Error-prone Translesion Synthesis Past DNA-Peptide Cross-links Conjugated to the Major Groove of DNA via C5 of Thymidine*

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Background: DNA-protein conjugates can be induced by reactive oxygen species and proteolytically cleaved to the corresponding peptide conjugates.

Results: Polymerase bypass past C5-dT peptide conjugates catalyzed by human polymerases η and κ gives rise to base substitutions and deletions.

Conclusion: Replication past C5-T peptide conjugates is mutagenic.

Significance: This study provides the first evidence for error-prone replication of DPCs cross-linked to pyrimidines in DNA.

DNA-protein cross-links (DPCs) are exceptionally bulky, structurally diverse DNA adducts formed in cells upon exposure to endogenous and exogenous bis-electrophiles, reactive oxygen species, and ionizing radiation. If not repaired, DPCs can induce toxicity and mutations. It has been proposed that the protein component of a DPC is proteolytically degraded, giving rise to smaller DNA-peptide conjugates, which can be subject to nucleotide excision repair and replication bypass. In this study, polymerase bypass of model DNA-peptide conjugates structurally analogous to the lesions induced by reactive oxygen species and DNA methyltransferase inhibitors was examined. DNA oligomers containing site-specific DNA-peptide conjugates were generated by copper-catalyzed [3 + 2] Huisgen cyclo-addition between an alkyn-functionalized C5-thymidine in DNA and an azide-containing 10-mer peptide. The resulting DNA-peptide conjugates were subjected to steady-state kinetic experiments in the presence of recombinant human lesion bypass polymerases κ and η, followed by PAGE-based assays to determine the catalytic efficiency and the misinsertion frequency opposite the lesion. We found that human polymerase κ and η can incorporate A, G, C, or T opposite the C5-dT-conjugated DNA-peptide conjugates, whereas human polymerase η preferentially inserts G opposite the lesion. Furthermore, HPLC-ESI−-MS/MS sequencing of the extension products has revealed that post-lesion synthesis was highly error-prone, resulting in mutations opposite the adducted site or at the +1 position from the adduct and multiple deletions. Collectively, our results indicate that replication bypass of peptides conjugated to the C5 position of thymine by human translesion synthesis polymerases leads to large numbers of base substitution and frameshift mutations.

DNA-protein cross-links (DPCs)2 are exceptionally bulky DNA lesions that form when cellular proteins become covalently trapped on DNA strands in the presence of exogenous and endogenous bis-electrophiles, ionizing radiation, and free radicals (1). Because of their unusual size, significant heterogeneity, and the ability to disrupt normal DNA-protein interactions (2–6), DPCs are hypothesized to block DNA replication, transcription, and repair and to induce cytotoxic and mutagenic effects (7–9). However, the exact biological consequences of these inherently complex DNA lesions are not fully understood due to their structural diversity and the difficulty in generating site-specific DPCs for biological studies.

Because of their bulky nature, DPCs can block human replicative DNA polymerases δ and ε, stalling cellular replication (10). A specialized group of translesion synthesis (TLS) polymerases (human Y family polymerases η, κ, ι, and Rev1, B family polymerase ξ, and human A family polymerase ν) can be recruited to blocked replication forks (11–18). TLS polymerases have large and flexible active sites that can accommodate a range of bulky nucleobase adducts such as exocyclic lesions, cyclobutane dimers, and DNA-DNA cross-links. However, DNA synthesis by TLS polymerases is inherently inefficient and error-prone, probably due to the expanded size and flexibility of their active sites and the lack of 3’ → 5’-proofreading activity (19, 20).

Previous investigations have revealed that the ability of DNA polymerases to bypass DNA-peptide conjugates is dependent on the lesion size, the attachment site within the DNA, and polymerase identity (10, 21–29). Lloyd and co-workers (26) have reported that human polymerase (hpol) κ and its Escherichia coli orthologue pol IV were able to catalyze error-free

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primer extension past DNA templates containing tetra- and dodecapeptides conjugated to the N² position of guanine via a trimethylene linker. hpollν was capable of bypassing both 4- and 12-mer peptides conjugated to N° of adenine via a trimethylene linkage, with correct base (dT) inserted opposite the adduct (27). In contrast, analogous adducts at the N° of guanine completely blocked DNA replication (27). Yamanaka et al. (27) proposed that small major groove DPC adducts have sufficient conformational flexibility to be accommodated within the active site of TLS polymerases without disturbing primer-template-enzyme interactions, although the corresponding minor groove adducts block replication. More recently, Guengerich et al. (28) reported that human polymerases α and ι, as well as bacterial polymerases pol T7 and DPO4, were capable of replicating DNA containing S-[4-(N°-deoxyadenosinoyl)-2,3-dihydroxybutyl] glutathione adducts (N°-da-(OH)₂butyl-Gly-Cys γGlu). However, to our knowledge, no systematic studies have been performed to examine replication bypass of DPCs conjugated to pyrimidine bases on DNA, despite their potential significance in vivo.

