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Effects of Tet-Induced Oxidation Products of 5-Methylcytosine on DNA Replication in Mammalian Cells

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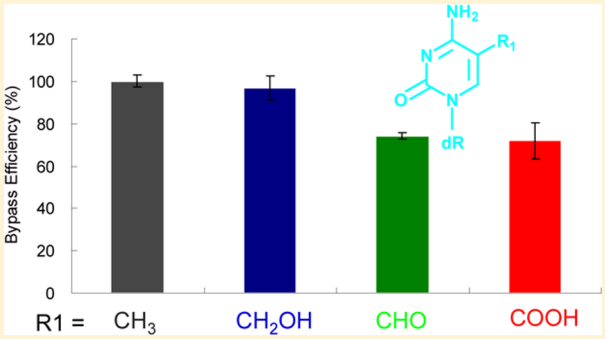
Supporting Information

ABSTRACT: Recently 5-hydroxymethyl-2′-deoxycytidine (5hmdC), 5-formyl-2′-deoxycytidine (5fdC), and 5-carboxyl-2′-deoxycytidine (5cadC) were discovered in mammalian DNA as oxidation products of 5-methyl-2′-deoxycytidine (5mdC) induced by the ten-eleven translocation family of enzymes. These oxidized derivatives of 5mdC may not only act as intermediates of active cytosine demethylation in mammals but also serve as epigenetic marks on their own. It remains unclear how 5hmdC, 5fdC, and 5cadC affect DNA replication in mammalian cells. Here, we examined the effects of the three modified nucleosides on the efficiency and accuracy of DNA replication in HEK293T human kidney epithelial cells. Our results demonstrated that a single, site-specifically incorporated 5fdC or 5cadC conferred modest drops, by approximately 30%, in replication bypass efficiency without inducing detectable mutations in human cells, whereas replicative bypass of 5hmdC is both accurate and efficient. The lack of pronounced perturbation of these oxidized 5mdC derivatives on DNA replication is consistent with their roles in epigenetic regulation of gene expression.



INTRODUCTION

Faithful transmission of genetic information during cellular differentiation and across generations is essential for the well being and survival of an organism. Different cells in a multicellular organism contain the same genes, but they exhibit significant differences in expression of their genome. Mammalian cells regulate gene expression through multiple mechanisms, such as sequence-specific DNA-binding proteins, post-translational modifications of histones, chromatin remodeling, and methylation of cytosine residues in DNA.[1,2]

Methylation at the C5 position of cytosine residues at CpG dinucleotide sites is the best-studied covalent modification of DNA.[3] The resulting 5-methyl-2′-deoxycytidine (5mdC) is frequently clustered around gene promoters, which results in transcriptional silencing.[4,5] Recent studies showed that 5mdC can be further modified by 10-11 translocation (Tet) family of enzymes to give 5-hydroxymethyl-2′-deoxycytidine (5hmdC), 5-formyl-2′-deoxycytidine (5fdC) and 5-carboxyl-2′-deoxycytidine (5cadC) and that these modified nucleosides could be detected in mammalian DNA (Figure 1).[6−9] For instance, 5hmdC, 5fdC and 5cadC are present in genomic DNA of HeLa cells at frequencies of 31, 0.67, and 0.27 per 10^6 nucleosides, respectively.[10] The amounts are much higher than (for 5hmdC) or comparable to (for 5fdC and 5cadC) those of some DNA lesions induced by endogenous reactive oxygen species, such as 8,5′-cyclopurine-2′-deoxynucleosides.[11,12] It was also observed that 5fdC, 5cadC, and the deaminated derivative of 5hmdC (i.e., 5-hydroxymethyl-2′-deoxyuridine) formed at CpG dinucleotide sites could be recognized and removed by thymine

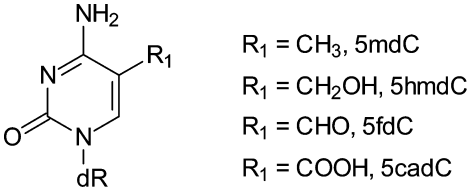


Figure 1. Chemical structures of modified 2′-deoxycytidine derivatives found in mammalian DNA. "dR" represents 2-deoxyribose.

