1012–1018 *Nucleic Acids Research, 2015, Vol. 43, No. 2* *Published online 8 January 2015*
doi: 10.1093/nar/gku1391

Transcriptional inhibition and mutagenesis induced by *N*-nitroso compound-derived carboxymethylated thymidine adducts in DNA

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Received November 26, 2014; Revised December 20, 2014; Accepted December 23, 2014

ABSTRACT

***N*-nitroso compounds represent a common type of environmental and endogenous DNA-damaging agents. After metabolic activation, many *N*-nitroso compounds are converted into a diazoacetate intermediate that can react with nucleobases to give carboxymethylated DNA adducts such as *N*3-carboxymethylthymidine (*N*3-CMdT) and O^4-carboxymethylthymidine (O^4-CMdT). In this study, we constructed non-replicative plasmids carrying a single *N*3-CMdT or O^4-CMdT, site-specifically positioned in the transcribed strand, to investigate how these lesions compromise the flow of genetic information during transcription. Our results revealed that both *N*3-CMdT and O^4-CMdT substantially inhibited DNA transcription mediated by T7 RNA polymerase or human RNA polymerase II *in vitro* and in human cells. In addition, we found that *N*3-CMdT and O^4-CMdT were miscoding lesions and predominantly directed the misinsertion of uridine and guanosine, respectively. Our results also suggested that these carboxymethylated thymidine lesions may constitute efficient substrates for transcription-coupled nucleotide excision repair in human cells. These findings provided important new insights into the biological consequences of the carboxymethylated DNA lesions in living cells.**

INTRODUCTION

The human genome is constantly threatened by DNA-damaging agents including *N*-nitroso compounds (NOCs), which can form from red meat consumption and be present in tobacco smoke as well as other environmental and endogenous sources (1–5). Multiple lines of evidence support a strong link between the exposure to NOCs and an elevated risk of gastrointestinal cancer and other human diseases (1,3–7). Metabolic activation of many NOCs results in the generation of a common reactive intermediate, diazoacetate, which can induce carboxymethylation of nucleobases in DNA (8–12). It has been suggested that diazoacetate-induced DNA carboxymethylation, but not methylation, may lead to the signature mutations in *p*53 gene found in human gastrointestinal cancer (13,14). In this respect, a yeast-based functional assay revealed that diazoacetate-induced mutation spectrum in human *p*53 gene at non-CpG sites is strikingly similar to those observed in mutated *p*53 in human stomach and colorectal tumors (13). Recent studies showed that *N*3-carboxymethylthymidine (*N*3-CMdT) and O^4-carboxymethylthymidine (O^4-CMdT) are the major carboxymethylated adducts of thymidine formed in isolated DNA upon exposure to diazoacetate (Figure 1), and these lesions may contribute to *p*53 mutations at non-CpG sites induced by diazoacetate (8,13,15,16).

Understanding the biological consequences of DNA lesions in living cells necessitates the examination about how these lesions compromise the transmission of genetic information during DNA replication and transcription (17–20). In the respect, it has been shown that *N*3-CMdT and O^4-CMdT are both blocking and miscoding during DNA replication *in vitro* and in *Escherichia coli* cells (15,16). In addition, *E. coli* DNA polymerase V or its yeast ortholog (i.e. polymerase η) is involved in translesion synthesis of *N*3-CMdT and O^4-CMdT (15,16). To date, the effects of carboxymethylated thymidine adducts on DNA transcription have not yet been studied. Herein, we placed a single *N*3-CMdT or O^4-CMdT at a defined position in the transcribed DNA strand of non-replicative double-stranded plasmids and assessed how these carboxymethylated DNA lesions perturb DNA transcription *in vitro* and in human cells.

MATERIALS AND METHODS

Materials and cell culture conditions

All enzymes, chemicals, [γ-^{32}P]ATP and unmodified oligodeoxyribonucleotides (ODNs), unless otherwise specified, were purchased from New England BioLabs, Sigma-Aldrich, Perkin-Elmer and Integrated DNA Technologies, respectively. All siRNAs used in this study were obtained from Thermo Scientific Dharmacon: *CSB*

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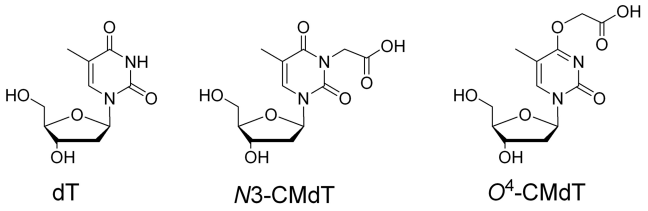

Figure 1. Chemical structures of dT, *N*3-CMdT and O^4-CMdT.

