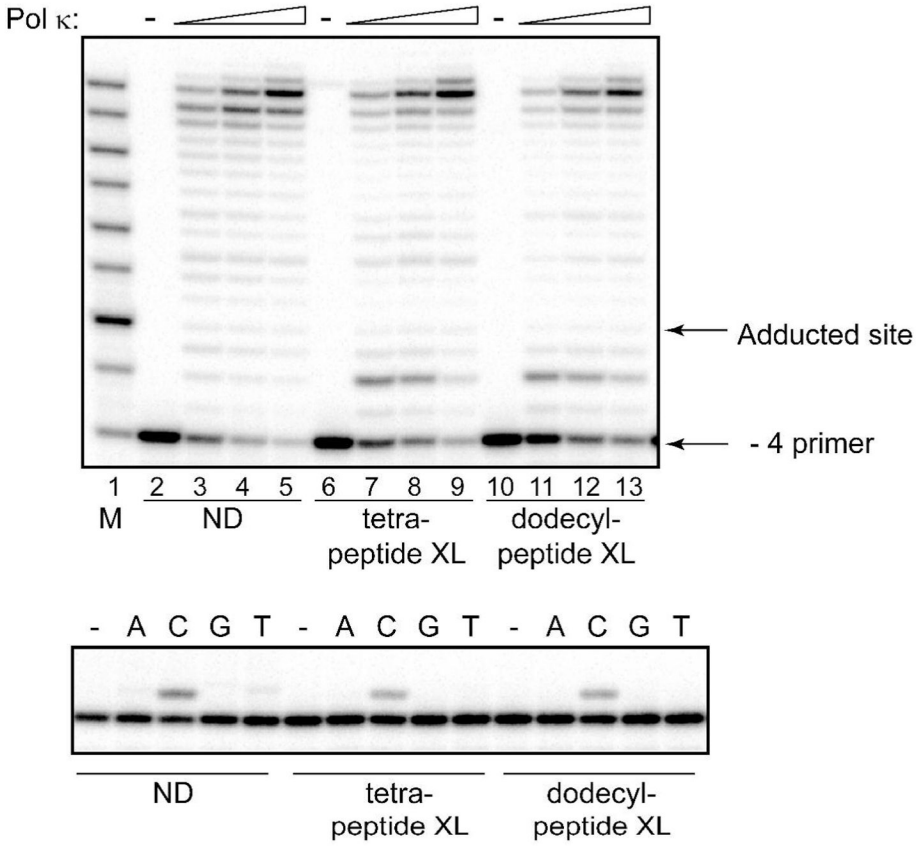

Fig. 2.

Replication bypass of DNA–peptide cross-links by human pol κ. Upper panel: Primer extensions by pol κ were carried out for 20 min in the presence of 100 μM dNTPs at 1.25 nM (lanes 3, 7, and 11), 2.5 nM (lanes 4, 8, and 12) or 5 nM (lanes 5, 9, and 13) protein concentrations. Lower panel: Single nucleotide incorporations by pol κ (1 nM) were carried out for 5 min in the presence of 20 μM individual dNTPs. M – oligodeoxynucleotide sizing markers; ND – non-damaged template; XL – cross-link-containing template.