DNA glycosylase (TDG) to yield abasic sites, which may be subsequently converted to 2′-deoxycytidine through the base excision repair (BER) pathway, thereby giving rise to active cytosine demethylation in mammals.[13−15] Apart from being considered as intermediates of active cytosine demethylation, emerging experimental findings suggested that 5hmdC, 5fdC and 5cadC may serve as stable epigenetic marks and possess unique regulatory functions.[13,16,17] In this vein, a proteome-wide analysis revealed many cellular proteins capable of binding 5fdC- and 5cadC-containing DNA, suggesting that 5fdC and 5cadC may recruit unique proteins for specific functions.[18]

Not much is known about how these oxidized 5mdC derivatives affect DNA replication and transcription in human cells. 5fdC and 5cadC were found to reduce the rate and substrate specificity of transcription mediated by yeast and mammalian RNA polymerase II.[19] Additionally, 5hmdC, 5fdC

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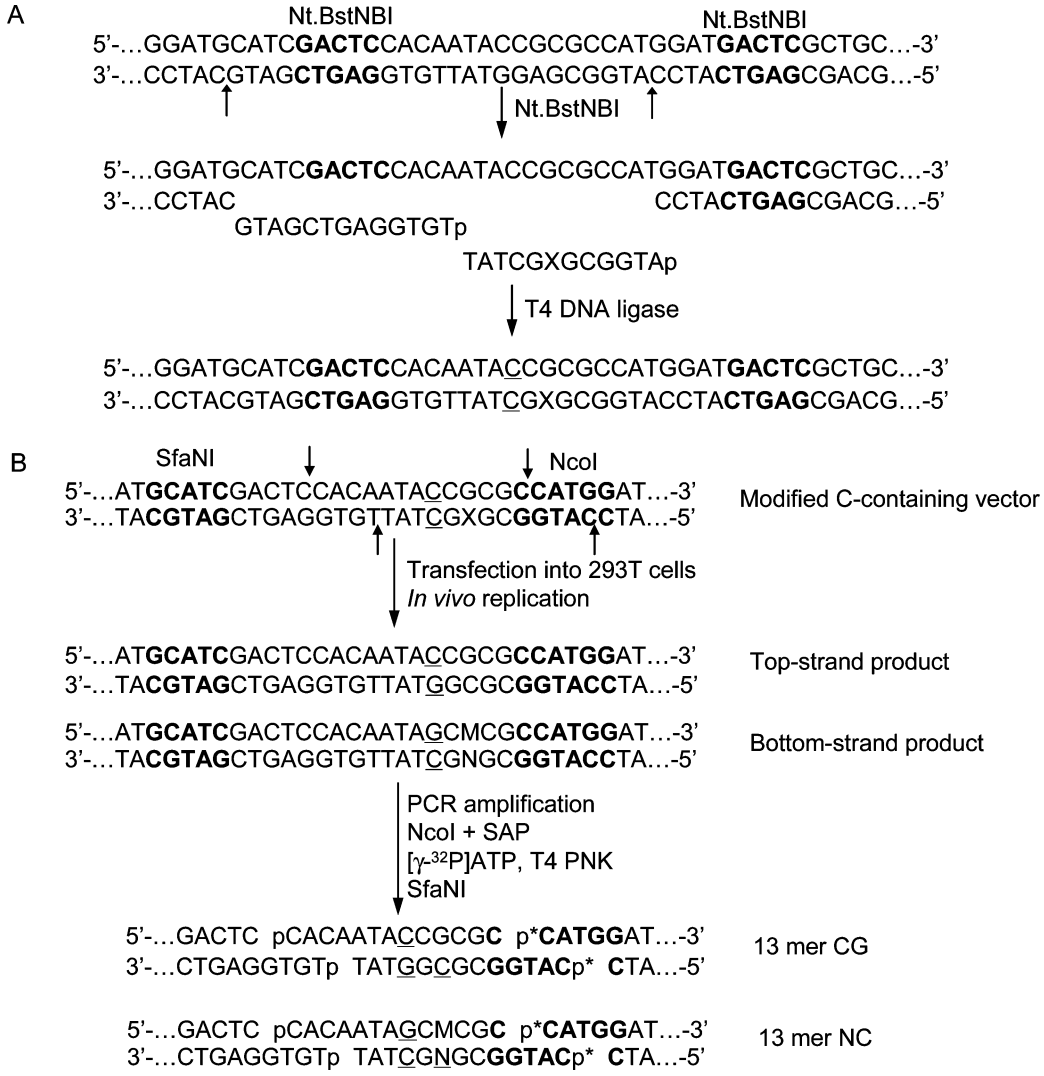