ON-TARGETplus SMARTpool (L-004888), *XPC* ON-TARGETplus SMARTpool (L-016040) and non-targeting control siRNA (D-001210). The 293T human embryonic kidney epithelial cells (ATCC) and human fibroblast cells (XP12RO and GM15876A) (21) were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin, and 100 μg/ml streptomycin (ATCC).

Transcription template preparation

The undamaged control and competitor plasmid substrates, which were derived from a non-replicating pTGFP-Hha10 plasmid (22,23), were constructed in a recent study (24). The *N*3- and O^4-CMdT-bearing plasmids were prepared following a well-established method as described elsewhere (Figure 2a) (22,23,25). Briefly, two nicks were produced in the transcribed strand of undamaged control plasmid by Nt.BstNBI, and the resulting 25-mer single-stranded ODN was removed from the nicked plasmid by annealing with an 'excess amount' of complementary ODN. The gapped vector was purified and ligated with a 13-mer ODN (5′-AATTGAGTCGATG-3′) and a 12-mer lesion-containing ODN (5′-ATGGCGXGCTAT-3′, X = *N*3-CMdT or O^4-CMdT) (8). The ligated products were incubated with ethidium bromide and the supercoiled lesion-bearing constructs were gel purified as described previously (25). The lesion-bearing or undamaged control plasmids were premixed with the competitor vector at a molar ratio of 3:1 (lesion or control/competitor) and used as DNA templates for all transcription assays in this study.

Transcription assay

In vitro transcription reactions mediated by T7 RNA polymerase (T7 RNAP) or human RNA polymerase II (hRNAPII) as well as transcription assays in human cells were conducted as described elsewhere (24). The human cells used in this study included XPA-deficient (XP12RO) and XPA-complemented (GM15876A) cell lines, and 293T cells that were treated with non-targeting control or siRNAs targeting CSB or XPC.

RNA extraction and reverse transcription-polymerase chain reaction

The RNA products arising from *in vitro* and *in vivo* transcription were extracted with Total RNA Kit I (Omega) and were further processed using a DNA-free kit (Ambion) following the manufacturer's instructions. The transcripts of interest were then reverse transcribed and polymerase chain reaction (PCR) amplified as described previously (23). The efficiency of siRNA knockdown was evaluated by real-time reverse transcription-PCR (RT-PCR) as described previously (23).

