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Cytotoxic and Mutagenic Properties of C1′ and C3′-Epimeric Lesions of 2′-Deoxyribonucleosides in Human Cells

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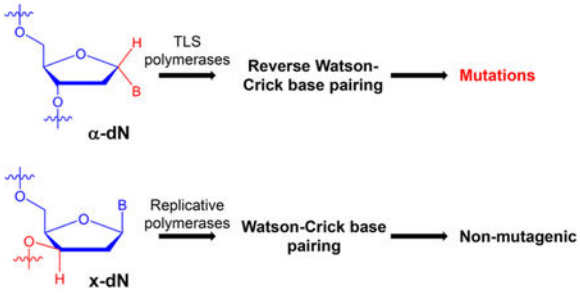
Abstract

Genomic integrity is constantly challenged by exposure to environmental and endogenous genotoxic agents. Reactive oxygen species (ROS) represent one of the most common types of DNA damaging agents. While ROS mainly induce single-nucleobase lesions, epimeric 2-deoxyribose lesions can also be induced upon hydrogen atom abstraction from the C1′, C3′, or C4′ carbon and the subsequent incorrect chemical repair of the resulting carbon-centered radicals. Herein, we investigated the replicative bypass of the C1′- and C3′-epimeric lesions of the four 2′-deoxynucleosides in HEK293T human embryonic kidney epithelial cells. Our results revealed distinct bypass efficiencies and mutagenic properties of these two types of epimeric lesions. Replicative bypasses of all C1′-epimeric lesions except α-dA are mutagenic in HEK293T cells, and their mutagenic properties are further modulated by translesion synthesis (TLS) DNA polymerases. By contrast, none of the four C3′-epimeric lesions are mutagenic, and the replicative bypass of these lesions is not compromised upon depletion of polymerase η, ι, κ, or ζ. Together, our results provide important new knowledge about the cytotoxic and mutagenic properties of C1′ and C3′ epimeric lesions, and reveal the roles of TLS DNA polymerases in bypassing these lesions in human cells.

Graphical Abstract



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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b01126.

PAGE and LC-MS and MS/MS results for monitoring the restriction fragments of PCR products for progeny genomes arising from replication in cultured HEK293T and the isogenic polymerase-deficient cells (PDF)

Notes

The authors declare no competing financial interest.

The genomic DNA in human cells is constantly subjected to damage by environmental and endogenous genotoxic agents, which challenges genomic integrity.[1] Reactive oxygen species (ROS) are required for normal physiological functions; however, they can also react with DNA to generate covalent modifications in DNA.[2,3] Nucleobase modifications constitute the major type of oxidative damage in DNA, which gives rise to a myriad of DNA lesions including 8-oxo-7,8-dihydro-2′-deoxyguanosine and thymidine glycol.[4,5] Aside from attacking nucleobases, the hydroxyl radical can also abstract a hydrogen atom from 2-deoxyribose, leading to the formation of carbon-centered radicals.[6,7] When the hydrogen atom is removed from the C1′, C3′, or C4′ position, a unique set of DNA lesions, i.e., epimeric 2-deoxyribose lesions, can form from incorrect chemical repair of the resulting carbon-centered radicals (Figure 1).[7]

Previous studies suggested that both the C1′ and C3′-epimeric lesions can be induced in DNA. α-2′-Deoxyadenosine (α-dA) is a major damage product when poly(dA) and poly(dA-dT) are exposed to γ-rays under anaerobic conditions.[8,9] In addition, a recent study showed the presence of appreciable levels of α-dG in calf thymus DNA and in DNA isolated from mouse pancreatic tissues.[10] Moreover, under anaerobic conditions, xylose-thymidine (x-dT) was found to form in oligodeoxyribonucleotides (ODNs) from the independently generated C3′ radical in the presence of glutathione (GSH).[11] The chemical modifications at the C1′ and C3′ positions of 2-deoxyribose lead to alterations in sugar puckering and backbone distortion, which result in perturbations of DNA stability and structure.[12,13] Although various repair proteins can remove DNA lesions, the structural similarities of epimeric nucleosides to their unmodified counterparts may render their recognition challenging because it requires the repair enzyme to be sensitive to relatively subtle perturbations in DNA structure[14,15]

To avoid a sustained stalling of replication fork by unrepaired DNA lesions, cells are equipped with translesion synthesis (TLS) polymerases to bypass DNA lesions. Although TLS polymerases can employ damaged DNA as a template, nucleotide insertions mediated by these polymerases are sometimes inaccurate, thereby leading to mutations in the genome. 16

Several shuttle vector studies have been conducted for assessing how site-specifically incorporated epimeric DNA lesions compromise DNA replication in *Escherichia coli* cells. [10,17] In this vein, all α-dN lesions except α-dA display replication blockage and mutagenic effects,[10,18] which are further influenced by genetic ablation of TLS polymerases (i.e., Pol II, Pol IV, and Pol V). Most x-dN lesions, on the other hand, were found not to be mutagenic,[17] revealing that the inversions of the stereochemical configurations at the C1′ and C3′ of 2-deoxyribose confer distinct effects on DNA replication.[10]

It remains unknown how the epimeric lesions are recognized by DNA replication machinery in mammalian cells. Herein, we constructed double-stranded plasmids containing site-specifically inserted C1′ and C3′ epimeric lesions and investigated how these lesions impair the efficiency and accuracy of DNA replication in HEK293T human embryonic kidney cells. We also examined the functions of TLS polymerases in the replicative bypass of these lesions.