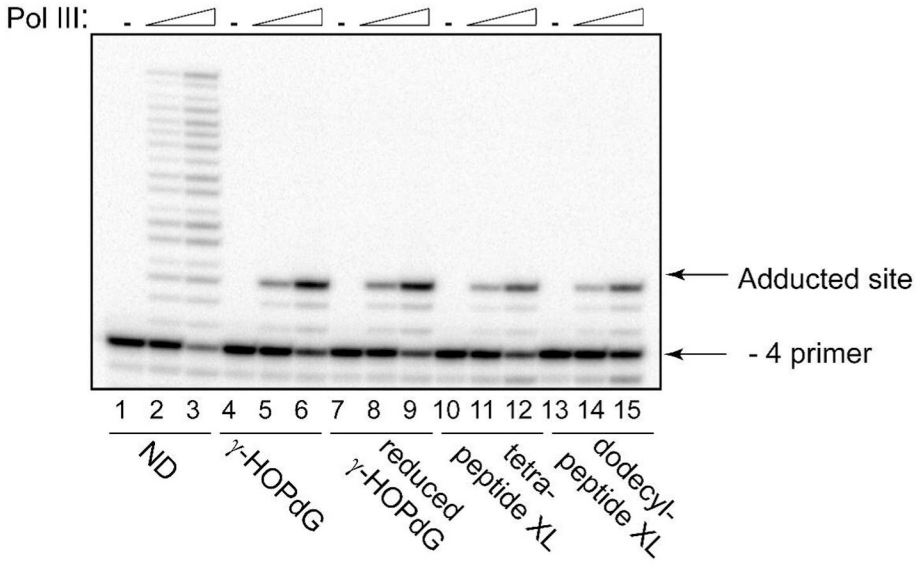

Fig. 3.

Replication bypass of γ-HOPdG, reduced γ-HOPdG, and DNA–peptide cross-links by *E. coli* pol III. Primer extensions by the pol III replicase were carried out for 20 min in the presence of 200 μM dNTPs at 0.01 nM (lanes 2, 5, 8, 11, and 14), or 0.05 nM (lanes 3, 6, 9, 12, and 15) protein concentrations. ND – non-damaged template; XL – cross-link-containing template.