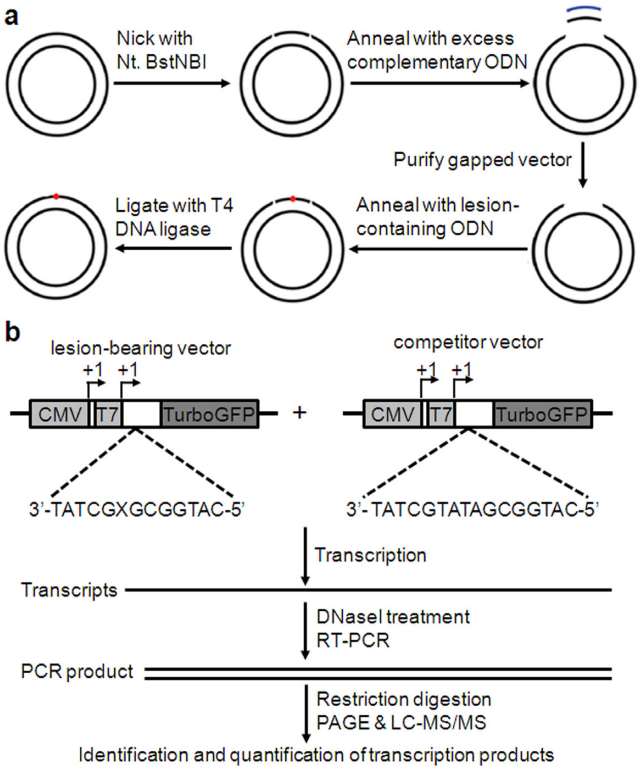

Figure 2. Experimental outline. (**a**) A schematic diagram illustrating the procedures for the construction of the plasmids harboring a site-specifically incorporated *N*3-CMdT or O^4-CMdT. (**b**) Strategy for assessing the impact of the CMdT lesions on DNA transcription. The non-lesion competitor vector has the same sequence as the lesion-bearing vector except for three more bases near the lesion region. 'X' indicates dT, *N*3-CMdT or O^4-CMdT, and the arrowheads indicate the +1 transcription start sites of the cytomegalovirus (CMV) and T7 promoters.

Polyacrylamide gel electrophoresis analysis

We employed NcoI/SfaNI-mediated restriction digestion/postlabeling assay, as described recently (24), to resolve the 13-mer non-mutagenic product d(CATGGCGAGCTAT) from the DNA fragments d(CATGGCGCGCTAT) and d(CATGGCGTGCTAT), which correspond to the transcription products carrying A→C and A→U mutations opposite the lesion, respectively (Figure 3a and b). Briefly, the RT-PCR products were treated with NcoI and shrimp alkaline phosphatase. After heat inactivation, the dephosphorylated restriction fragments were radiolabeled with the use of [γ-^{32}P]ATP and T4 polynucleotide kinase (T4 PNK) and were further digested with SfaNI. The products were separated by 30% native acrylamide:bisacrylamide (19:1) gel and analyzed by a phosphorimager (23,25). Similarly, we employed MluCI/Cac8I-mediated restriction digestion/postlabeling assay to separate the 10-mer fragment d(AATTATAGCG) (i.e. with A→G mutation) from the corresponding non-

mutagenic product or the products with an A→C or A→U mutation (Figure 2c and d). The relative bypass efficiency (RBE) and the frequency of base misincorporation were determined as described previously (23,26).

Liquid chromatography-tandem mass spectrometry analysis

We employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the identification of transcription products, as described elsewhere (24). Briefly, the RT-PCR products were treated with SfaNI and shrimp alkaline phosphatase. After heat inactivation, the dephosphorylated restriction fragments were further digested by NcoI. The proteins in the restriction mixture were removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), and the aqueous portion was desalted with HPLC, and subjected to LC-MS/MS analysis (23,25).

RESULTS

We employed a well-established competitive transcription and adduct bypass assay (23) to systematically examine how *N*3-CMdT and O^4-CMdT compromise the efficiency and fidelity of DNA transcription *in vitro* and in human cells. For this purpose, we incorporated site-specifically a single *N*3-CMdT or O^4-CMdT into a non-replicative double-stranded plasmid using a previously described method (Figure 2a) (22,23,25). The lesions were placed on the template strand 39 and 58 nucleotides downstream from the transcriptional start sites of the T7 promoter and cytomegalovirus promoter, respectively (Figure 2b). We premixed the lesion-carrying or undamaged control plasmid with a lesion-free competitor construct and used them as DNA templates for *in vitro* or *in vivo* transcription. The transcripts of interest were amplified using RT-PCR, and the resulting RT-PCR products were digested with suitable restriction enzymes. The DNA fragments released from the restriction digestion reactions were subjected to polyacrylamide gel electrophoresis (PAGE) and LC-MS/MS analyses (Figure 2b).

We performed the *in vitro* transcription assays using single-subunit T7 RNA polymerase (T7 RNAP) or multi-subunit human RNA polymerase II (hRNAPII) in HeLa cell nuclear extract. In this regard, because of the structural homology between T7 RNAP and eukaryotic mitochondrial RNAPs (27), we chose T7 RNAP as a model to assess the potential effects of *N*3-CMdT and O^4-CMdT on mitochondrial transcription in eukaryotic cells. After *in vitro* transcription, the RNA products were purified, reverse-transcribed and amplified with PCR.

By digesting the RT-PCR products with two sets of restriction enzymes, i.e. NcoI/SfaNI and MlucI/Cac8I, we were able to unequivocally distinguish the restriction fragments with a single nucleotide difference at the lesion site by PAGE analysis (Figure 3a–d).