MATERIALS AND METHODS

All chemicals, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO), and all enzymes were from New England Biolabs (Ipswich, MA). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was obtained from Oakwood Products Inc. (West Columbia, SC), and [γ-^{32}P]ATP was acquired from PerkinElmer (Piscataway, NJ). All unmodified ODNs were obtained from Integrated DNA Technologies (Coralville, IA). HEK293T cells deficient in *POLH, POLI, POLK,* and *REV3L* genes, which encode DNA polymerases η, ι, κ, and the catalytic subunit of Pol ζ, respectively, were produced previously by using the CRISPR-Cas9 genome editing method.[19]

Construction of Lesion-Containing and Control Plasmids.

The lesion-containing as well as the lesion-free control and competitor genomes were prepared, following the previously published procedures[20]. Briefly, the parental vector was digested with Nt.BstNBI to generate a gapped plasmid, followed by removal of the resultant 25-mer single-stranded ODN through annealing with a 25-mer complementary ODN that is in 100× excess. The gapped plasmid was then isolated from the mixture by using 100 kDa cutoff ultracentrifugal filter units (Millipore). The purified gapped plasmid was annealed with a 5′-phosphorylated 13-mer lesion-free ODN (5′-AATTGAGTCGATG-3′) and a 5′-phosphorylated 12-mer lesion-carrying or lesion-free control ODN, 5′-ATGGCGXGCTAT-3′ (X = α-dN, x-dN, or dN), followed by incubation with T4 DNA ligase and ATP in the ligation buffer (Figure 2).

Cell Culture, Transfection, and Plasmid Isolation.

Replication experiments of the above-constructed genomes were conducted using the previously reported strand-specific PCR-competitive replication and adduct bypass (SSPCR-CRAB) assay.[21] The lesion-containing and the respective control plasmids were premixed individually with the competitor genome and transfected into HEK293T cells or the isogenic polymerase-deficient cells. The molar ratios of the competitor to control and lesion-bearing genome were 1:3 and 1:9, respectively. The cells (1×10^5) were seeded in 24-well plates and cultured overnight at 37 °C in a 5% CO_2 atmosphere, after which they were transfected with 300 ng of the mixed genomes by using TransIT-2020 (Mirus Bio), following the manufacturer's recommended procedures. The cells were harvested at 24 h following transfection, and the progenies of the plasmid were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The residual unreplicated plasmid was further removed by DpnI digestion and exonuclease III cleavage, as described previously.[22]

PCR Amplification and Polyacrylamide Gel Electrophoresis (PAGE) Analyses.

The progeny genomes were amplified by nested PCR following previously published procedures.[23] The final PCR amplicons were separated using 1% agarose gel, purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, San Jose, CA), and stored at −20 °C until use. For PAGE analysis, 150 ng of the PCR fragments were treated with 5 units of NcoI-HF and 1 unit of shrimp alkaline phosphatase (SAP) at 37 °C in 10 μL of NEB buffer 3 for 1 h, followed by heating at 80 °C for 20 min to deactivate the SAP. To the above mixture were then added 1.25 μCi (0.5 pmol) of [γ-^{32}P]ATP and 5 units of T4

polynucleotide kinase (PNK). The reaction was continued at 37 °C for 30 min, followed by heating at 75 °C for 20 min to deactivate the T4 PNK. To the above mixture was subsequently added 2.0 units of SfaNI, and the solution was incubated at 37 °C for 1.5 h. The digestion was subsequently terminated by the addition of 20 μL of formamide gel-loading buffer. The above restriction digestion yielded a 16-mer radiolabeled fragment for the competitor genome and 13-mer fragments for the control or lesion-carrying genome (Figure 3A). However, the 13-mer fragment with a T→C mutation (13-mer C) cannot be separated from the nonmutagenic 13-mer T with native PAGE. Therefore, we used a different restriction digestion method, where the PCR amplicon was treated with MluCI followed by Cac8I. This alternative digestion procedure yielded 10-mer radiolabeled fragments for the opposite strand that permits the differentiation of the nonmutagenic product from the product with a T→C mutation (Figure 3C). The digestion products were separated using 30% native polyacrylamide gel (acrylamide/bis-acrylamide 19:1) and quantified via phosphorimager analysis (Figure 3B and D). The relative bypass efficiency (RBE) and mutation frequency (MF) were used to characterize the effects of DNA lesions on replication efficiency and fidelity, respectively, where the RBE values were calculated from the ratios of (lesion signal/competitor signal)/(nonlesion control signal/competitor signal).