Proteins can become covalently conjugated to cytosine and thymine bases of DNA via either enzymatic or chemical reactions. Cytosine methylation by DNA methyltransferases is initiated by nucleophilic attack of an active site cysteine at the C6 position of cytosine, which activates the neighboring C5 position for methyl transfer (30, 31). The transient protein-DNA intermediate can be irreversibly trapped in the presence of antitumor DNA methyltransferase inhibitors such as aza-dC (30, 31). Furthermore, reactive oxygen species formed endogenously and induced by ionizing radiation can also cause protein cross-linking to the C5 and the C6 positions of pyrimidine bases on DNA (32–35).

We have recently developed an efficient methodology for generating site-specific DNA-protein and DNA-peptide conjugates using 1,3-dipolar cycloaddition between azide-containing proteins/peptides and alkyne-functionalized oligodeoxynucleotides in the presence of copper (37). This approach generates covalent conjugates between the C5 position of thymine in DNA and azide-functionalized polypeptides (Scheme 1). The resulting conjugates are structurally related to DPCs induced by reactive oxygen species (32–36, 38). Our preliminary polymerase bypass studies with substrates containing site-specific C5-dT conjugates to polypeptides of increasing size (a 10-mer, 23-mer, and a 28.4-kDa protein) have revealed that although DPC lesions containing larger polypeptides completely blocked replication, 10-mer peptide conjugates were bypassed by hpoll η and κ (37). These results suggest that large DPC lesions may undergo proteolytic degradation, followed by the bypass of the resulting DNA-peptide adducts by translesion DNA polymerases.

In this work, we examined the fidelity of translesion synthesis past model DNA-peptide conjugates containing a 10-mer peptide (EQKLISEEDL) conjugated to the C5 position of thymidine in DNA (Scheme 1) using a combination of gel electrophoresis and mass spectrometry-based methodologies. Our results provide evidence for the highly error-prone nature of replication past C5-dT-peptide adducts by hpoll η and κ, which gives rise to the large numbers of base substitutions and frameshift mutations.

**EXPERIMENTAL PROCEDURES**

**Materials—**C8-Alkyne-dT-CE phosphoramidite, protected 2′-deoxyribonucleoside-3′-phosphoramidites (dA-CE, Ac-dC-CE, dmf-dG-CE, and dT-CE), Ac-dC-CPG ABI columns, and all other reagents for automated DNA synthesis were purchased from Glen Research (Sterling, VA). Recombinant hpoll η and hpoll κ were either purchased from Enzymax (Lexington, KY) or expressed and purified as described previously (39, 40). T4 polynucleotide kinase (T4-PNK) and E. coli uracil DNA glycosylase (UDG) were obtained from New England Biolabs (Beverly, MA), whereas [γ³²P]ATP was purchased from PerkinElmer Life Sciences. 40% 19:1 acrylamide/bis solutions and micro BioSpin 6 columns were purchased from Bio-Rad. The unlabeled dNTPs were obtained from Omega Bio-Tek (Norcross, GA). Illustra NAP-5 desalting columns and Sep-Pak C18 SPE cartridges were purchased from GE Healthcare and Waters Associates (Milford, MA), respectively. All other chemicals and solvents were purchased from Sigma and were of the highest grade available.

**Synthesis and Characterization of Oligodeoxynucleotides—**Synthetic 18-mer oligodeoxynucleotides (5′-TCA TXG AAT CCT TCC CCC-3′) containing native thymidine or 5-(octa-1,7-diylnyl)-uracil (C8-alkyne-dT) at position X were synthesized by solid phase synthesis using an ABI 394 DNA synthesizer (Applied Biosystems, CA). The modified nucleotide was added using an off-line manual coupling protocol. A biotinylated 23-mer primer (biotin-5′-(T)₉GGG GGA AGG AUT C-3′) and 13-mer primer (5′-GGG GGA AGG ATT C-3′) were purchased from Integrated DNA Technologies (Coralville, IA). All oligodeoxynucleotides were purified by semi-preparative HPLC, desalted by Illustra NAP-5 columns, characterized by
HPLC-ESI-MS, and quantified by UV spectrophotometry as described previously (37).

Synthesis and Characterization of DNA-Peptide Conjugates—

Synthetic DNA 18-mers (5'-TCA TXG AAT CCT TCC CCC-3') containing C8-dT-alkyne at position X were conjugated to the 10-mer azide-containing peptide (N6(Ch3)2CO-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Leu-NH2) via copper-catalyzed [3 + 2] Huisgen cyclo-addition (click reaction, Scheme 1) (37). Site-specific DNA-peptide conjugates were isolated using 20% (v/v) denaturing polyacrylamide gels containing 7 M urea, followed by gel elution and desalting via Sep-Pak C-18 SPE. To assess the purity of the isolated conjugates, an aliquot of the purified sample was radiolabeled with [γ-32P]ATP and resolved on a 20% (v/v) denaturing polyacrylamide gel containing 7 M urea, followed by visualization using a Typhoon FLA 7000 phosphorimager (GE Healthcare). A separate aliquot was subjected to alkaline phosphate/phosphodiesterase digestion, and the resulting nucleoside-peptide conjugates were characterized by nano-LC-nanospray-MS/MS (37).