Figure 2. Experimental procedures for the construction of modified cytosine-containing duplex vectors (A) and for determining the effects of the modified cytosine derivatives on DNA replication in cells (B). "X" represents 5mdC, 5hmdC, 5fdC, or 5cadC, and the C:C mismatch site is underlined. "p*" and "p" designate ^{32}P-labeled and unlabeled phosphate, respectively. The restriction recognition sites are highlighted in bold, and cleavage sites are indicated by arrows.

and 5cadC were observed to be weakly mutagenic in *Escherichia coli* cells, with the C → T transition mutation occurring at frequencies of 0.17%−1.12%.[20] This is in line with a previous *in-vitro* mutagenesis assay showing that 5fdC is only marginally mutagenic (1% C → T transition).[21] In this article, we assessed how these cytosine derivatives perturb the efficiency and fidelity of DNA replication in cultured human cells. Our results demonstrated that 5fdC and 5cadC but not 5hmdC could modestly inhibit DNA replication, though none of them could induce detectable mutations during replication in HEK293T cells; our results are in agreement with the roles of these modified nucleosides in epigenetic regulation.

EXPERIMENTAL PROCEDURES

Materials. Unmodified oligodeoxyribonucleotides (ODNs) used in this study were purchased from Integrated DNA Technologies (Coralville, IA). [γ-^{32}P]ATP was obtained from PerkinElmer (Piscataway, NJ). Shrimp alkaline phosphatase (SAP) was obtained from USB Corporation (Cleveland, OH). All other enzymes unless otherwise specified were purchased from New England BioLabs (Ipswich, MA). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from TCI America (Portland, OR). Chemicals unless otherwise noted were obtained from Sigma-Aldrich (St. Louis, MO). The HEK293T human embryonic kidney epithelial cells were purchased from ATCC (Manassas, VA). The siRNAs used in this study were purchased from Thermo Dharmacon: *human-TDG SMART*pool (L-003780) and siRNA Control Non-Targeting pool (D-001210).

Modified cytosine-containing ODNs (5′-ATGGCG**X**GCTAT-3′, X = 5mdC, 5hmdC, 5fdC, or 5cadC) were synthesized previously. The HPLC traces for monitoring the purities of these ODNs are shown in Figure S1 (Supporting Information), and the identities of these ODNs were confirmed by electrospray ionization−mass spectrometry (ESI-MS) and tandem MS (MS/MS) analyses.[22]

Construction of Modified Cytosine-Bearing Double-Stranded Shuttle Vectors. We constructed the modified cytosine-containing double-stranded shuttle vectors by using a previously described method (Figure 2A).[23−25] The parent vector was constructed by modifying the sequence of the original pTGFP-Hha10 plasmid.[23] The parent vector was subsequently nicked with Nt.BstNBI, and the resulting 25-mer single-stranded ODN was removed from the nicked plasmid by annealing the cleavage mixture with its complementary ODN in 50-fold molar excess. The gapped plasmid was then isolated from the mixture by using 100 kDa-cutoff ultracentrifugal filter units (Centricon 100 from Millipore). The gapped vector was filled with a 12-mer-modified cytosine-bearing