Identification of Mutagenic Products by LC-MS/MS.

The replication products were also identified by using LC-MS/MS analysis. Briefly, 2 μg of the above-described PCR products were digested with 30 units of SfaNI and 15 units of SAP in 150 μL of NEB buffer 3 at 37 °C for 2 h, followed by deactivating the phosphatase through heating at 80 °C for 20 min. To the mixture was added 50 units of NcoI, and the solution was incubated at 37 °C for another 2 h. The resulting solution was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and to the mixture were subsequently added 2.5 volumes of ethanol and 0.1 volume of 3.0 M sodium acetate to precipitate the DNA. The DNA pellet was then reconstituted in water and subjected to LC-MS and MS/MS analysis. An Agilent 1200 capillary HPLC system (Agilent Technologies, Santa Clara, CA) and an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) were used for all the LC-MS and MS/MS experiments. The mass spectrometer was set up for acquiring the higher-resolution “zoom-scan” MS and the MS/MS for the $[M-3H]^{3-}$ ions of the 13-mer 5′-AATTACAGCNCGC-3′, where “N” represents A, T, C, or G (Figures S3–S10).

RESULTS

The major goal of this study was to examine the impact of the C1′- and C3′-epimeric lesions on the efficiency and fidelity of DNA replication in human cells. We synthesized a series of ODNs containing site-specifically incorporated epimeric lesions (Figure 2) and ligated the ODNs into a double-stranded plasmid carrying an SV40 replication origin, which allows for its replication in large T antigen-transformed HEK293T cells. The lesion-containing plasmid was transfected together with the competitor plasmid into HEK293T cells. We employed a previously published SSPCR-CRAB assay[21] to assess the replication blockage effects and mutagenic properties of the epimeric lesions in human cells. We also examined how the replicative bypass of these lesions is affected by TLS DNA polymerases

by conducting replication experiments using isogenic HEK293T cells with DNA polymerase η, ι, κ or ζ being knocked out by CRISPR-Cas9.[19]

Replication Blockage Effects of C1′- and C3′-Epimeric Lesions and the Roles of TLS Polymerases in the Replicative Bypass of These Lesions.

Our PAGE analysis results revealed that all the C1′-epimeric lesions except α-dG significantly blocked DNA replication in HEK293T cells (Figure 4A and B). Although α-dA only moderately decreases the bypass efficiency (to 68%), α-dT and α-dC constitute strong impediments to DNA replication, with the bypass efficiencies being 38.2% and 36.1%, respectively. Genetic ablation of any of the four TLS polymerases (Pol η, Pol ι, Pol κ, or Pol ζ) did not exert any pronounced effect on the bypass efficiency for α-dA, whereas genetic depletion of some TLS polymerases led to apparent decreases in bypass efficiencies for the other three C1′-epimeric lesions. In particular, knockout of Pol η or Pol ζ gave rise to reduced bypass efficiencies for α-dT. Ablation of Pol η, Pol κ, or Pol ζ led to the diminished bypass efficiencies for α-dC. On the other hand, depletion of Pol η or Pol κ appreciably attenuated the bypass efficiency of α-dG (Figure 4A and S1). These results reveal the involvement of multiple TLS polymerases in bypassing the C1′-epimeric lesions of dC, dG, and dT.

For all the C3′-epimeric lesions, x-dA, x-dT, and x-dG exhibited similar effects on blocking DNA replication, with the bypass efficiencies being 35.6%, 36.4%, and 39.4%, respectively. x-dC, however, did not display any appreciable blockage to DNA replication in HEK293T cells (Figure 4B). Furthermore, depletion of Pol η, ι, κ, or ζ did not alter the bypass efficiencies for any of the C3′-epimeric lesions; thus, none of these TLS polymerases assume an appreciable role in bypassing x-dN lesions.

The Effects of C1′- and C3′-Epimeric Lesions on the Fidelity of DNA Replication in Mammalian Cells.

Among all the C1′-epimeric lesions, α-dA was not mutagenic in HEK293T or the isogenic TLS polymerase-deficient cells (Figure 4C and S1). In contrast, α-dT induced mainly T→G transversion (29.3%), which was accompanied by a low frequency (8.8%) of T→A transversion. Loss of Pol η gave rise to elevated T→G transversion, together with a decreased frequency of T→A mutation. Deletion of Pol ζ, however, led to diminished T→G transversion and augmented T→A mutation.

α-dC and α-dG induced C→G transversion and G→A transition at frequencies of 29.3% and 12.0%, respectively. For α-dC, elevated C→G mutation frequencies were observed upon removal of Pol κ (to 54.7%) or Pol ζ (to 44.3%; Figures 4C, S1, and S2). With respect to α-dG, depletion of Pol η resulted in a marked increase in mutation frequency (to 32.7%; Figure 4C and S1), and a drastic elevation of G→A mutation was found in Pol κ-deficient cells (to 93.9%), demonstrating a major role of Pol κ in minimizing the misincorporation of dTMP opposite the lesion. It is worth noting that, apart from the correct nucleotides, pyrimidine nucleotides are preferentially inserted opposite C1′-epimeric lesions instead of purine nucleotides. In contrast to α-dN lesions, none of the four C3′-epimeric x-dN lesions were mutagenic in HEK293T or any of the isogenic polymerase-deficient HEK293T cells,

further supporting the lack of involvement of Pol η, ι, κ, or ζ in bypassing this family of lesions.