Our results showed that both *N*3-CMdT and O^4-CMdT markedly inhibited DNA transcription catalyzed by T7 RNAP *in vitro*, with the RBE values being ∼46 and 29%, respectively (Figure 3e). Similarly, *N*3-CMdT and O^4-CMdT exhibited strong inhibitory effects on the hRNAPII-mediated transcription *in vitro*, with the RBE values being ∼26 and 19%, respectively (Figure 3e). Moreover, we found that *N*3-CMdT was highly mutagenic during *in vitro* transcription mediated by T7 RNAP and hRNAPII, with uridine misincorporation opposite the lesion (A→U) occurring at frequencies of ∼83 and 70%, respectively (Figure 3f). Transcriptional bypass of O^4-CMdT by T7 RNAP and hRNAPII was also mutagenic, with guanosine misincorporation opposite the lesion (A→G) occurring at frequencies of ∼33 and 17%, respectively (Figure 3g). The identities of the above mutant products were also confirmed by LC-MS/MS analysis (Supplementary Figures S1 and S2).

We next asked how *N*3-CMdT and O^4-CMdT perturb the efficiency and fidelity of DNA transcription in human cells. To this end, we co-transfected the lesion-containing or lesion-free control plasmids together with a competitor vector into XPA-deficient XP12RO and XPA-complemented GM15876A cells. After a 24-h incubation, we extracted the RNA from the cells and analyzed the transcription products as described above (Figures 2b and 3a and c).

Our results showed that *N*3-CMdT and O^4-CMdT substantially impeded DNA transcription in GM15876A cells, with the RBE values being ∼33 and 37%, respectively (Figure 4a and Supplementary Figure S4a). Moreover, we observed significantly lower RBE values for both *N*3-CMdT and O^4-CMdT in XPA-deficient XP12RO cells (Figure 4a and Supplementary Figure S4a). Transcriptional bypass of *N*3-CMdT was observed to induce A→U mutation opposite the lesion site at a frequency of ∼40% in GM15876A cells, whereas such mutation occurred at a significantly higher frequency (∼65%) in XP12RO cells (Figure 4b and Supplementary Figure S4a and b). We also found that O^4-CMdT induced a markedly higher degree of A→G mutation in XP12RO cells than in GM15876A cells (Figure 4c and Supplementary Figure S4a and b). These results indicated that XPA, a core component of the nucleotide excision repair (NER) pathway (28,29), is involved in the removal of *N*3-CMdT and O^4-CMdT in human cells.

Because CSB and XPC are specifically required for transcription-coupled NER (TC-NER) and global-genome NER (GG-NER), respectively (28,29), we investigated the effects of their depletion on transcriptional alternations induced by *N*3-CMdT and O^4-CMdT in human cells. Our results showed that the siRNA-mediated downregulation of CSB resulted in a significant decrease in transcriptional bypass efficiency of *N*3-CMdT in human 293T cells (Figure 4d and Supplementary Figures S4 and S5a). In addition, depletion of CSB caused a significant elevation in transcriptional mutagenesis induced by *N*3-CMdT, with the frequency of A→U mutation being increased from ∼23% in control siRNA-treated cells to ∼39% in CSB-knockdown cells (Figure 4e and Supplementary Figure S5a and b). Similarly, we found that siRNA knockdown of CSB elicited a significant change in the effects of O^4-CMdT on the efficiency and fidelity of transcription in human 293T cells (Figure 4d and f and Supplementary Figure S5a and b). On the other hand, siRNA-mediated downregulation of XPC did not significantly alter the transcriptional bypass efficiency or mutation frequency of *N*3-CMdT and O^4-CMdT in 293T cells (Figure 4e and f and Supplementary Figures S4 and S5a and b). Taken together, these results indicated that TC-NER may play a major role in the removal of