Preparation of Primer-Template Duplexes—For single nucleotide insertion assays, 13-mer DNA primers (5'-GGGGGGA-GGATTC-3', 1 nmol) were radiolabeled by incubating with T4 PNK (20 units) and [γ-32P]ATP (20 μCi) in the presence of T4 PNK reaction buffer (total volume of 20 μl) at 37 °C for 1 h. The mixture was heated at 65 °C for 10 min to inactivate the enzyme and passed through Illustra Microspin G-25 columns (GE Healthcare) to remove excess [γ-32P]ATP. 5', 2'-P-Labeled primers (50 pmol) were mixed with 2 eq. of template strands (5'-TCA TXG AAT CCT TCC CCC-3') containing either 2'-deoxythymidine (dT) or dT-10-mer peptide conjugate (dT-peptide) in 10 mM Tris buffer (pH 8) containing 50 mM NaCl. The primer and the template strands were annealed by heating at 95 °C for 10 min and cooled slowly overnight to afford the corresponding primer-template complexes.

Single Nucleotide Incorporation Assays—Initial single nucleotide insertion assays were conducted to determine which nucleotides can be incorporated opposite the DNA-peptide conjugates upon in vitro replication. 32P-End-labeled primer-template duplexes containing either dT or dT-peptide conjugate at position X (2 eq.) were used for HPLC-ESI-MS/MS sequencing experiments were generated analogously by annealing biotinylated 23-mer primer (5'-biotin-5'-T(T)10 GGG GGA AGG AUT C-3', 100 pmol) to template strands (5'-TCA TXG AAT CCT TCC CCC-3') containing either unmodified dT or the dT-peptide conjugate at position X (2 eq.).

Steady-state Kinetic Analyses—Steady-state kinetics for incorporation of individual nucleotides opposite native dT and dT-peptide conjugate was investigated by performing single nucleotide incorporation assays. Experiments were conducted with 0.25–1.25 nM hpol η or 2.5–100 nM hpol κ in the presence of increasing concentrations of individual dNTPs (0–800 μM) for specified time periods (0–180 min). The extension products were visualized after electrophoretic fractionation using a Typhoon FLA 7000 phosphorimager (GE Healthcare) and quantified by volume analysis using the ImageQuant TL 8.0 software (GE Healthcare). The 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 and Quantitation of Primer Extension Products by Liquid Chromatography-Tandem Mass Spectrometry—

Primer extension assays were conducted by incubating biotinylated primer-template duplexes (100–150 pmol) with recombinant hpol η or θ (40–60 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 1 mM each of the four dNTPs at 37 °C for 6 h. Following the polymerase reaction, 400 μl of 20 mM sodium phosphate (pH 7.0) buffer containing 150 mM NaCl were added to the reaction. Streptavidin-Sepharose high performance beads (0.2 ml, GE Healthcare) were prepared by centrifugation and washing with 500 μl of 20 mM sodium phosphate (pH 7.0) buffer containing 150 mM NaCl. The beads were added to the polymerase reaction mixture, and the resulting suspension was incubated at room temperature for 2 h with tapping every 10 min to promote the binding of biotinylated DNA to the beads. The supernatant was removed, and the beads were washed three times with water (300 μl). A solution of Tris-HCl buffer (50 mM, pH 7.5) containing uracil DNA glycosylase (UDG, 20 units), 1 mM EDTA, and 1 mM DTT (500 μl) was added to the streptavidin-coated bead suspension, and the mixture was incubated for 4 h at 37 °C to allow for uracil excision from the extended primer strands. The liquid was removed, and the beads were washed with water (three times in 300 μl). An aqueous solution of 250 mM piperidine (400 μl) was added to the streptavidin-coated beads, and the mixture was heated at 95 °C for 60 min to cleave the resulting abasic sites. The liquid from the piperidine cleavage was collected, and the beads were washed with water (three times in 200 μl). The piperidine cleavage fraction was combined with the water washes and dried in vacuo, and the residue was reconstituted in 25 μl of water containing 14-mer internal standard (5'-biotin-5'-pCTT CAC GAG CCC CC-3', 40 pmol).

Capillary HPLC-ESI-MS/MS analyses were conducted on an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) coupled to a Thermo LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The instrument was operated in the negative ion ESI-FMS/MS mode. Primer extension products were resolved on an Agilent Zorbax 300SB-C18 (0.5 × 150 mm, 5 μm) column using a gradient of 15 mM ammonium acetate (buffer A) and acetonitrile (buffer B). The column was eluted at a flow rate of 15 μl/min. Solvent composition was linearly changed from 1 to 10% B in 24

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Electrospray ionization conditions were as follows: ESI source voltage, 3.5 kV; source current, 6.7 A; auxiliary gas flow rate setting, 0; sweep gas flow rate setting, 0; sheath gas flow setting, 30; capillary temperature, 275 °C; and S-lens RF level, 50%. The most abundant ions from the ESI-FTMS spectra were selected and subjected to collision-induced dissociation (CID) analysis using a linear ion trap. The MS/MS conditions were as follows: normalized collision energy, 35%; activation Q, 0.250; activation time, 10 ms; product ion scan range, m/z 300–2000. Relative quantitation of primer extension products was done by comparing peak areas corresponding to each product in extracted ion chromatograms with the peak area of the internal standard. Product sequences were confirmed by comparing the observed MS/MS fragments to the expected CID fragmentation patterns of oligonucleotides obtained using the Mongo Oligo mass calculator version 2.06 (The RNA Institute, College of Arts and Sciences, State University of New York at Albany).

