DNA-protein cross-links (DPCs) are bulky DNA lesions that form both endogenously and following exposure to bis-electrophiles such as common antitumor agents. The structural and biological consequences of DPCs have not been fully elucidated due to the complexity of these adducts. The most common site of DPC formation in DNA following treatment with bis-electrophiles such as nitrogen mustards and cisplatin is the N7 position of guanine, but the resulting conjugates are hydrolytically labile and thus are not suitable for structural and biological studies. In this report, hydrolytically stable structural mimics of N7-guanine-conjugated DPCs were generated by reductive amination reactions between the Lys and Arg side chains of proteins/peptides and aldehyde groups linked to 7-deazaguanine residues in DNA. These model DPCs were subjected to in vitro replication in the presence of human translesion synthesis DNA polymerases. DPCs containing full-length proteins (11–28 kDa) or a 23-mer peptide blocked human polymerases \( \eta \) and \( \kappa \). DPC conjugates to a 10-mer peptide were bypassed with nucleotide insertion efficiency 50–100-fold lower than for native G. Both human polymerase (hPol) \( \kappa \) and hPol \( \eta \) inserted the correct base (C) opposite the 10-mer peptide cross-link, although small amounts of T were added by hPol \( \eta \). Molecular dynamics simulation of an hPol \( \kappa \) ternary complex containing a template-primer DNA with dCTP opposite the 10-mer peptide DPC revealed that this bulky lesion can be accommodated in the polymerase active site by aligning with the major groove of the adducted DNA within the ternary complex of polymerase and dCTP.

Covalent entrapment of cellular proteins on genomic DNA is a common process that occurs upon exposure to a variety of endogenous and exogenous bis-electrophiles, heavy metals, and free radicals (1–7). Specifically, common chemotherapeutics such as nitrogen mustards, platinum compounds, and alkylating agents induce bulky DNA lesions that selectively induce DPC lesions cause toxicity and mutations in human cells (18). In a more recent study, we have shown that human cells deficient in nucleotide excision repair provided evidence that DPCs are subject to proteolytic cleavage by the corresponding DNA-peptide lesions, which are subsequently removed from DNA in a replication-dependent repair process. Thus, it is critically important to investigate the activ-

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This article contains a supplemental movie.
ity and accuracy of DNA polymerases on DNA-protein and DNA-peptide lesions.

The most common site on DNA that participates in DNA-protein cross-linking mediated by bis-electrophiles is the N7 atom of guanine (3–5, 10). However, to our knowledge, no reports are available in the literature in regard to the replication bypass of N7-guanine adducts, probably because of their labile nature. Alkylation at the N7 atom of 2′-deoxyguanosine creates a positive charge at the affected nucleobase, destabilizing the N-glycosidic bond and leading to spontaneous depurination and abasic site formation (23, 24).

In the present work, we generated a range of hydrolytically stable model DPCs structurally resembling N7-guanine cross-links. Template-primer complexes containing model DPCs were subjected to in vitro polymerization in the presence of human lesion bypass DNA polymerases under steady-state kinetic conditions, and the resulting primer extension products were characterized by gel electrophoresis and HPLC-ESI-MS/MS. Finally, molecular dynamics (MD) simulations were conducted to examine how DNA-peptide lesions can be accommodated within the active site of human lesion bypass polymerase κ.

Results

Synthesis and Characterization of DNA-Protein and DNA-Peptide Cross-links—Model DNA-protein and DNA-peptide conjugates were prepared by reductive amination reactions between the Lys/Arg side chains of proteins/peptides and 7-deazaguanine residues of DNA (Fig. 1). The latter were formed in situ from 7-deazaguanine-containing DNA as described in our earlier publication (25). In the resulting model cross-links, Lys or Arg side chains of proteins and peptides are conjugated to the 7-deazaguanine residues of DNA via a two-carbon linker, creating a structure that is analogous to DPC lesions induced in cells by antitumor nitrogen mustards (3) and ethylene dibromide (26).

DNA sequences (5′-G TCA CTG GTA XCA AGC ATT G-3′ and 5′-GAA AGA AGX ACA GAA GAG GGT ACC ATA GAG TCA GTG-3′ where X is the modified base; Table 1) were selected to minimize self-annealing and were used previously in studies of DNA-DNA cross-links (27, 28). Two peptides of increasing size, a 10-mer peptide derived from c-Myc protein (EQKLISEEDL) and a 23-mer derived from tetanus toxoid.
(N$_3$(CH$_2$)$_3$CO-PDAQLVPINGKAHLVNNESSE) were used. The same two peptides were used in our earlier publication investigating replication bypass of DPCs conjugated to the C5 position of thymidine using Cu(I)-catalyzed azide-alkyne cycloaddition (29). In addition, histone H2A (14 kDa), histone H4 (11.5 kDa), and AlkB (22.9 kDa) were chosen for these experiments based on the documented ability of histones and DNA repair proteins to engage in the formation of DNA-protein cross-links (3, 8, 10). We also included enhanced green fluorescent protein (eGFP; 28 kDa), which was used in our earlier study of pyrimidine-conjugated DPCs (29). DNA-peptide and DNA-protein conjugates were isolated by gel electrophoresis, and their purity was confirmed by PAGE (see representative gel image in Fig. 2). Synthetic DNA-protein and DNA-peptide cross-links were structurally characterized by nano-HPLC-nanoelectrospray ionization-MS/MS (25).