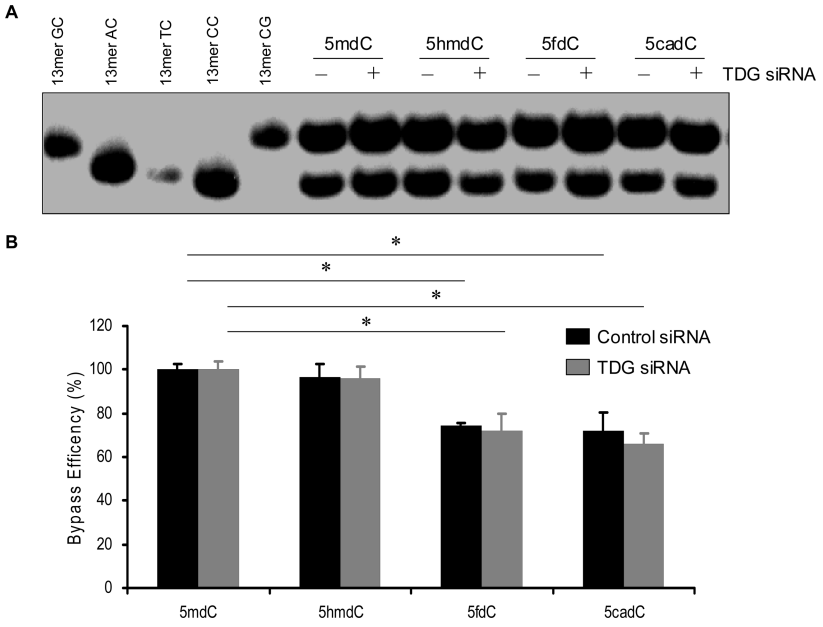

Figure 3. *In-vivo* replication studies of 5mdC and its oxidized derivatives in HEK293T cells. (A) Representative PAGE gel image showing the restriction fragments of PCR products of the progeny genome emanating from the replication of 5mdC-, 5hmdC-, 5fdC-, and 5cadC-containing plasmids in HEK293T cells treated with human TDG siRNA or control nontargeting siRNA. (B) The bypass efficiencies of the modified cytosines in HEK293T cells. The black bars represent the data from cells treated with control siRNA, whereas the gray bars designate the data from cells treated with hTDG siRNA. The data represent the mean and standard deviation of results from three independent replication experiments. "*", $p < 0.05$. The p values were calculated by using two-tailed, unpaired Student's t test. The horizontal lines above the bar graph indicate the pairs of data for which the p values were calculated.

ODN (5′-ATGGCG**X**GCTAT-3′, X = 5mdC, 5hmdC, 5fdC, or 5cadC) and a 13-mer unmodified ODN (5′-CTCTGAGTCGATG-3′) by T4 DNA ligase. The ligation mixture was incubated with ethidium bromide for 10 min, and the resulting supercoiled-modified cytosine-bearing plasmid was isolated by agarose gel electrophoresis.

siRNA Treatment and Quantitative Real-Time PCR. The HEK293T cells were seeded in 6-well plates at 40% confluence level and transfected with approximately 100 pmol siRNAs for TDG or nontargeting control siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommended procedures. Total RNA was extracted from the cells at 48 h after siRNA transfection using the Total RNA Kit I (Omega). cDNA was generated by using M-MLV reverse transcriptase (Promega) and a mixture of an oligo$(dT)_{16}$ primer. Relative quantification of gene expression was conducted by using qRT-PCR on a Bio-Rad iCycler system (Bio-Rad). The experiment was performed in an optical 96-well plate, including iQ SYBR Green Supermix Kit (Bio-Rad), 1 μL of the cDNA sample, and 0.2 μM of each gene-specific primers, in a final volume of 25 μL. The *GAPDH* gene was used as the endogenous control. The qRT-PCR primers for the *TDG* gene were 5′-TATGATCCAGGTTATGAGG-3′ and 5′- ATGCAGCAGTGAACCTTG-3′. The qRT-PCR primers for the *GAPDH* gene were 5′-TTTGTCAAGCTCATTTCCTGGTATG-3′ and 5′-TCTCTTCCTCTTGTGCTCTTGCTG-3′. The reactions followed the temperature profile 95 °C for 3 min; 45 cycles of 95 °C for 15 s; 55 °C for 30 s; and 72 °C for 45 s. The comparative cycle threshold method ($\Delta\Delta Ct$) was used for the relative quantification of gene expression.[26]

***In-Vivo* Replication in siRNA-Treated Cells.** The HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (ATCC), and incubated at 37 °C in 5% CO_2 atmosphere. The cells were seeded in 6-well plates at 40% confluence level and transfected with approximately 100 pmol hTDG siRNAs or control nontargeting siRNA using Lipofectamine 2000 (Invitrogen). After a 24-h incubation, 500 ng of modified cytosine-containing plasmids were independently transfected into the cells together with another 100 pmol of siRNA using Lipofectamine 2000. The cells were harvested at 24 h after transfection, and the progenies of the plasmid were isolated by using an alkali lysis method. The residual unreplicated plasmid was removed by DpnI digestion overnight, followed by digesting the resulting linear DNA with exonuclease III for 0.5 h as described elsewhere.[27,28] In this vein, the parent plasmid carried 25 DpnI recognition sites, and cleavage at any of these sites would result in the degradation of the entire plasmid by exonuclease III and prevent the subsequent PCR amplification of the parent vector.

PCR and Polyacrylamide Gel Electrophoresis (PAGE) Analyses. The progeny plasmids arising from *in-vivo* replication were amplified by PCR with the use of Phusion high-fidelity DNA polymerase (New England Biolabs). The two primers were 5′-CTTTCCAAAATGTCGTAACAACTCC-3′ and 5′-CAACACTC-AACCCTATCTCGGTCTAT-3′. The PCR amplification consisted of 98 °C 30 s; 36 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and stored at −20 °C until use.