RESULTS

Synthesis of Primer-Template Duplexes Containing Site-specific DNA-Peptide Conjugates—Synthetic DNA strands containing site-specific DNA-peptide conjugates were generated by copper-catalyzed [3+2] Huisgen cycloaddition between the 5-(octa-1,7-diynyl)-uracil (C8-alkyne-dT) base (X) positioned within DNA 18-mer (5'-TCA X G AAT CCT TCC CCC-3') and the N-terminal azide moiety appended to a 10-mer c-Myc peptide (N3(CH2)3CO-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-NH2) (Scheme 1) (37). The resulting DNA-peptide conjugates (Scheme 1) were isolated by denaturing PAGE (37), and their purity was confirmed by sequencing PAGE. Only DNA templates that were at least 99% pure were employed for primer extension assays. DNA-peptide conjugates were further characterized by nanoLC-nanospray-FTMS/MS (37) and quantified by UV spectrophotometry using the calculated value of the extinction coefficient ε260 (DNA-peptide conjugate) = ε260 (DNA) + ε260 (peptide) = 220.1 nm⁻¹ cm⁻¹.

Single Nucleotide Incorporation Assays—Lesion-containing DNA 18-mers (5'-TCA X G AAT CCT TCC CCC-3') were annealed to 32P-end-labeled 13-mer primers (5'-GGG GGA AGG ATT C-3'). In the resulting primer-template complexes, the 3'-primer terminus is positioned immediately prior to the lesion site (~1 primer, Scheme 2A). Initial single nucleotide incorporation assays were conducted to determine what nucleotides can be inserted opposite the adducted site by human translesion synthesis polymerases, hpol η and κ, which were selected due to their documented ability to catalyze replication past other bulky DNA lesions (26, 29). The resulting primer-template duplexes were incubated with recombinant polymerases in the presence of individual dNTPs for 0–90 min. Denaturing PAGE followed by phosphor-imaging analysis has revealed that each of the four dNTPs can be incorporated, indicative of error-prone replication of DNA containing dT-peptide conjugates (Fig. 1). Under our experimental conditions (substrate/enzyme ratio = 2:1), full incorporation opposite the DPC lesion by hpol η was achieved within 5 min (Fig. 1). hpol κ-catalyzed dNTP incorporation opposite the peptide conjugate was less efficient, catalyzing <60% nucleotide insertion opposite the lesion.
following 90 min of incubation at substrate/enzyme = 1:5 (data not shown). These initial experiments suggested that hpol η is much more efficient than hpol κ at catalyzing replication past peptide-DT conjugates. Multiple nucleotide insertions observed in the single nucleotide incorporation experiments (Fig. 1) may be due to the low fidelity of these two TLS polymerases (29, 41, 42).

Steady-state Kinetic Analyses—Steady-state kinetic experiments were conducted to determine the catalytic efficiency for incorporation of individual dNTPs opposite the DPC lesion by hpol η and hpol κ. Primer-template complexes containing unmodified dT (positive control) or dT-peptide conjugate (X in Scheme 2A) were incubated with hpol η or hpol κ in the presence of individual dNTPs (0–800 μM), and the reactions were quenched at specified time points (0–180 min). Conditions were chosen so that the maximum percentage of products was ≥35% of the starting substrate concentration. Kinetic parameters (k_{cat} and K_{m} in Table 1) were calculated by plotting reaction velocity against the concentrations of individual dNTP (43). The specificity constant (k_{cat}/K_{m} in Table 1) is a measure of the catalytic efficiency of incorporation of each dNTP, whereas the misinsertion frequency \( f = (k_{cat}/K_{m})_{incorrect \ dNTP}/(k_{cat}/K_{m})_{correct \ dNTP} \) in Table 1) provides a quantitative measure of incorporating an incorrect versus a correct dNTP opposite the lesion (44).

According to our steady-state kinetics data (Table 1), the specificity constants (k_{cat}/K_{m}) for the incorporation of the correct base (A) opposite the dT-peptide conjugate were 0.04 and 0.09 μM \(^{-1}\) min \(^{-1}\) for hpol κ and η, respectively. These values were 14–37-fold lower than those obtained for dATP insertion opposite the unmodified dT (0.54 and 3.31 μM \(^{-1}\) min \(^{-1}\), Table 1). This is not unexpected because it may be difficult to accommodate a bulky peptide adduct in the polymerase active site, and this process is likely to be both kinetically and thermodynamically disfavored. The calculated k_{cat}/K_{m} values for hpol η were greater than those for hpol κ (Table 1), suggesting that hpol η is more efficient in the replication bypass of the dT-peptide conjugate as compared with hpol κ.