**Replication Bypass of DNA-Protein Cross-links**—To examine the influence of DNA-protein cross-links conjugated to the C7 position of 7-deaza-G in DNA on DNA replication, template–primer complexes containing site-specific DPC lesions (10-mer peptide EQKLISEEDL derived from c-Myc protein (30, 31), 23-mer peptide PDAQLVPINGKAHLVNNESSE derived from tetanus toxoid, AlkB, histones H4 and H2A, and GFP protein) were subjected to starting start and running start primer extension in the presence of human translesion synthesis (TLS) polymerases $\eta$ and $\kappa$. Templates containing native dG and deaza-DHP-dG served as negative controls.

Our "standing start" assays used a 9-mer primer P1 (5'-CAA FAMTG GC TTG-3') extending to the penultimate position of the modified site (−1 primer) on the 20-mer templates T1−T9 (5'-G TCA CTG GTA XCA AGC ATT G-3' where X is unmodified dG, deaza-DHP-dG, or a DPC adduct; Table 1 and Fig. 3A). Starting stand primer extension past dG- and deaza-DHP-dG-containing templates resulted in complete extension products (20-mers) by hPol $\eta$ and $\kappa$ (Figs. 4, A and B, and 5, A and B). We found that the 10-mer peptide DPC was bypassed by hPol $\eta$ and $\kappa$ albeit with low efficiencies (Figs. 4C and 5C). In contrast, the 23-mer peptide cross-link and all three protein conjugates completely blocked primer extension (Figs. 4, D–G, and 5, D–F). The ability of polymerase $\eta$ to copy the damaged strand was restored when the protein portion of DPC was digested with proteinase K (Fig. 4H). Taken together, these results indicate that the ability of TLS polymerases to bypass DPC lesions is a function of peptide size with lesions $\geq$23 amino acids long causing a complete replication block.

Because bulky DPC lesions may affect polymerase loading on the primer, in vitro replication experiments were repeated under "running start" conditions. Running start experiments were conducted using 13-mer primer P2 (5'-CTA TGA FAMTG GC TAC C-3) annealed to 39-mer templates T10−T16 (5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' where X is unmodified dG, deaza-DHP-dG, or DNA-protein conjugate; Table 1 and Fig. 3B). In this primer-template complex, the 3'-end of the primer is placed 10 nucleotides upstream from the adducted site (−10 primer; Fig. 3B).

**TABLE 1** Synthetic DNA sequences used in the present study

| Abbreviation | Sequence |
|--------------|----------|
| P1           | 5'-CAA FAMTG GC TTG-3' |
| P2           | 5'-CTA TGA FAMTG GC TAC C-3' |
| P3           | 5'-P-D-A CAA TCC TTG-3' |
| P4           | Biotin-5'-T$_3$-CA TGG UTG-3' |
| T1           | 5'-G TCA CTG GTA GCA AGC ATT G-3' |
| T2           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = deaza-DHP-dG) |
| T3           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = N$_3$(CH$_2$)$_3$CO-EQKLISEEDL-NH$_2$) |
| T4           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = Ac-EQKLISEEDL-NH$_2$) |
| T5           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = azido-(CH$_2$)$_3$CO-PDAQLVPINGKAHLVNNESSE) |
| T6           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = AlkB protein) |
| T7           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = histone H4) |
| T9           | 5'-G TCA CTG GTA XCA AGC ATT G-3' (X = histone H2A) |
| T10          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' |
| T11          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = deaza-DHP-dG) |
| T12          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = N$_3$(CH$_2$)$_3$CO-EQKLISEEDL-NH$_2$) |
| T13          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = N$_3$(CH$_2$)$_3$CO-PDAQLVPINGKAHLVNNESSE) |
| T14          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = AlkB protein) |
| T15          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = histone H4) |
| T16          | 5'-GAA AGA AGX ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3' (X = eGFP) |
We found that under running start conditions, both 10-mer and 23-mer peptide conjugates were partially bypassed by hPols β/H9257 and γ/H9260 to generate full-length products. For both peptide lesions, pronounced pausing was observed 1–2 nucleotides ahead of the adducted site and downstream from the lesion, suggesting inefficient polymerase translocation along the template (Figs. 6, C and D, and 7, C and D). Although the deaza-DHP-dG control caused some degree of polymerase pausing, it was less blocking than the peptide lesions (Figs. 6B and 7B). In contrast, all three DPC lesions completely blocked nucleotide addition, yielding no extension products (Figs. 6, E–G, and 7E). Similar results were observed when the primer was moved farther upstream from the lesion site (Fig. 8). As was the case with standing start experiments, proteinase K-digested cross-links were bypassed (Fig. 6H). Interestingly, replication past proteinase K-digested lesions and peptide cross-links generated large numbers of −3, −2, and −1 products (Fig. 6, C, D, and H).