DISCUSSION

Human cells are continuously exposed to endogenous and exogenous sources of genotoxic agents that give rise to chemical modifications in cellular DNA. ROS can result in stereochemical inversions at the C1′, C3′, and C4′ carbons in 2-deoxyribose to yield epimeric lesions.[8–10,24] We examined previously the cytotoxic and mutagenic properties of the C1′ and C3′-epimeric lesions in *E. coli*,[10,17] which revealed completely different bypass efficiencies and mutagenic properties of these two types of lesions. Some TLS polymerases (e.g., Pol IV and Pol V) assume important roles in the accurate bypass of the C1′-epimeric lesions.[10] Although functional conservation of TLS polymerases is known, mounting evidence also indicates the different properties of prokaryotic and eukaryotic TLS polymerases in bypassing some DNA lesions.[25,26] The major objective of the present study is to gain a comprehensive understanding about the impact of C1′ and C3′ epimeric lesions on DNA replication and to uncover the roles of TLS polymerases in bypassing these lesions in human cells.

Although α-dA moderately blocked DNA replication, our results revealed that the lesion can be accurately bypassed by DNA replication machinery in human cells. Moreover, a loss of any of the four TLS polymerase did not alter the efficiency or fidelity of DNA replication across α-dA (Figure 4A and S1). This observation, which is reminiscent of the findings made from our previous replication studies in *E. coli*,[10] underscores that the lesion may be readily bypassed by replicative polymerases. It is worth noting that one-nucleotide deletion (−1 deletion) at the α-dA site was previously observed;[18] however, this mutation was not detected in our previous study in *E. coli* cells[10] or the present study in human cells. This discrepancy might be attributed to the difference in the lesion being placed in a single- versus double-stranded plasmid, or the different flanking nucleoside(s) of α-dA employed in these studies.

In contrast to α-dA, all three of the other C1′-epimeric lesions were mutagenic in HEK293T cells, where depletion of some TLS polymerases significantly decreased the bypass efficiencies of these lesions. α-dT exerted a strong blockage effect on DNA replication, with the bypass efficiency being 38.2% in HEK293T cells. Genetic depletion of Pol η or Pol ζ further diminished the bypass efficiencies of the lesion (to 29.6% and 27.4%, respectively), though a loss of Pol κ or Pol ι did not appreciably alter the bypass efficiency (Figure 4A and S1). A 29.3% T→G transversion and an 8.8% T→A transversion were observed for α-dT in HEK293T cells. On the other hand, our previous study showed that α-dT elicits nearly exclusively T→A transversion in *E. coli*,[10] suggesting the difference between the replication machineries of bacterial and mammalian cells in bypassing the lesion. A loss of Pol η significantly stimulated T→G mutation (to 38.8%), though T→A mutation was substantially decreased (to 5.1%; Figure 4C). These results revealed that, relative to other polymerases involved in nucleotide incorporation opposite α-dT, Pol η has a higher tendency to incorporate dTMP but a lower tendency to insert dCMP. A previous solution-phase NMR study unveiled that α-dA remains largely intrahelical in duplex DNA and it

forms a base pair with the opposing thymine base in a reverse Watson–Crick configuration.[14] A recent study also suggested that α-dT could pair with correct/incorrect nucleotides through reverse/standard Watson–Crick base pairing.[10] On the basis of these previous studies, we proposed base pairing models for nonmutagenic α-dA and mutagenic α-dT (Figure 5A–D).

Interestingly, although deletion of Pol ζ did not affect the overall mutation frequency (T→A/G: 37.8% in Pol ζ-depleted versus 38.1% in WT cells), the distribution of the two types of mutations altered significantly, where similar frequencies of T→G (20.6%) and T→A (17.2%) mutations were observed in a Pol ζ-deficient background (Figure 4C). Multiple studies support that human Pol ζ mainly serves as an effective extender during lesion bypass.[27,28] Our results suggest that this polymerase may have different efficiencies in extending the nascent strand from the α-dT:dT and α-dT:dC mispairs (Figure 4D). Thus, upon a loss of Pol ζ, other TLS polymerase(s) may be able to extend from the two mispairs at similar efficiencies.