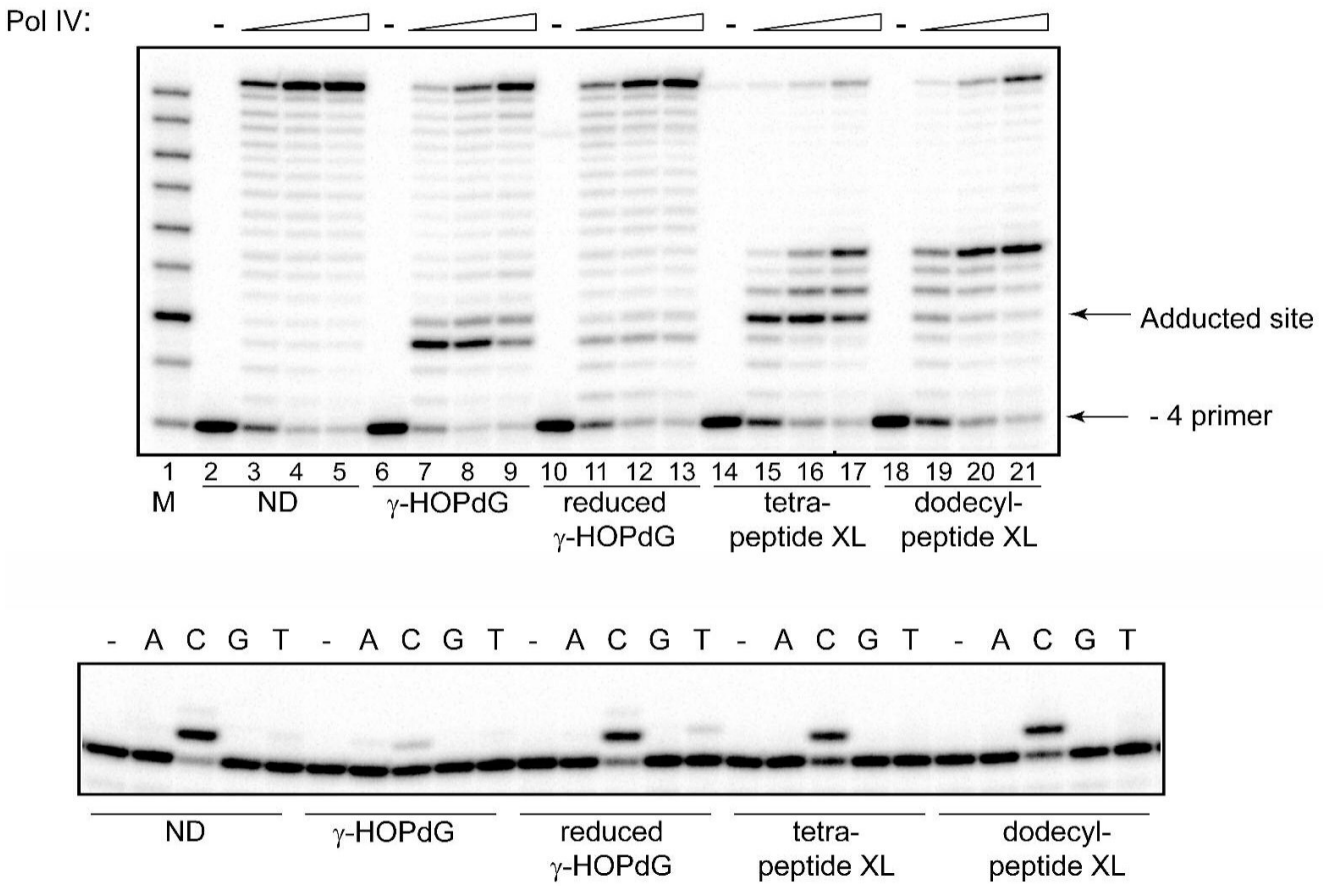

Fig. 4.

Replication bypass of γ-HOPdG, reduced γ-HOPdG, and DNA–peptide cross-links by *E. coli* pol IV. Upper panel: Primer extensions by pol IV were carried out for 20 min in the presence of 100 μM dNTPs at 1.25 nM (lanes 3, 7, 11, 15, and 19), 2.5 nM (lanes 4, 8, 12, 16, and 20) or 5 nM (lanes 5, 9, 13, 17, and 21) protein concentrations. Lower panel: Single nucleotide incorporations by pol IV (1 nM) were carried out for 20 min in the presence of 20 μM individual dNTPs. M – oligodeoxynucleotide sizing markers; ND – non-damaged template; XL – cross-link-containing template.

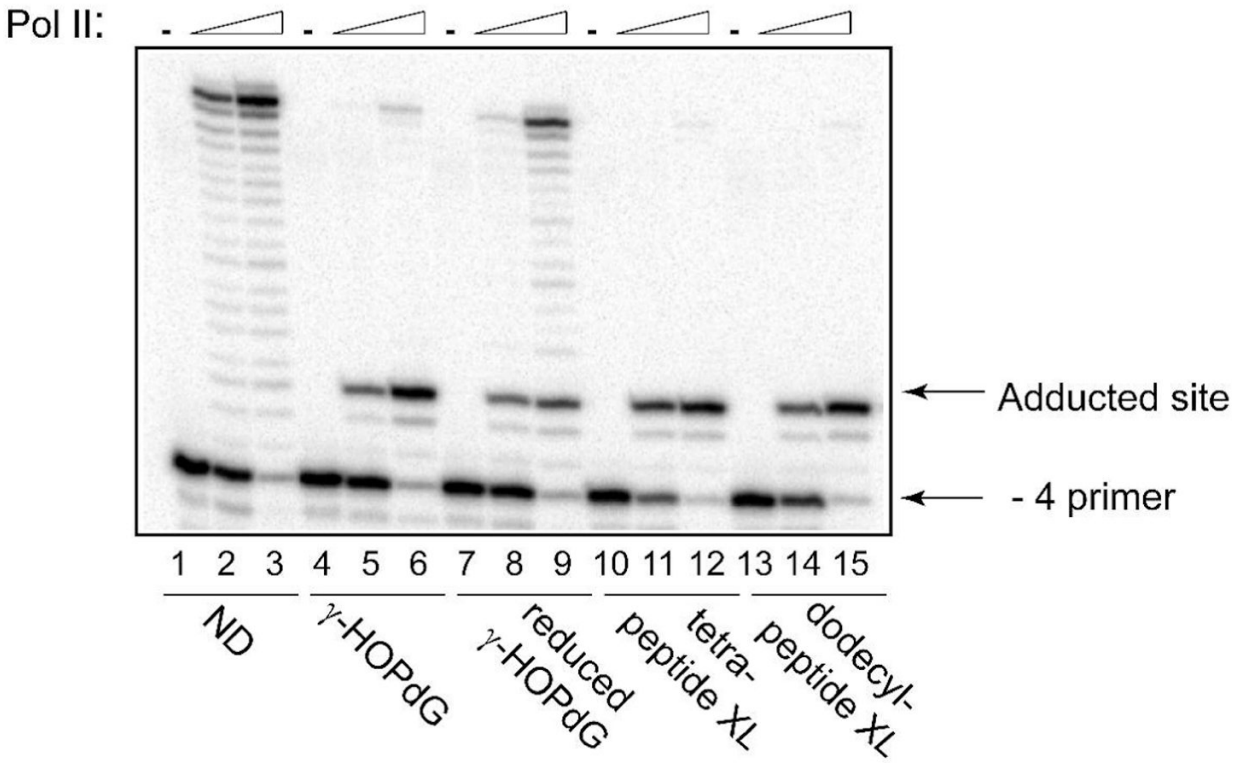

Fig. 5.