Taken together, our running start experiments are consistent with the standing start results (Figs. 4 and 5), indicating that although peptide DPCs can be bypassed by TLS polymerases β/H9257 and γ/H9260 DPCs to full-length proteins completely block DNA replication. In cells, such replication blockage may be relieved by proteolytic degradation of the protein component of DPC (20–22).

Kinetics of Single Nucleotide Incorporation Opposite 10-mer Peptide Cross-link—To examine the fidelity of nucleotide insertion opposite DNA-peptide lesions by TLS polymerases β and γ, we conducted single nucleotide incorporation assays. Template-primer complexes created by annealing radiolabeled 9-mer primers to 20-mer templates containing unmodified dG (A), deaza-DHP-dG (B), or covalent cross-links to 10-mer peptide EQKLISEEDL (C), 23-mer peptide PDAQLVPGINGKAHLVNYRESSE (D), or full-sized protein AlkB (E), histone (His) H4 (F), or eGFP (G). The resulting primer-template duplexes (0.15 μM) were incubated in the presence of hPol β (0.3 μM). Reactions were initiated by the addition of a mixture of dNTPs (500 μM) and quenched at the indicated time points. Extension products were separated by 20% denaturing PAGE and visualized by autoradiography. Panel H shows results for DPC to histone (His) H4 following digestion with Proteinase K.

We found that under running start conditions, both 10-mer and 23-mer peptide conjugates were partially bypassed by hPols β and γ to generate full-length products. For both peptide lesions, pronounced pausing was observed 1–2 nucleotides ahead of the adducted site and downstream from the lesion, suggesting inefficient polymerase translocation along the template (Figs. 6, C and D, and 7, C and D). Although the deaza-DHP-dG control caused some degree of polymerase pausing, it was less blocking than the peptide lesions (Figs. 6B and 7B). In contrast, all three DPC lesions completely blocked nucleotide addition, yielding no extension products (Figs. 6, E–G, and 7E). Similar results were observed when the primer was moved farther upstream from the lesion site (Fig. 8). As was the case with standing start experiments, proteinase K-digested cross-links were bypassed (Fig. 6H). Interestingly, replication past proteinase K-digested lesions and peptide cross-links generated large numbers of −3, −2, and −1 products (Fig. 6, C, D, and H).

Taken together, our running start experiments are consistent with the standing start results (Figs. 4 and 5), indicating that although peptide DPCs can be bypassed by TLS polymerases β and γ, DPCs to full-length proteins completely block DNA replication. In cells, such replication blockage may be relieved by proteolytic degradation of the protein component of DPC (20–22).
correct base (dC) opposite native G as expected (see representative data in Figs. 9 and 10). Furthermore, hPol/H9257 inserted a single nucleotide opposite the 10-mer peptide cross-link with high fidelity (Fig. 10). hPol/H9257 also favored the incorporation of the correct base (C), although a small amount of T was also incorporated (Fig. 9).

To determine the catalytic efficiency and to calculate the misinsertion frequency for incorporation of individual dNTPs opposite the 10-mer peptide cross-link, primer-template duplexes containing unmodified dG or 10-mer peptide cross-link (Fig. 3C) were incubated with hPol/H9257 or H9260 in the presence of increasing concentrations of individual dNTPs (0–500 μM), and the reactions were quenched at preselected time points (0–60 min). Polymerase concentrations and the time points were selected such that the extent of product formation was ~35% of the starting substrate concentration (see representative gel image in Fig. 11). The specificity constant \( (k_{\text{cat}}/K_{\text{m}}) \) was calculated to evaluate the catalytic efficiency of incorporation of each dNTP and the frequency of incorporating an incorrect versus correct dNTP opposite the lesion \( (f) \) (32) were calculated by plotting the reaction velocity against dNTP concentration.

Our steady-state kinetic studies have revealed that the specificity constants \( (k_{\text{cat}}/K_{\text{m}}) \) for nucleotide insertion by hPol η were 4–150-fold higher compared with those of hPol κ, indicating that replication of DNA-peptide lesions is more efficient in the presence of hPol η (Table 2). The specificity constants for the insertion of the correct base (dCTP) opposite the adduct were 0.03 and 0.0002 μM\(^{-1}\) min\(^{-1}\) for hPols η and κ, respectively (Table 2). These values were 49- and 1850-fold lower than those obtained for dCTP insertion opposite native G (1.5 and 0.37 μM\(^{-1}\) min\(^{-1}\), respectively, Table 2), reflecting the difficulty of accommodating the bulky peptide adduct in the polymerase active site.