The bypass efficiency of α-dC (36.1%) was comparable to that of α-dT, where three out of four TLS polymerases were involved in bypassing the lesion. Loss of Pol η, Pol κ, or Pol ζ decreased the bypass efficiencies to 21.8%, 26.8%, and 23.9%, respectively (Figure 4A and S2). This finding parallels the observations made in *E. coli*, which showed pronounced attenuated bypass efficiency of α-dC upon depletion of Pol IV or Pol V (orthologs of human Pol κ and Pol η, respectively).[10] Although both C→A and C→G mutations were found in *E. coli*,[10] α-dC induced exclusively C→G transversion in HEK293T cells. Moreover, Pol η promotes the bypass of α-dC without changing the mutation frequency, indicating a similar fidelity of Pol η in nucleotide insertion opposite α-dC to other TLS polymerases that are involved in bypassing this lesion. A loss of Pol κ or Pol ζ resulted in pronounced increases in frequencies for the two types of mutations (Figure 4C and S2). We reason that Pol κ preferentially inserts the correct dGMP opposite the α-dC, followed by extension of the nascent DNA strand via Pol ζ or by itself.[15,28–30]

In contrast to the other three C1′-epimeric lesions, α-dG does not impede DNA replication in HEK293T cells (Figure 4A). The bypass efficiency is slightly attenuated upon a loss of Pol η but markedly diminished in the absence of Pol κ (to 61.1%). Consistent with the bypass efficiency data, we also observed markedly elevated frequencies of G→A mutation upon depletion of Pol η or Pol κ (Figure 4C). This observation revealed the relatively high fidelities of these two polymerases in bypassing α-dG. Interestingly, neither the bypass efficiency nor the mutagenic property of α-dG was affected by the depletion of Pol ζ, indicating that Pol ζ may not be the main extender in bypassing the α-dG lesion in human cells. The modest, yet statistically significant reductions in the bypass efficiencies of α-dT, α-dC, and α-dG in different polymerase-deficient backgrounds suggest the cooperative bypass of these lesions by TLS polymerases. This differs from the specific role of polymerase η in bypassing the *cis–syn* cyclobutane thymine–thymine (TT) dimer, whose bypass efficiency in human cells was substantially diminished upon the genetic depletion of Pol η.[31]

It is worth noting that the existence of Pol κ is important for the accurate bypass of both α-dC and α-dG (Figure 4C). Previous studies indicated that minor-groove modifications in template DNA and incoming guanine nucleotide are well tolerated by Pol κ.[32–34] For example, Pol κ inserts preferentially the correct dCMP opposite the minor-groove N^2-(1-carboxyethyl)-2′-deoxyguanosine (N^2-CEdG) lesion, with the efficiency being higher than that opposite the unmodified dG.[35] Meanwhile, Pol κ also carried out efficient extension after a cytosine nucleotide is incorporated opposite the minor groove γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γHOP-dG) adduct by Pol ι.[36] Additionally, the X-ray crystal structure of Pol κ revealed the topological difference in the thumb domain between Pol κ and other Y-family polymerases, which elucidates the unique ability of Pol κ in mismatch extension and in promoting replication through various minor-groove DNA lesions. On the basis of these results, we propose reverse Watson–Crick base pairing models for α-dC:dG and α-dG:dC (Figure 5E and F), where the N^4 amino group of α-dC and the carbonyl group on C6 of α-dG is positioned in the minor groove. In addition, we surmise that this minor-groove protrusion can be well tolerated by Pol κ, but not by other TLS polymerase, which may explain the role of Pol κ in supporting the accurate bypass of α-dC and α-dG by Pol κ. Future structural studies are needed to reveal whether this is the case.

Among the four C3′-epimeric lesions, x-dA, x-dT, and x-dG constitute strong blockades to DNA replication, with the bypass efficiencies being 35.6%, 36.4%, and 39.4%, respectively, whereas x-dC exhibits no blockage effect on DNA replication. Removal of polymerase η, ι, κ, or ζ did not alter the bypass efficiencies for any of the x-dN lesions, indicating that the replicative bypass of the C3′-epimeric lesions does not involve these polymerases (Figure 4B). Moreover, our PAGE and LC-MS/MS results showed that none of the C3′-epimeric lesions were mutagenic. Together, these results indicate that all C3′-epimeric lesions can be bypassed by replicative polymerases. On the basis of these observations, we reason that the inversion of the stereochemical configuration at C3′ of 2-deoxyribose may not disrupt Watson–Crick base pairing; nevertheless, the alteration of stereochemical configuration at the C3′ carbon may compromise the efficiency of nucleotide incorporation at or near the lesion site.