### Table 1

| Polymerase | Template | Incurring nucleotide | k_{cat} | K_{m} | k_{cat}/K_{m} | f |
|------------|----------|----------------------|--------|------|--------------|--|
| hpol κ     | dT       | dATP                 | 2.75 ± 0.18 | 5.11 ± 1.96 | 0.54 | 1 |
|            |          | dCTP                 | 0.19 ± 0.02 | 145.6 ± 48.55 | 0.0013 | 0.002 |
|            |          | dGTP                 | 1.93 ± 0.22 | 94.84 ± 31.27 | 0.02 | 0.04 |
|            |          | dTTP                 | 0.51 ± 0.07 | 371.8 ± 98.51 | 0.0014 | 0.003 |
|            | dT-peptide | dATP             | 0.67 ± 0.04 | 16.61 ± 5.51 | 0.04 | 1 |
|            |          | dCTP                 | 0.07 ± 0.007 | 90.81 ± 42.90 | 0.0007 | 0.02 |
|            |          | dGTP                 | 0.16 ± 0.008 | 152.5 ± 25.61 | 0.001 | 0.03 |
|            |          | dTTP                 | 0.03 ± 0.003 | 361.8 ± 84.11 | 0.00008 | 0.002 |
| hpol η     | dT       | dATP                 | 9.44 ± 0.26 | 2.85 ± 0.70 | 3.31 | 1 |
|            |          | dCTP                 | 2.26 ± 0.52 | 72.60 ± 54.38 | 0.031 | 0.009 |
|            |          | dGTP                 | 9.00 ± 1.67 | 13.36 ± 8.22 | 0.67 | 0.2 |
|            |          | dTTP                 | 1.04 ± 0.16 | 58.82 ± 27.89 | 0.018 | 0.005 |
|            | dT-peptide | dATP             | 2.81 ± 0.37 | 31.16 ± 22.91 | 0.09 | 1 |
|            |          | dCTP                 | 2.67 ± 0.36 | 65.85 ± 29.08 | 0.091 | 0.45 |
|            |          | dGTP                 | 13.44 ± 1.91 | 33.42 ± 11.60 | 0.402 | 4.5 |
|            |          | dTTP                 | 1.21 ± 0.14 | 32.60 ± 16.55 | 0.037 | 0.41 |

### Table 2

| Polymerase | X | Extension product | Percent product | Base opposite | Comment |
|------------|---|-------------------|-----------------|--------------|---------|
| hpol κ     | dF | T C4A TGA         | 87 A            | error-free   |         |
|            |    | T CFA TGA         | 9 T             | substitution opposite adduct |         |
|            |    | T CGA TGA         | 3 G             | substitution opposite adduct |         |
|            |    | T C_T GGA         | 1 A             | one-base deletion |         |
|            |    | T CCA TGA         | 1 C             | substitution opposite adduct |         |
|            |    | T C_G TGA         | 1 G             | one-base deletion |         |
| dT-peptide | dF | T C4 _ TGA        | 89 A            | one-base deletion |         |
|            |    | T C_G GGA         | 5 G             | one-base deletion |         |
|            |    | T C4A TGA         | 2 A             | error-free   |         |
|            |    | T C_G TGA         | 2 T             | two-base deletion |         |
|            |    | T C4A TGA         | 1 T             | substitution opposite adduct |         |
|            |    | T C_T GGA         | 1 G             | substitution opposite adduct |         |
|            |    | T C_G TGA         | 1 C             | one-base deletion |         |
| hpol η     | dF | T C4G TGA         | 36 A            | substitution at +1 position |         |
|            |    | T C4C TGA         | 36 A            | substitution at +1 position |         |
|            |    | T C4A TGA         | 28 A            | error-free   |         |
| dT-peptide | dF | T C4G TGA         | 23 A            | substitution at +1 position |         |
|            |    | T C4A TGA         | 19 A            | error-free   |         |
|            |    | T C_G TGA         | 16 G            | one-base deletion |         |
|            |    | T C_T GGA         | 15 A            | substitution at +1 position |         |
|            |    | T C_G TGA         | 15 A            | one-base deletion |         |
|            |    | T C_G TGA         | 12 C            | one-base deletion |         |
|            |    | T C_G TGA         | 1 C             | one-base deletion |         |

**Scheme 3.** Streptavidin capture in combination with capillary HPLC-ESI−MS/MS methodology for sequencing and quantitation of primer extension products.
Furthermore, hpol η was more likely to incorporate incorrect bases opposite the DNA-peptide conjugates when compared with hpol κ.

The misinsertion frequencies (f) calculated from kinetic data (Table 1) suggest that both hpol κ and η bypass dT-peptide conjugates in an error-prone manner. The order of nucleotide insertion opposite the adducted template was A > G > C > T for hpol κ, with 33-, 50-, and 500-fold preference for the incorporation of dATP over dGTP, dCTP, and dTTP, respectively. The order of nucleotide insertion opposite the adducted base by hpol η was G > A > C > T with 4.5-, 10-, and 11-fold preference for the incorporation of dGTP, an incorrect base, over dATP, dCTP, and dTTP. These data indicate that replication bypass of the DNA-peptide conjugates by hpol η is much
more error-prone as compared with bypass of the same adduct by hpol $\kappa$.