Nucleotide insertion opposite the 10-mer peptide conjugate by hPols η and κ was characterized by high fidelity, resulting in incorporation of C opposite the adduct. The insertion frequency of dTMP \( (f) \) was 500-fold lower compared with the frequency of incorporation of the correct nucleotide (dCMP) (Table 2). Overall, our steady-state kinetic data were indicative of low efficiency, high fidelity nucleotide insertion opposite the 10-mer peptide cross-linked to 7-deaza-G in DNA.

**Sequence Analysis of Primer Extension Products by Liquid Chromatography-Tandem Mass Spectrometry**—A mass spectrometry-based strategy reported previously (33–36) was used to sequence primer extension products of the translesion synthesis past peptide-dG conjugates. This method allows for detection of insertion, deletion, and point mutation products at the site of damage as well as any postlesion synthesis errors (34). Primer extension reactions were carried out with DNA duplexes containing native dG or the 10-mer peptide cross-link in the template strand (Fig. 3D). A uracil base was incorporated 3 nucleotides upstream from the primer end. Streptavidin capture of biotinylated primer extension products and treatment with UDG and hot piperidine allow for the release of short
FIGURE 6. Running start assays for replication bypass of DNA-protein cross-links by hPol η. 13-mer FAMdT primers were annealed with 39-mers containing unmodified dG (A), deaza-DHP-dG (B), or covalent cross-links to 10-mer peptide EQKLISEEDL (C), 23-mer peptide PDAQLVPQGKENDL (D), or full-sized protein AlkB (E), histone H2A (F), or eGFP (G). The resulting primer-template complexes (0.15 μM) were incubated in the presence of hPol η (0.30 μM). Reactions were initiated by the addition of the four dNTPs (500 μM) and quenched at the indicated time points. Extension products were separated by 20% denaturing PAGE and visualized by fluorescence imaging. Panel H shows results for DPC to histone H2A following digestion with Proteinase K.

FIGURE 7. Running start assays for replication bypass of DNA-peptide and DNA-protein cross-links by hPol κ. 13-mer FAMdT primers were annealed with 39-mers containing unmodified dG (A), deaza-DHP-dG (B), or covalent cross-links to 10-mer peptide (C), 23-mer peptide (D), or AlkB protein (E). The resulting primer-template complexes (0.15 μM) were incubated in the presence of hPol κ (0.30 μM). Reactions were initiated by the addition of the four dNTPs (500 μM) and quenched at the indicated time points. Extension products were separated by 20% denaturing PAGE and visualized by fluorescence imaging.
Translesion Synthesis Past 7-Guanine DNA-Protein Conjugates

Lesion bypass is an important mechanism of cellular DNA damage tolerance as it allows for DNA replication past bulky nucleobase adducts that block DNA polymerases (39–43). Specialized TLS polymerases can be recruited to blocked replication forks and insert nucleotides opposite damaged bases (39–41, 43–49). TLS polymerases are catalytically less efficient than replicative enzymes and are much more error-prone because of their open and flexible active sites that accommodate large DNA adducts (43, 49–51). TLS polymerases include Y family polymerases hPols η, κ, and ι and Rev1, A family Pol ν, and B family Pol ζ (41, 43–49). Polymerase switching is thought to be regulated, at least in part, by posttranslational modifications of the proliferating cell nuclear antigen sliding clamp, which acts as a scaffold for polymerase enzymes (41, 43–49).

DPCs represent a special challenge to DNA replication because of their large size and their ability to distort DNA structure. DPCs had been hypothesized to completely block DNA polymerases, leading to stalling of replication forks (43, 56). In our earlier study, we found that model DPCs generated by copper-catalyzed [3 + 2] Huisgen cycloaddition (click reaction) between an alkyne group from 5-(octa-1,7-diynyl)-uracil in DNA and an azide group within engineered proteins/polypeptides completely blocked DNA replication, whereas the corresponding 10-mer peptide conjugate was passed in an error-prone manner (29, 36). In these model DPCs, peptides were conjugated to the C5 position of thymidine via a six-carbon linker (29). However, the majority of DNA–protein conjugates induced by antitumor nitrogen mustards such as mechlorethamine and chlorambucil, platinum compounds, dibromoethane, and diepoxides such as 1,2,3,4-diepoxybutane involve the N7 atom of G residues in DNA (3–5, 8, 10, 57), and the cross...

FIGURE 8. Running start assays for replication bypass of DNA-peptide and DNA-protein cross-links by hPol η. 12-mer FAMdT primers were annealed with 39-mers containing unmodified dG (negative control) or AlkB-DNA cross-link. The reaction mixture of primers was incubated in the presence of AlkB η (0.30 μM). Reactions were initiated by the addition of the four dNTPs (500 μM) and quenched at the indicated time points. Extension products were separated by 20% denaturing PAGE and visualized by fluorescence imaging.

Oligodeoxynucleotide products containing the region of interest that are amenable for sequencing by HPLC-ESI-MS/MS (Fig. 12).