For PAGE analysis, a portion of the PCR products was treated with 5 U of NcoI and 1 U 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 30 min to deactivate SAP. The above mixture was then treated in 15 μL of NEB buffer 3 with 5 mM DTT, ATP (50 pmol cold, premixed with 1.66 pmol [γ-^{32}P]ATP), and 5 U of T4 polynucleotide kinase (T4 PNK). The reaction was continued at 37 °C for 1 h, followed by heating at 65 °C for 20 min to deactivate the polynucleotide kinase. To the reaction mixture was subsequently added 5 U of SfaNI, and the solution was incubated at 37 °C for 1 h, followed by quenching with 15 μL of formamide gel loading buffer containing xylene cyanol FF and bromophenol blue dyes. The mixture was purified using 30% polyacrylamide gel (acrylamide/bis-acrylamide = 19:1), and the gel band intensities were quantified by phosphorimager analysis. We then determined the ratio of band intensity observed for the restriction fragment of the PCR product of the progeny from the modified 5mdC-containing strand over that of its complementary strand, and this ratio was normalized to the corresponding ratio obtained for the 5mdC-bearing plasmid to give the bypass efficiency.[29,30]

LC-MS/MS Analysis. To identify the replication products arising from modified cytosine-bearing substrates using LC-MS/MS, PCR products from 200 μL of PCR reaction mixture were treated with 50 U of NcoI and 20 U of SAP in 200 μL of NEB buffer 3 at 37 °C for 4 h, followed by heating at 80 °C for 20 min. To the resulting solution was added 50 U of SfaNI, and the reaction mixture was incubated at 37 °C for 4 h followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v, Figure S2, Supporting Information). The aqueous portion was dried with Speed-vac, desalted with HPLC, and dissolved in 12 μL of water. The ODN mixture was subjected to LC-MS/MS analysis. A 0.5 × 150 mm Zorbax SB-C18 column (5 μm in particle size, Agilent Technologies) was used for the separation, and the flow rate was 8.0 μL/min, which was delivered by using an Agilent 1100 capillary HPLC pump. A 5 min gradient of 5−25% methanol followed by a 40 min of 25−50% methanol in 400 mM HFIP (pH was adjusted to 7.0 by the addition of triethylamine) was employed for the separation. The effluent from the LC column was coupled directly to an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA), which was set up for monitoring the fragmentation of the $[M-3H]^{3-}$ ions of the 13-mer ODNs [d(CATGGCGXGCTAT), where "X" designates A, T, C, or G].

RESULTS AND DISCUSSION

We employed our previously described shuttle vector method to investigate how the modified 5mdC derivatives perturb the efficiency and fidelity of DNA replication in human cells.[23−25] To this end, we first constructed the 5mdC-, 5hmdC-, 5fdC- and 5cadC-containing double-stranded plasmids, as well as the control vector housing a 5mdC at the corresponding site (Figure 2A). We incorporated a C/C mismatch two nucleotides away from the modified nucleoside site (Figure 2A) so as to differentiate the replication products of the modified cytosine-containing strand from that of the unmodified complementary strand. The modified nucleoside-bearing plasmids and the 5mdC-containing control plasmid were transfected individually into HEK293T cells (Figure 2B). After *in-vivo* replication for 24 h, the progenies of the plasmids were isolated from human cells, and residual unreplicated plasmids were removed by a combined treatment with DpnI and exonuclease III as described previously.[27,28] The progeny genomes were subsequently amplified using a pair of PCR primers spanning the modified cytosine site. The resulting PCR products were digested with restriction enzymes (i.e., NcoI and SfaNI) and subjected to PAGE and LC-MS/MS analyses for product identification and quantification (Figure 2B). The bypass efficiencies were calculated from the ratio of the restriction products from the modified 5mdC-containing strand over that of the unmodified complementary strand and normalized to the corresponding ratio obtained for the 5mdC-containing plasmid (Figure 3A).[24]

Our results revealed that the bypass efficiency for 5hmdC is almost identical to that of 5mdC (Figure 3B). However, the bypass efficiencies for 5fdC and 5cadC are approximately 74% and 72% relative to that of 5mdC, respectively (Figure 3B). Thus, 5fdC and 5cadC constitute modest blocks to DNA replication machinery in human cells. Along this line, it was observed recently that 5fdC and 5cadC moderately blocked yeast and mammalian RNA polymerase II-mediated transcription *in vitro*, though these two modified nucleosides did not block DNA replication in *E. coli* cells.[20]