**Sequencing and Quantitation of Primer Extension Products by Liquid Chromatography-Tandem Mass Spectrometry—**A mass spectrometry-based strategy similar to the approach developed by Christov et al. (45) (Scheme 3) was employed to sequence the products of translesion synthesis past the peptide-dT conjugates. This methodology allows for the detection of insertion and/or deletion products and point mutations at the site of damage, as well as any postlesion synthesis errors (46). A biotinylated primer (biotin-5'-T)$_{10}$ GGG GGA AGG AUT C-3' was annealed to the 18-mer template containing the site-specific DPC at position T (Scheme 2B). The primer group on the 5'-end of the oligodeoxynucleotide was used to purify the extension products via affinity capture prior to HPLC-ESI$^-$-FTMS and MS/MS analysis (45). Thymine residue three nucleotides from the site of damage, as well as any postlesion synthesis errors were released by treatment with UDG and hot piperidine (Scheme 3). The products of in vitro replication were sequenced by HPLC-ESI$^-$-FTMS and MS/MS on 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 extracted ion chromatograms were used to determine the relative quantities of each extension product as compared with the internal standard. Each sample was subsequently analyzed in the HPLC-ESI$^-$-MS/MS mode to allow for nucleotide sequence determination.

Capillary HPLC-ESI$^-$-FTMS analysis of the hpol $\kappa$-catalyzed reactions conducted with the unmodified template revealed one main peak corresponding to the expected error-free product (5'-pT CAA TGA-3'; m/z 1090.68; [M - 2H]$^{2-}$) (Table 2 and Fig. 2A). In contrast, HPLC-ESI$^-$-FTMS of the corresponding primer extension reaction conducted with dT-peptide conjugate revealed eight different extension products (Table 2 and Fig. 3). The main extension product (89% of total products) corresponded to a single base deletion product (5'-pT C_A TGA-3'; m/z 934.15; [M - 2H]$^{2-}$), and only 2% of the primer extension products yielded the error-free replication product (5'-pT CA TGA-3'; m/z 1090.68; [M - 2H]$^{2-}$). Other major MS peaks at m/z 777.62, 1086.17, and 1098.68 corresponded to doubly charged ions of 5'-pT C_G TGA-3' (one-base deletion, 15%), 5'-pT C_G TGA-3' (two-base deletion, 2%), 5'-pT C_A TGA-3' (1%), and 5'-pT C_C TGA-3' (1%) (Fig. 3). Oligodeoxynucleotide sequences were determined using the MS/MS spectra obtained by collision-induced dissociation of the corresponding doubly charged ions (Figs. 2B and 4).

HPLC-ESI$^-$-FTMS analysis of the primer extension products generated upon hpol $\eta$-catalyzed primer extension using the unmodified template revealed three main peaks present in roughly equal amounts at m/z 1090.68 (28%), 1098.68 (36%), and 1078.67 (36%), which correspond to the doubly charged ions of 5'-pT CA A TGA-3', 5'-pT CAG TGA-3', and 5'-pT CAC TGA-3', respectively (Table 2). MS/MS sequencing has confirmed that hpol $\eta$ inserts the correct base (A) opposite the unmodified T, but insertion of the succeeding nucleotide is error-prone (Fig. 5). hpol $\eta$-catalyzed primer extension using the dT-peptide-containing template gave rise to seven different products (Table 2 and Fig. 6). 19% of the total extension products corresponded to the error-free full extension product (5'-pT CAA TGA-3'; m/z 1090.68; [M - 2H]$^{2-}$), while 23 and 15% were G and C substitutions at the +1 position (5'-pT CAG TGA-3'; m/z 1098.68; [M - H]$^{2-}$ and 5'-pT CAC TGA-3'; m/z 1078.67; [M - 2H]$^{2-}$), respectively (Table 2 and Figs. 6 and 7). Other major MS peaks at m/z 942.15, 934.15, and 922.14 corresponded to the doubly charged ions of 5'-pT C_G TGA-3' (one-base deletion and an A $\rightarrow$ G mutation at +1, 16%), 5'-pT C_A TGA-3' (one-base deletion, 15%), and 5'-pT C_C TGA-3' (one-base deletion and an A $\rightarrow$ C mutation at +1, 12%) (Table 2 and Figs. 6 and 7). These results indicate that replication
bypass of dT-peptide conjugates by TLS polymerases is highly error-prone and that the extension products vary dramatically depending on DNA polymerase involved. Large numbers of deletion mutations observed upon primer extension past the peptide lesions are consistent with previous reports that TLS polymerases can skip the adducted site during replication and continue the polymerization reaction one base past the lesion (46, 48–50).