The extension products from hPol η-catalyzed primer extension reactions were sequenced by HPLC-ESI-FTMS/MS using an Orbitrap Velos mass spectrometer. The samples were initially analyzed in the full scan mode to detect all extension products. HPLC-ESI−FTMS peak areas in the extracted ion chromatograms corresponding to each product were compared with that of an internal standard to determine the relative quantities of each extension product.

Capillary HPLC-ESI−FTMS analysis of in vitro replication products resulting from primer extension reactions of template T4 containing 10-mer dG-peptide conjugate revealed one main peak corresponding to the accurate replication product (m/z 1002.16, [M − 4H]4−). The MS/MS spectrum of this oligomer was consistent with the theoretical sequence (5′-pTG CTA CCA GTG AC-3′; Fig. 12). Targeted HPLC-ESI−FTMS analyses failed to detect any point mutations, single nucleotide deletions, or postlesion synthesis errors. Taken together, our HPLC-ESI−FTMS/MS sequencing results are in agreement with the data from steady-state kinetic experiments (Table 2), indicating that nucleotide insertion opposite the 10-mer peptide conjugate by hPol η is largely accurate.

Modeling and Simulation—To better understand how the 10-mer peptide cross-link can be accommodated in the active site of TLS polymerases and copied with high fidelity, molecular dynamics simulations were carried out using a model for hPol κ developed earlier (37) based on the crystal structure of the hPol κ ternary complex by Lone et al. (38). The C7 atom of 7-deazaguanine is located in the DNA major groove. Modeling of the Ac-EQKLISEEDL-NH₂ cross-link was achieved by extending the longer N-terminal end of the peptide toward the 3′-end of the replicating DNA template (Fig. 13A and supplemental movie). Inspection of the trajectory showed an average Ca RMSD of 1.9 Å over the course of the 100-ns MD simulation, suggesting that the presence of the 10-mer cross-link within the major groove does not significantly affect the overall structure of hPol κ (Fig. 13B). The largest Ca RMSD of 3.6 Å occurred at Lys-204 in a turn of the palm region more than 30 Å away from the active site (Fig. 13C). All the interatomic distances between the incoming dCTP and the two Mg2⁺ ions were found to be conserved in the final structure of the simulation. The three Watson–Crick hydrogen bonds between 7-deazaguanine and the dCTP were present throughout the simulation, which would allow hPol κ to catalyze high fidelity nucleotide addition opposite the DNA-peptide lesion. However, the simulation also revealed that the Watson–Crick pairing was distorted because of the rigidity imposed on the damaged base by the cross-link and that there was steric crowding between the peptide lesion and the N-clasp domain of hPol κ (Fig. 13A and supplemental movie). This may contribute to inefficient lesion bypass and polymerase stalling at the peptide cross-link (Figs. 6 and 7).

Discussion

Lesion bypass is an important mechanism of cellular DNA damage tolerance as it allows for DNA replication past bulky nucleobase adducts that block DNA polymerases (39–43). Specialized TLS polymerases can be recruited to blocked replication forks and insert nucleotides opposite damaged bases (39–41, 43–49). TLS polymerases are catalytically less efficient than replicative enzymes and are much more error-prone because of their open and flexible active sites that accommodate large DNA adducts (43, 49–51) and their lack of intrinsic 3′ → 5′ proofreading activity (41, 46, 52–55). In humans, TLS polymerases include Y family polymerases hPols η, κ, and ι and Rev1, A family Pol ν, and B family Pol ζ (41, 43–49). Polymerase switching is thought to be regulated, at least in part, by posttranslational modifications of the proliferating cell nuclear antigen sliding clamp, which acts as a scaffold for polymerase enzymes (41, 43–49).
Translesion Synthesis Past 7-Guanine DNA-Protein Conjugates

linking site within DNA duplex is likely to modulate the effects of DPC lesions on DNA replication. 

$N_7$-Guanine-conjugated DPCs are induced by many DNA cross-linking drugs such as nitrogen mustards, haloethylnitrosoureas, and platinum antitumor agents commonly used in the clinic to treat leukemia; lymphoma; ovarian adenocarcinoma; and breast, lung, and testicular cancers (58–62). The majority of $N_7$-G lesions are labile to hydrolysis (23), making it difficult to synthesize site-specific DNA substrates containing such adducts for polymerase bypass assays. We recently developed a reductive amination methodology to prepare hydrolytically stable model 7-deaza-G DPC substrates that resemble DPCs formed by nitrogen mustards and dibromoethane (25). In the present study, this new methodology was used to prepare DNA-peptide and DNA-protein substrates of increasing size (1.3–28 kDa; Fig. 1). The resulting template-primer complexes were subjected to steady-state kinetics and polymerization experiments to investigate replication bypass of these bulky lesions by human TLS polymerases $\eta$ and $\kappa$.