The denaturing PAGE gel analysis did not allow us to resolve the product with C → T mutation from the unmutated counterpart. To identify potential mutagenic products, we digested the PCR product with restriction enzymes and subjected the digestion mixture to LC-MS/MS analysis. The LC-MS/MS result revealed the absence of mutagenic product for all the modified cytosine derivatives (Figures S2−S6, Supporting Information). Along this line, our previous results showed that LC-MS/MS could quantify a molar ratio of ODNs that is as low as 0.2%.[31] These results, therefore, suggest that the mutation rate introduced by the modified cytosines should be less than 0.2% in HEK293T cells.

In mammalian cells, 5fdC and 5cadC in double-stranded plasmids could be potentially removed by the TDG-mediated BER pathway.[13−15] To explore this possibility, we knocked down the expression of TDG in HEK293T cells by employing siRNA and assessed the effect of the knockdown on the replicative bypass of 5fdC and 5cadC. Real-time PCR results showed that the knockdown efficiency of TDG was approximately 70% (Figure S7, Supporting Information). Relative to control-siRNA treated cells, knockdown of TDG did not give rise to significant alterations in the bypass efficiency for 5hmdC, 5fdC, or 5cadC (Figure 3). Furthermore, the LC-MS/MS data showed that no obvious mutation was introduced during replication after TDG knock-down.

DNA functions in the storage and transmission of genetic information. The genetic information must be transmitted with high fidelity; thus, the epigenetic modifications on DNA should not compromise the fidelity of DNA replication. 5hmdC, 5fdC, and 5cadC as the oxidized products of epigenetic mark 5mdC not only act as the intermediates of DNA demethylation[6,22,32−36] but also serve as epigenetic marks.[17] The same as 5mdC, all these modification groups are attached to the C5 position of cytosine, which is not involved in pairing with guanine. Therefore, these modification groups do not disrupt Watson−Crick base pairing, which may account for the lack of mutation during the replicative bypass of these modified cytosine derivatives in human cells. This is largely consistent with the very low frequencies of C → T transition mutation (0.17−1.12%) observed for 5hmdC, 5fdC, and 5cadC when replicated in *E. coli* cells.[20] The observation of detectable levels of mutations for these three modified 5mdC derivatives in *E. coli* cells, but not in human cells, may reflect the use of different assay systems (single- vs double-stranded plasmids were used for the replication experiments in *E. coli* and human cells, respectively) and the involvement of different polymerases in these two organisms.

CONCLUSIONS

In summary, we investigated the effects of Tet-mediated oxidation products of 5mdC on DNA replication in human cells. Although 5fdC and 5cadC constituted modest blocks to DNA replication, replication of 5hmdC, 5fdC, and 5cadC displayed high fidelity. The high fidelity and relatively high efficiency in replication of 5hmdC, 5fdC, and 5cadC are in keeping with the roles of these modified 5mdC derivatives in epigenetic regulation. Our recent LC-MS/MS/MS quantification studies revealed that 5hmdC, 5fdC, and 5cadC are present in genomic DNA of HeLa cells at frequencies of 31, 0.67, and 0.27 per 10^6 nucleosides, respectively.[10] The lack of any apparent deleterious effects on DNA replication may justify, in part, for human cells the possession of a relatively high level of 5hmdC in the their genome. However, aside from serving as important intermediates for active cytosine demethylation in mammals, the blockage to DNA replication may constitute another rationale for the efficient removal of 5fdC and 5cadC from the mammalian genome.

■ ASSOCIATED CONTENT

Supporting Information

Real-time PCR results, procedures for restriction digestion of replication products for LC-MS/MS analysis, and representative LC-MS/MS results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

5mdC, 5-methyl-2′-deoxycytidine; 5hmdC, 5-hydroxymethyl-2′-deoxycytidine; 5fdC, 5-formyl-2′-deoxycytidine; 5cadC, 5-carboxyl-2′-deoxycytidine; Tet, 10-11 translocation; TDG, thymine DNA glycosylase; BER, base excision repair; ODN, oligodeoxyribonucleotide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; ESI-MS, electrospray ionization−mass spectrometry; MS/MS, tandem MS; PAGE, polyacrylamide gel electrophoresis; SAP, shrimp alkaline phosphatase; T4 PNK, T4 polynucleotide kinase

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