In summary, our results demonstrate that the alteration of stereochemical configurations at C1′ and C3′ of 2-deoxyribose confer markedly different mutagenic properties. In addition, replicative bypass of all the C1′-epimeric lesions except α-dA requires TLS polymerases, whereas the Y-(i.e., Pol η, ι, and κ) and B-family (i.e., Pol ζ) TLS polymerases we studied did not elicit any apparent effects on bypassing the C3′-epimeric lesions. Taken together, our comprehensive replication study provides new insights into the impact of the two classes of epimeric 2-deoxyribose lesions on DNA replication in human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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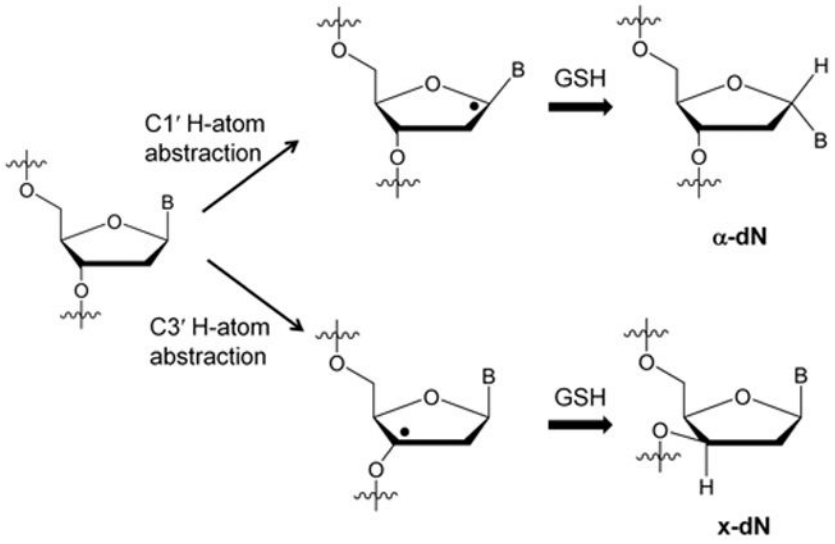

Figure 1.

Formation of α- and x-dN lesions through the improper chemical repair of the C1′ and C3′ radicals with a hydrogen atom donor. “B” indicates a nucleobase.

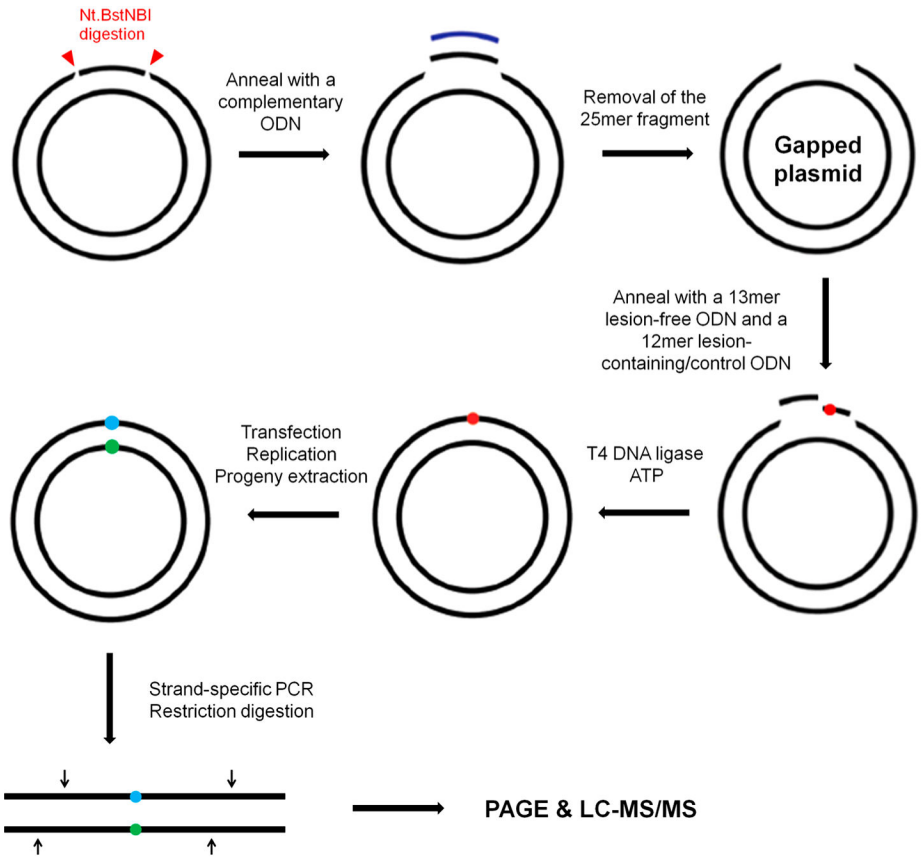

Figure 2.

A schematic diagram depicting the procedures for the construction of the lesion-containing plasmid and the SSPCR-CRAB assay. The original pTGFP-Hha10 vector was digested by Nt.BstNBI, and the resulting gapped plasmid was purified. The lesion-carrying plasmid was subsequently generated by ligating lesion-containing ODN into the gapped plasmid with T4 DNA ligase, where the red circle designates the epimeric DNA lesions. The progeny plasmids were harvested at 24 h following transfection, and the fragment containing the original lesion site was amplified by strand-specific PCR The PCR amplicon was digested and postlabeled with [γ-^{32}P]ATP, and the restriction fragments were analyzed using native PAGE. The PCR amplicon was also digested with restriction enzymes and analyzed by LC-MS/MS.