We found that human TLS polymerases $\eta$ and $\kappa$ were completely blocked by full size DPCs conjugated to the $7$-deazaguanine within DNA (histone H4, 11.5 kDa; histone H2A, 14 kDa, AlkB, 23 kDa, and eGFP, 28 kDa) but were able to bypass the corresponding small peptide lesions (Figs. 4–7). DPC-induced replication blocks were observed in both standing start and running start experiments but were removed when the protein component of DPCs was digested with proteinase K (Figs. 4 and 6). Importantly, the presence of DPC lesions affects polymerization at least 18 nucleotides upstream from the lesion, probably by interfering with polymerase loading (Fig. 8). These results are in agreement with our earlier primer extension studies of DPCs conjugated to the C5 position of thymidine (29). In both cases, the presence of TLS polymerases cannot overcome the replication block imposed by bulky protein-DNA conjugates. It should be noted that the C7 of G and the C5 of T occupy similar positions in the major groove of DNA.

Primer extension results shown here are consistent with published reports of polymerase blockage by DPCs in vivo (56, 58, 59).

FIGURE 9. Single nucleotide insertion opposite unmodified guanine (dG) and the 10-mer peptide cross-linked to C7 of 7-deazaguanine (10-mer peptide) by TLS polymerase $hPol$ $\eta$. Primer-template duplexes (50 nM) were incubated with 50 nM polymerase in the presence of 100 $\mu$M individual dNTPs. The reactions were quenched at predetermined time points (0–60 min), and the products were separated by 20% denaturing PAGE and visualized by phosphorimaging.

FIGURE 10. Single nucleotide insertion opposite unmodified guanine (dG) and the 10-mer peptide cross-linked to C7 of 7-deazaguanine (10-mer peptide) by TLS polymerase $hPol$ $\kappa$. 50 nM primer-template duplexes were incubated with 150 nM polymerase in the presence 100 $\mu$M individual dNTPs. The reactions were quenched at predetermined time points (0–60 min), and the products were separated by 20% denaturing PAGE and visualized by phosphorimaging.
Translesion Synthesis Past 7-Guanine DNA-Protein Conjugates

To examine the influence of 7-deaza-G DNA-peptide lesions on replication efficiency and accuracy, we investigated primer extension past 10-mer and 23-mer peptides (EQKLISEEDL and PDAQLPINGKAHLNVSSE) conjugated to a 7-deaza-G in an oligonucleotide. We found that both peptide conjugates were bypassed under running start conditions (Figs. 6, C and D, and 7, C and D), although the 23-mer peptide cross-link blocked replication in standing start experiments (see Figs. 4, C and D, and 5, C and D). Polymerase pause sites were observed 1–3 nucleotides prior to the adducted site (Fig. 6, C and D). Furthermore, both nucleotide insertion opposite DPC and postlesion synthesis were extremely inefficient (Figs. 6 and 7).

To our knowledge, this is the first investigation of the effects of 7-guanine-conjugated peptides on DNA replication in the presence of TLS polymerases. Previous studies have shown that Pol ν was able to catalyze primer extension past tetrapeptides and dodecapeptides cross-linked to the N⁶ position of adenine (69), whereas the same peptides conjugated to the N⁷-guanine position were bypassed by hPol κ and E. coli Pol IV (70). In our earlier study, the same 10-mer peptide conjugated to C5 of thymine via a six-carbon linker was bypassed by hPol κ and hPol η (29). Taken together, these results indicate that unlike DPCs containing full size proteins peptide conjugates can be tolerated by human TLS polymerases.

Steady-state kinetic experiments have revealed that the efficiencies of dCMP insertion opposite 7-deaza-G DNA-peptide...
FIGURE 12. Collision-induced dissociation mass spectra of the error-free extension products observed following hPol η-catalyzed replication past the 10-mer peptide cross-linked to 7-deazaguanine.

FIGURE 13. A, MD snapshot at 100 ns of the hPol η-DNA ternary complex containing the 10-mer peptide cross-linked to 7-deazaguanine. The complex is displayed in schematic mode except for the dCTP and the cross-link-containing template base, which are in stick. The peptide is shown in red surface. The three Watson-Crick hydrogen bonds are shown with dotted lines. The inset, rotated about 90° along the y axis, highlights the Watson-Crick pair distortion. A rotating view is given in the supplemental movie. Cα RMSD (B) and Cα RMSF (C) of the hPol η over the course of the 100-ns MD simulation for the hPol η-DNA ternary complex containing the lesion peptide.
conjugates by hPol η and hPol κ were 49- and 1850-fold lower than for the template containing native G (Table 2). As observed previously for C5-thymine lesions (36), hPol η was more efficient than hPol κ. Higher catalytic efficiency of hPol η than hPol κ is consistent with previous reports for other bulky nucleobase lesions such as exocyclic adducts (35, 71–73) and DNA-glutathione conjugates (74).

Unlike C5-thymine-peptide lesions, which induce high numbers of frameshift and base substitution mutations upon primer extension in the presence of hPol κ and hPol η (36), C7-G conjugated DPCs were not miscoding. Our gel electrophoresis and mass spectrometry experiments have shown that replication bypass of the 10-mer peptide cross-linked to C7-G by hPol κ was essentially error-free, whereas hPol η incorporated the correct base (dc) with 500-fold preference over the mispair (dT) (Table 2 and Figs. 9 and 10). These results indicate that the biological outcomes of DNA-lesion bypass are largely defined by the cross-linking site on DNA (C7 of deazaguanine versus C5 of thymine).