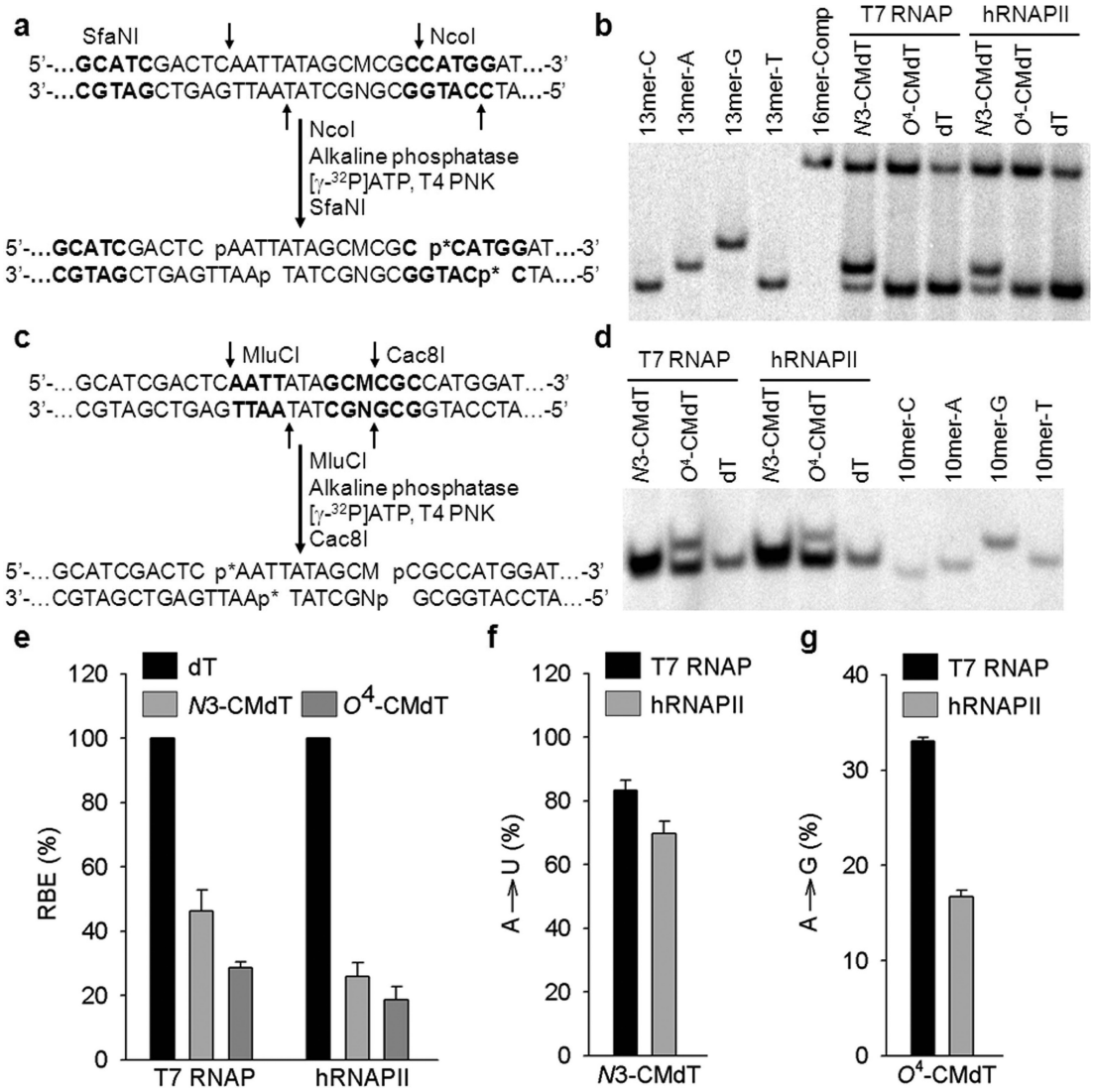

Figure 3. The effects of *N*3-CMdT and O^4-CMdT on DNA transcription mediated by T7 RNAP and HeLa nuclear extract (hRNAPII) *in vitro*. (**a**) Sample processing for NcoI/SfaNI-mediated restriction digestion/postlabeling assay (*P** indicates a ^{32}P-labeled phosphate group). (**b**) Representative gel images showing the NcoI/SfaNI-produced restriction fragments of interest. The restriction fragment arising from the competitor vector, i.e. d(CATGGCGATATGCTAT), is designated as '16mer-Comp', '13mer-C', '13mer-A', '13mer-G' and '13mer-T' represent the standard synthetic ODNs d(CATGGCGNGCTAT), where 'N' is C, A, G and T, respectively. (**c**) Sample processing for MluCI/Cac8I-mediated restriction digestion/postlabeling assay. (**d**) Representative gel images showing the MluCI/Cac8I-generated restriction fragments of interest. '10mer-C', '10mer-A', '10mer-G' and '10mer-T' represent the standard synthetic ODNs d(AATTATAGCM), where 'M' is C, A, G and T, respectively. (**e**) The RBE values of *N*3-CMdT and O^4-CMdT in *in vitro* transcription systems. (**f** and **g**) Mutagenic properties of *N*3-CMdT (f) and O^4-CMdT (g) in *in vitro* transcription systems. The data represent the mean and standard error of results from three independent experiments.

*N*3-CMdT and O^4-CMdT from the transcribed strand of active genes in human cells.

DISCUSSION

Accurate and efficient transmission of genetic information is essential for maintaining normal cellular functions (17–20,30). Unrepaired DNA damage, apart from perturbing DNA replication, may also elicit cytotoxic effects by blocking transcription and/or confer genome instability by inducing aberrant transcripts during DNA transcription (17,18,30,31). Notably, emerging evidence suggests that transcriptional mutagenesis may cause stable phenotypic changes in cells and may ultimately contribute to carcinogenesis or other adverse health effects (17,32–35). Due to the abundant presence of carboxymethylating agents in the environment and within cells, carboxymethylation of nucleobases in DNA is generally unavoidable (2–4,8–12). In this vein, O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG) was detected, by an immunoslot blot assay, at a level of three to eight lesions per 10^7 nucleosides in the blood DNA of healthy human subjects (12), and humans consuming red meat have elevated levels of O^6-CMdG in colonic exfoliated cells than control group consuming vegetarian diet (36). Hence, it is important to examine how