FIGURE 4. Representative MS/MS spectra of the extension products observed following hpol α-catalyzed replication past the 10-mer peptide cross-linked to C5 of thymidine. CID spectra of extension products: A, 5’-pT CTA TGA-3’, a substitution mutation; B, 5’-pT C_G TGA-3’; C, 5’-pT C_T TGA-3’, one-base deletion followed by a substitution; D, 5’-pT C__TGA-3’, double deletion.
DISCUSSION

DPCs are a ubiquitous form of DNA damage found in normal cells as a result of exposure to endogenous and exogenous reactive species (1, 5). These bulky, heterogeneous lesions can adversely affect cell viability and induce mutations by interfering with DNA transcription, replication, and repair (1, 5). There is increasing evidence for the involvement of DPCs in various human pathologies, including cancer, cardiovascular disease, and neurodegenerative conditions (5, 51–56). However, the biological fate of these lesions is not well understood.

Because of their large size as compared with other DNA lesions, DPCs are likely to block DNA replication. It has been proposed that proteins cross-linked to DNA can be proteolytically processed to generate less bulky DNA-peptide conjugates, which can be subsequently bypassed by TLS polymerases in an error-free or error-prone manner. DNA-peptide conjugates are preferable substrates for DNA repair over DNA-protein cross-links (57–61). Furthermore, proteasomal inhibitors have been reported to slow down the repair of formaldehyde-induced DPCs in cells (62) and to interfere with intracellular repair of DPC-containing plasmids (58). In addition, several in vitro replication studies using site-specific substrates containing small peptides conjugated to the major or minor groove of DNA have provided direct evidence for the ability of lesion bypass polymerases to catalyze nucleotide incorporation opposite the DNA-peptide lesions (25–27). Huang et al. (63) reported NMR structure of the KWKK peptide conjugated to the N\textsuperscript{2} position of guanine, which was shown to orient in the minor groove. However, previous studies were conducted with model DPCs containing peptides conjugated to various positions within purine nucleobases of DNA (the N\textsuperscript{6} of adenine and the N\textsuperscript{2} of guanine), and no information is available on polymerase bypass of the corresponding lesions involving pyrimidine residues in DNA. Such lesions can be generated upon exposure to endogenous free radicals, UV light, and ionizing radiation (32–36). Furthermore, irreversible trapping of DNA methyltransferases on genomic DNA can form DPCs at the C6 position of cytosine (30, 31).

Our primer extension results reported here (Tables 1 and 2) provide the first evidence for error-prone bypass of peptides conjugated to the major groove of DNA through a pyrimidine nucleobase by human lesion bypass polymerases. Model lesions containing a 10-mer peptide (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-NH\textsubscript{2}) conjugated to the C5 position of thymidine were generated via click chemistry (Scheme 1) (37). This peptide is derived from the c-Myc protein, a transcription factor that plays a key role in cell cycle progression, apoptosis, and cellular transformation. c-Myc regulates the expression of >15% of all human genes and is up-regulated in many types of cancer (64, 65). Because of its strong affinity for DNA and the presence of nucleophilic amino acids in its structure, c-Myc is likely to participate in DNA-protein cross-linking upon exposure to bis-electrophiles and reactive oxygen species.

We found that both hpol \(\kappa\) and \(\eta\) can incorporate either of the four deoxynucleotides opposite the DNA-tripeptide conjugate \(N^6\text{-dA-(OH)\textsubscript{2}butyl-GSH}\) (29). The extremely low accuracy observed with hpol \(\kappa\) in bypassing the dT-peptide conjugate in this work is of interest because hpol \(\kappa\) has been
FIGURE 7. Representative MS/MS spectra of extension products observed following *in vitro* replication past the 10-mer peptide cross-linked to C5-T by *hpol* h. CID spectra of extension products: A, 5'-pT CAA TGA-3'; error-free product; B, 5'-pT CAG TGA-3'; and C, 5'-pT CAC TGA-3'; substitutions opposite 1-mer. D, 5'-pT C_A TGA-3'; one-base deletion; E, 5'-pT C_G TGA-3'; F, 5'-pT C_C TGA-3'; one-base deletion followed by base substitution.
reported to exclusively incorporate the correct base, dC, opposite peptides of comparable size (12-mers) conjugated to \(N^2\) of dG (26). This discrepancy may arise because the dT-peptide lesion is conjugated to the major groove of DNA, while the \(N^2\)-dG-peptide is localized in the minor groove (26) and is consistent with the notion that the particular site of cross-linking within DNA is an important determinant in modulating the ability of polymerase to bypass specific DNA adducts (27).

It should be noted that the efficiency of replication bypass of adducted DNA is as important as the fidelity of replication. From single nucleotide insertion assays, we found that hpol \(\eta\) is more efficient at bypassing DNA-peptide conjugates when compared with hpol \(\kappa\) (Fig. 1). These results were confirmed by quantitative data obtained from steady-state kinetics (Table 1). Catalytic efficiencies \(k_{\text{cat}}/K_m\) of dNTP incorporation opposite the dT-peptide conjugate by hpol \(\eta\) were 2.3–463-fold higher than that of hpol \(\kappa\). Furthermore, higher misinsertion frequencies \([b]_j\) were observed for hpol \(\eta\) as compared with hpol \(\kappa\) (Table 1). Taken together, these data suggest that hpol \(\eta\) is more efficient than hpol \(\kappa\) in replication bypass in the context of the sequence and adduct studied in this work. The order of bypass efficiency, hpol \(\eta > \) hpol \(\kappa\), is consistent with previously reported values for 1,\(N^6\)-etheno-dA (49), 1,\(N^6\)-(2-hydroxy-3-hydroxymethylpropan-1,3-diy1)-2'-deoxyadenosine (66), \(N^2\)-3-etheno-dG (67), and \(N^6\)-dA-(OH)\(_2\)butyl-GSH (29).