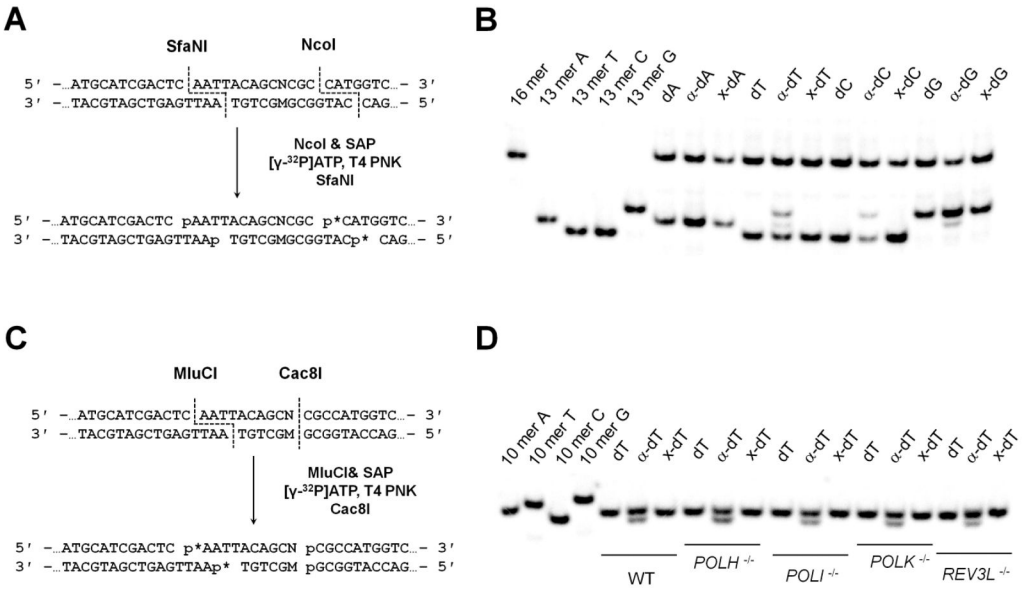

Figure 3.

Restriction digestion and postlabeling method for determining the bypass efficiencies and mutation frequencies of the lesions in HEK293T cells and the isogenic TLS polymerase-deficient cells. (A) Digestion of PCR products using NcoI and SfaNI restriction endonucleases and postlabeling assay. (B) Representative gel image showing the NcoI/SfaNI-produced restriction fragments of interest. The standard synthetic ODN representing the restriction fragment arising from the competitor vector (i.e., 5′-CATGGCGATATGCTGT-3′) is designated as “16-mer;” “13-mer A,” “13-mer G,” “13-mer T,” and “13-mer C” designate the standard synthetic ODNs 5′-CATGGCGMGCTGT-3′, where “M” is A, G, T, and C, respectively. (C) Sample processing for restriction cleavage using MluCI and Cac8I and postlabeling assay. (D) Representative gel image showing the MluCI/Cac8I-generated restriction fragments of PCR products amplified from the progenies of α-dT-containing plasmid. “10-mer A,” “10-mer G,” “10-mer T,” and “10-mer C” designate the standard synthetic ODNs 5′-AATTACAGCN-3′, where “N” is A, G, T, and C, respectively. “p*” indicates a ^{32}P-labeled phosphate group.