Replication bypass of γ-HOPdG, reduced γ-HOPdG, and DNA–peptide cross-links by *E. coli* pol II. Primer extensions by pol II were carried out for 20 min in the presence of 250 μM dNTPs at 0.5 nM (lanes 2, 5, 8, 11, and 14), or 5.0 nM (lanes 3, 6, 9, 12, and 15) protein concentrations. ND – non-damaged template; XL – cross-link-containing template.

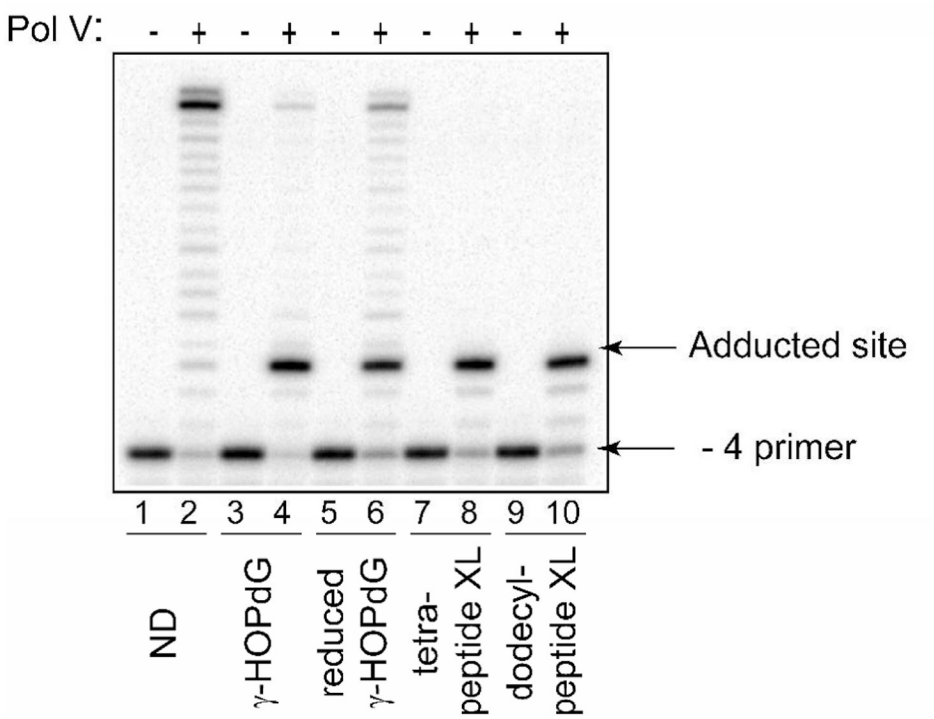

Fig. 6.

Replication bypass of γ-HOPdG, reduced γ-HOPdG, and DNA–peptide cross-links by *E. coli* pol V. Primer extensions by pol V (200 nM) were carried out for 20 min in the presence of 500 μM dNTPs. ND – non-damaged template; XL – cross-link-containing template.

Table 1

Efficiency of *E. coli* transformation using vector DNAs containing DNA–peptide cross-links

***E. coli* strain**	**Insert**	**Relative efficiency of transformation**	**Number of colonies tested**	**Colonies with inserts, %**	**Relative efficiency of transformation adjusted for number of colonies with inserts**	**Efficiency of transformation of pol IV-deficient mutant relative to wild type**
Wild type	Non-damaged	1	106	97	1	
	Tetra-peptide cross-link	0.45 ± 0.04	136	85	0.39 ± 0.035	1
	Dodecyl-peptide cross-link	0.39 ± 0.15	128	93	0.37 ± 0.14	1
Pol IV^{-}	Non-damaged	1	136	98.5	1	
	Tetra-peptide cross-link	0.05 ± 0.01	67	37	0.02 ± 0.004	0.05
	Dodecyl-peptide cross-link	0.12 ± 0.07	80	56	0.07 ± 0.04	0.19