Our model cross-links differ from lesions formed in cells by the absence of a positive charge on the guanine 7-position. The absence of a positive charge on the guanine 7-position. The 7-deazaguanine DNA substrates used here are structurally analogous to the major DNA-protein cross-links formed in vivo when cancer patients are treated with nitrogen mustard drugs (3). These results suggest that proteolytic degradation of toxic DPCs to smaller DNA-peptide cross-links allows for them to be bypassed in an accurate manner by human TLS polymerases.

**Experimental Procedures**

**Materials**—Fluorescein-dT phosphoramidite, protected 2′-deoxyribonucleoside 3′-phosphoramidites (da-CE, Ac-DC-CE, dmf-dG-CE, and dT-CE), Ac-dC-CPG ABI, dmf-dG-CPG ABI columns, and all other reagents required for automated DNA synthesis were purchased from Glen Research (Sterling, VA). Recombinant human DNA polymerases hPol η and κ were expressed and purified as described elsewhere (84, 85). Recombinant AKB protein was a generous donation from Prof. Chuan He (Department of Chemistry, University of Chicago). Expression of His6-eGFP protein (referred to as eGFP throughout) (86) and synthesis of 23-mer and 10-mer peptides (PDAQLVPINGKAIHLVNNESSE and EQKLISEEDL) (29) were carried out according to previously reported protocols. T4 polynucleotide kinase (PNK) and recombinant histones H2A and H4 were obtained from New England Biolabs (Beverly, MA); [γ-32P]ATP was purchased from PerkinElmer Life Sciences. 40% 19:1 acrylamide/bisacrylamide solution and Micro Bio-Spin 6 columns were purchased from Bio-Rad. Unlabeled dNTPs were obtained from Omaha Bio-Tek (Norcross, GA). Size exclusion columns (NAP-5 and Microspin G25) and Sep-Pak C18 solid phase extraction cartridges were obtained from GE Healthcare and Waters (Milford, MA), respectively. All other chemicals and solvents were purchased from Sigma-Aldrich and were of the highest grade available.

**Synthesis and Characterization of Oligodeoxynucleotides—**Synthetic oligonucleotides containing site-specific 7-deaza-7-(2,3-dihydroxypropan-1-yl)-2′-deoxyguanosine (5′-G TCA CGT GAA AGA AGG ACA GAA GAG GGT ACC ATC GC TTG-3′) and 5′-GAA AGA AGA deaza-DHP-dG ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3′) were prepared as described previously (27). Primers internally labeled with 5′-[N-(fluoresceinyl)-aminohexyl]-3-acycylimido]-2′-deoxyuridine (5′-C A A A FAMdTTG TTT-3′) and 5′-C T A G FMADt GG TAC C-3′) and native DNA strands (5′-G TCA CGT GCA GAC ATT G-3′, 5′-GAA AGA AGG ACA GAA GAG GGT ACC ATC ATA GAG TCA GTG-3′, and 5′-C A A A TGC TTG-3′) were prepared by standard solid phase synthesis using an ABI 394 DNA synthesizer (Applied Biosystems, Foster City, CA). Biotinylated 19-mer primers (biotin-5′-T16 CAG TGC UTG-3′) were purchased from Integrated DNA Technologies (Coralville, IA). All synthetic oligodeoxynucleotides were purified by semiprep-
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Primer Extension Assays—For standing start experiments, DNA complexes containing primer P1 annealed to templates T1–T9 in Table 1 (0.15 μM; Fig. 3A) were incubated with human DNA polymerase η or κ (0.30 μM) at room temperature in the presence of a buffered solution containing 50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM DTT, 100 μg/ml BSA, 10% glycerol (v/v), and 5 mM MgCl₂. Polymerization reactions were initiated by adding a solution containing all four dNTPs (0.5 mM each). Aliquots of the reaction mixtures (4 μl) were withdrawn at preselected time intervals (0, 5, 30, 60, 90, and 180 min) and quenched by the addition of 95% (v/v) formamide containing 10 mM EDTA (18 μl). Samples were loaded onto 20% (v/v) denaturing polyacrylamide gels and run at 80 watts for 3 h. The extension products were visualized using a Typhoon FLA 7000 in the fluorescence imaging mode. Running start experiments were conducted in a similar manner using 13-mer FAMdT primer-39-mer template DNA duplexes (Fig. 3B).