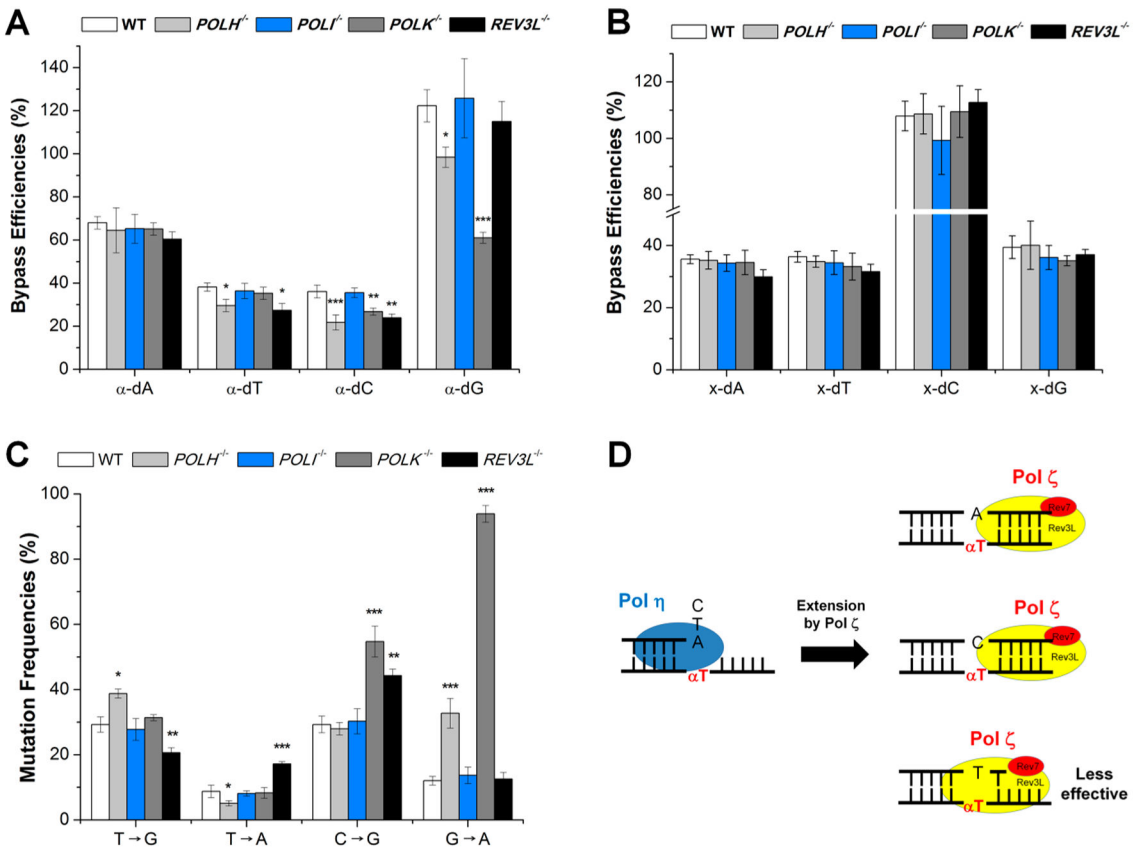

Figure 4.

Bypass efficiencies of α-dN (A) and x-dN (B) and the mutation frequencies of α-dN (C) in HEK293T cells and the isogenic TLS polymerase-deficient cells. Shown in D is a proposed model about the role of Pol ζ in modulating the mutagenic property of α-dT. The bypass efficiencies and the mutation frequencies were calculated from PAGE analysis, and the data represent the means and standard deviations of results from three independent replication experiments. *$0.01 \leq P < 0.05$; **$0.001 \leq P < 0.01$; ***$P < 0.001$. The P values were calculated by using unpaired two-tailed Student's t-test.

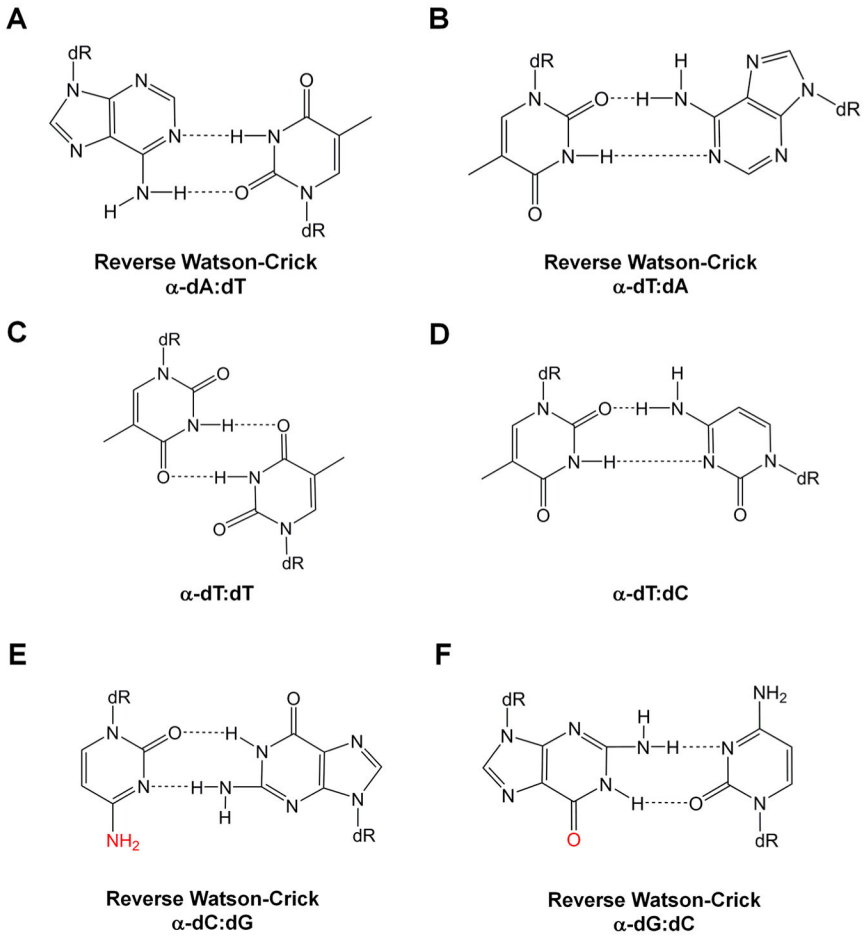

Figure 5.

Proposed base-pairings involved in correct nucleotide incorporations opposite α-dA (A) and α-dT (B), along with the misinsertion of dTMP (C) and dCMP (D) opposite α-dT. E and F show the correct nucleotide incorporations opposite α-dC and α-dG, respectively, where the groups that are located in the minor groove are labeled with a red color. “dR” designates 2-deoxyribose.