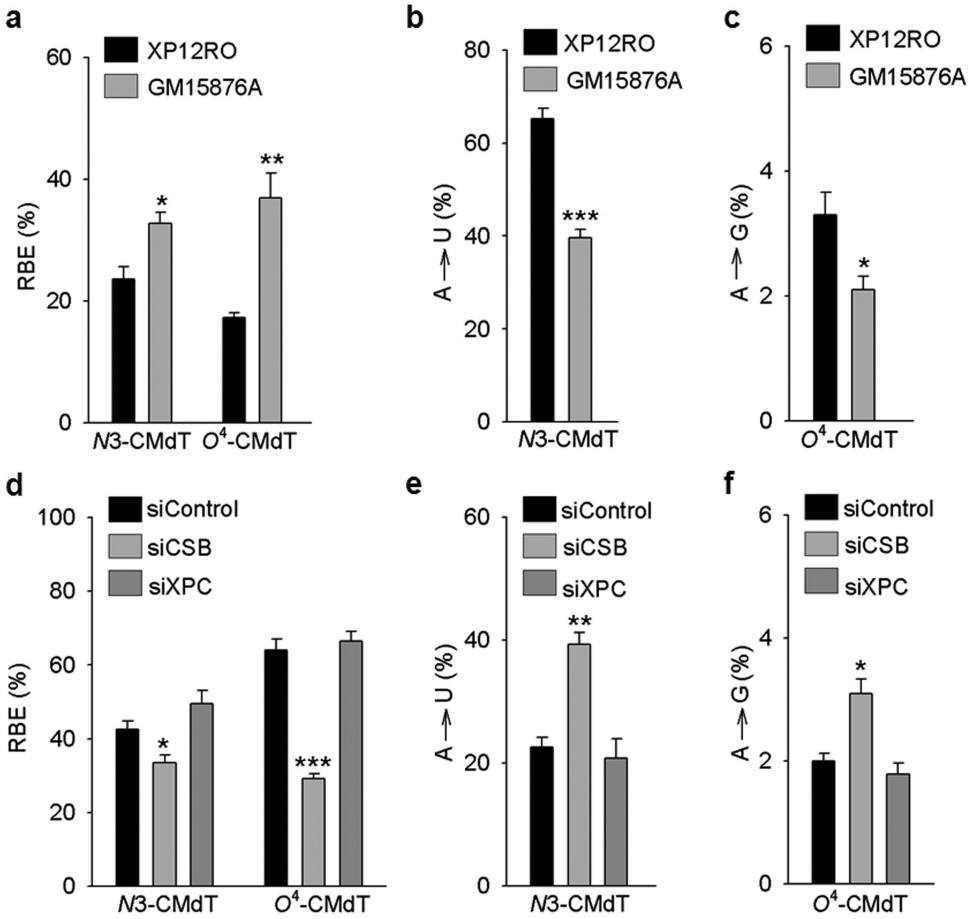

Figure 4. The effects of *N*3-CMdT and O^4-CMdT on DNA transcription in human cells. (**a**) The RBE values of *N*3-CMdT and O^4-EtdT in XPA-deficient XP12RO and XPA-complemented GM15876A cells. (**b** and **c**) Mutagenic properties of *N*3-CMdT (b) and O^4-CMdT (c) in XP12RO and GM15876A cells. (**d**) The RBE values of *N*3-CMdT and O^4-CMdT in HEK293T cells treated with control non-targeting, *CSB* or *XPC* siRNAs. (**e** and **f**) Mutagenic properties of *N*3-CMdT (e) and O^4-CMdT (f) in HEK293T cells treated with *CSB* or *XPC* siRNAs. The data represent the mean and standard error of results from three independent experiments. '*', $P < 0.05$; '**', $P < 0.01$; '***', $P < 0.001$. The *P* values were calculated by using unpaired two-tailed Student's *t*-test.

carboxymethylated DNA lesions compromise the efficiency and fidelity of DNA transcription.

It has been previously shown that two carboxymethylated thymidine lesions, i.e. *N*3-CMdT and O^4-CMdT, are strong blockages to DNA replication machinery in *E. coli* cells and primarily induced T→A and T→C mutations at the lesion sites, respectively (16). In keeping with these findings, we showed here that both *N*3-CMdT and O^4-CMdT markedly inhibited DNA transcription mediated by T7 RNA polymerase or human RNA polymerase II *in vitro*. In addition, we found that *N*3-CMdT was a strong miscoding lesion and predominantly induced mutant transcripts containing a uridine opposite the lesion, whereas O^4-CMdT was a moderately miscoding lesion that exclusively induced misinsertion of guanosine opposite the lesion during transcription *in vitro*.