Single Nucleotide Incorporation Assays—32P-End-labeled primer-template duplexes containing native dG or 10-mer peptide-deaza-dG conjugate (50 nM) at position X (Fig. 3C) were incubated with human TLS polymerases (50 nM hPol η or 150 nM hPol κ) in 50 mM Tris–HCl (pH 7.5) buffer containing 50 mM NaCl, 5 mM DTT, 5 mM MgCl₂, 100 μg/ml BSA, and 10% (v/v) glycerol at room temperature. Polymerization reactions were initiated by the addition of individual dNTPs (100 μM) in a final volume of 20 μl. Aliquots (4 μl) were withdrawn at preselected time points, and the reactions were quenched by the addition of a solution containing 10 mM EDTA, 0.03% (w/v) bromophenol blue, and 0.03% (w/v) xylene cyanol in 95% (v/v) formamide (8 μl). The extension products were resolved by 20% (v/v) denaturing PAGE containing 7 μm urea and visualized using a Typhoon FLA 7000 phosphorimaging system.

Steady-state Kinetic Analyses—The steady-state kinetics for incorporation of individual nucleotides opposite the native dG or the 10-mer peptide-deaza-dG cross-link were investigated by performing single nucleotide incorporation assays with 0.5–50 nM hPol η or 3–55 nM hPol κ in the presence of increasing concentrations of individual dNTPs (0–500 μM). Polymerization reactions were quenched at preselected time points (0–60 min). The product bands were visualized using a Typhoon FLA 7000 phosphorimaging system and quantified by volume analysis using Image Quant TL 8.0 software (GE Healthcare). Steady-state kinetic parameters were calculated by nonlinear regression analysis using one-site hyperbolic fits in Prism 4.0 (GraphPad Software, La Jolla, CA).

Sequence of hPol η Primer Extension Products by Liquid Chromatography-Tandem Mass Spectrometry—Biotinylated primer-template duplexes (150 pmol) prepared by annealing uracil-containing primer P4 with template T1 or T4 (Table 1 and Fig. 3D) were incubated with hPol η (60 pmol) in 50 mM Tris–HCl (pH 7.5) buffer containing 50 mM NaCl, 5 mM DTT, 5 mM MgCl₂, 100 μg/ml BSA, and a 1 mM concentration each of the four dNTPs. Following a 6-h incubation at room temperature, biotinylated DNA strands were captured on streptavidin beads. Briefly, 20 mM sodium phosphate (pH 7.0) buffer containing 150 mM NaCl (400 μl) was added to each reaction. Streptavidin-Sepharose high performance beads (0.2 ml; GE Healthcare) were washed with 500 μl of 20 mM sodium phos-
phate (pH 7.0) buffer containing 150 mM NaCl and then added to the polymerase reaction mixture. The resulting suspension was incubated at room temperature for 2 h with mixing every 10 min. Following binding of the biotinylated DNA to the beads, the supernatant was removed, and the beads were washed three times with water (300 μL). A solution of 50 mM Tris–HCl buffer (pH 7.5) containing uracil–DNA glycosylase (20 units), 1 mM EDTA, and 1 mM DTT (500 μL) was added followed by incubation at 37 °C for 4 h. The beads were washed three times with H2O (300 μL) and then incubated with 250 mM piperidine (400 μL) at 95 °C for 1 h to cleave the abasic sites resulting from uracil–DNA glycosylase-mediated excision of U. The resulting samples were dried in vacuo, and the residue was reconstituted in 25 μL of water containing a 14-mer internal standard (5′-pCTT CAC GAG CCC CC-3′; 40 pmol).

Capillary HPLC-ESI-MS/MS analyses were conducted as described previously (36). An Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) coupled to a Thermo LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) was operated in the negative ion ESI-FTMS/MS mode. HPLC separation was conducted using an Agilent Zorbax 300SB-C18 column (0.5 × 150 mm; 5 μm) eluted with a gradient of 15 mM NH4(CH3CO2) (buffer A) and CH3CN (buffer B) at a flow rate of 15 μL/min. The percentage of solvent B was changed linearly from 1% to 10% in 24 min, further to 75% in 1 min, held at 75% for 3 min, and finally brought back to 1% in 2 min (all v/v). Nucleotide sequences of the extension products were confirmed by comparing the observed MS/MS fragments to the expected collision-induced dissociation fragmentation patterns of oligodeoxynucleotides (Mongo Oligo mass calculator version 2.06, available online from The RNA Institute, College of Arts and Sciences, State University of New York at Albany).

Modeling and Simulation—All molecular modeling was carried out using the Schrödinger modeling package (Schrödinger LLC, New York, NY). The starting model of hPol κ in complex with unmodified DNA was based on our earlier publication. We placed the Ac-EQKLISEEDL-NH2 peptide lesion with its N terminus directed toward the 3′-end of the template strand; the opposite orientation, in the 5′-orientation places the peptide comfortably in the major groove. The templating G within the polymerase active site was replaced by a 7-deazaguanine and conjugated to the side chain of a single lysine residue via a two-carbon linker (Fig. 1). Individual amino acids were iteratively added and locally minimized with fixed protein backbone and DNA until the full sequence of the 10-mer peptide cross-link were assessed by evaluating molecular modeling results and generated figures for publication. S. W. and N. T. wrote the paper with the help of Y. Y. S., M. D., S. B., O. D. S., and F. P. G.

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