Additional information was obtained from capillary HPLC-ESI\(^–\)MS/MS sequencing of extension products (Table 2). We found that hpol \(\kappa\) replicated the unmodified template with good accuracy; 87% of the products correspond to the error-free full extension product (Table 2 and Fig. 3A). In contrast, replication bypass of the dT-peptide conjugate was highly error-prone, inducing a large number of one- and two-nucleotide deletions (98% of total products, see Table 2, Figs. 2, 3B, and 4). It has been previously reported that polymerases show particularly high single base insertion/deletion rates in repetitive sequences (68), which is applicable in this case (5'\text{-TCA TTG AAT CCT TCC CCC-3'}).

HPLC-ESI\(^–\)MS/MS sequencing of the extension products of hpol \(\eta\) using the unmodified template revealed that the replication was highly error-prone (Table 2). Specifically, hpol \(\eta\) incorporated the correct base (dA) opposite the first T in 5'\text{-TCA TTG AAT CCT TCC CCC-3'} but the subsequent nucleotide insertion opposite the second T was highly error-prone (Table 2). hpol \(\eta\)-catalyzed replication using DPC template gave rise to significant amounts of transition and transversion mutations (15 and 23%, respectively; Table 2 and Fig. 6).

Low fidelity of DNA replication by hpol \(\eta\) and \(\kappa\) is well documented and can be explained by large size and flexibility of their active sites, along with the lack of 3'→5' exonuclease activity (19, 20, 69–71). These enzymes catalyze error-prone bypass of 8-oxo-dG (72, 73), abasic sites (72–74), 1,\(N^6\)-(2-hydroxy-3-hydroxymethylpropan-1,3-diy1)-2'-deoxyadenosine (66), 1,\(N^6\)-etheno-dA (49), and \(N^2\)-3-etheno-dG (67). hpol \(\eta\) also reads past bulky benzo[a]pyrene-\(N^2\)-dG (72) in an error-prone manner, while hpol \(\kappa\) erroneously bypasses 2-acetylanilofluorene-dG (73, 74). Importantly, the highest substitution rates for hpol \(\eta\) were observed for templates containing undamaged dT and d(TT) (70, 71). In our study, the presence of the dT-peptide lesion at the first T of a TT repeat has led to a large number of one-base deletions (43% of the total replication products; Table 2 and Fig. 6).

Several mechanisms for the induction of deletion mutations by TLS polymerases have been proposed based on x-ray crystallographic structures of ternary complexes between the polymerase, DNA, and the incoming deoxynucleotides (46, 48–50). It has been shown that the adducted base alone, or in concert with the adjacent base, can misalign, forming a slipped or extrahelical conformation (46, 49). Alternatively, a slippage event can occur subsequent to nucleotide insertion opposite the adduct (46, 48, 49). Extension of the misaligned primer yields one- and two-base deletions (46, 48, 49), although realignment after nucleotide insertion and further extension cause substitution mutations (46, 49). These prominent mutations (98% of total products for hpol \(\kappa\)) may also form upon replication of other DNA-peptide conjugates, but would not be detected by gel electrophoretic methodologies employed in previous studies (25–27). Furthermore, it should be emphasized that single standard nucleotide insertion assays performed by monitoring incorporation directly opposite the damaged base cannot detect mutations occurring downstream from the adduct but can be observed using HPLC-ESI\(^–\)MS/MS sequencing (Table 2).

In summary, we investigated the replication bypass of 10-mer peptides conjugated to the major groove of DNA via a pyrimidine base (C5 position of T) using human lesion bypass polymerases \(\eta\) and \(\kappa\). Our gel electrophoresis and HPLC-ESI\(^–\)MS/MS results provide evidence for highly error-prone replication past these bulky adducts. Specifically, large numbers of −1 and −2 deletions were observed for replication catalyzed by hpol \(\kappa\) (Table 2). Low fidelity of bypass past the DNA-peptide conjugates by human polymerases is likely to contribute to the mutagenesis of endogenous and exogenous DPCs (7). To our knowledge, this is the first systematic study of the replication fidelity of DPCs involving pyrimidine residues of DNA. In the future studies, it would be interesting to determine how these peptide conjugates are accommodated in the active site of TLS polymerases. X-ray crystallographic or NMR structural studies of ternary complexes of these adducts in the presence of TLS polymerases and dNTPs should be able to provide details on why and how this error-prone replication occurs, especially in reference to the large amounts of deletion mutations observed in our study.

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