We further demonstrated that both *N*3-CMdT and O^4-CMdT substantially impeded DNA transcription in human cells. In addition, we found that *N*3-CMdT and, to a much lesser degree, O^4-CMdT were able to induce transcriptional mutagenesis in human cells. Moreover, our results revealed that depletion of TC-NER components could further exacerbate the deleterious effects of these carboxymethylated DNA lesions on transcriptional efficiency and fidelity in cells. In this respect, it was observed that NER-deficient cells are significantly more sensitive to carboxymethylating agents than repair-proficient cells, suggesting that one or more unidentified carboxymethylated DNA adduct(s) may serve as potential substrate(s) for NER, thereby conferring cytotoxic effects that are selective toward NER-deficient cells (37). Our results indicate that *N*3-CMdT and O^4-CMdT may constitute such carboxymethylated DNA lesions that elicit elevated sensitivities of NER-deficient cells toward carboxymethylating agents. In addition, previous studies showed that TC-NER plays an important role in the repair of several non-bulky lesions, including abasic sites, in cells (23,24,38,39). Our present work added *N*3-CMdT and O^4-CMdT to the pool of non-bulky DNA lesions that partially inhibit transcription and may trigger TC-NER, which is a subpathway of NER that efficiently removes transcription-blocking lesions in cells (24,28,29).

The *N*3 and O^4 positions of thymine are among the major sites for DNA modification induced by alkylating agents *in vitro* (40,41); it is therefore of interest to compare the sim-

ilar and distinct effects of *N*3-CMdT, O^4-CMdT and their structurally related alkylated lesions on the efficiency and fidelity of DNA transcription (24). Similar as *N*3-CMdT, it was recently reported that *N*3-ethylthymidine (*N*3-EtdT) is both blocking and miscoding during transcription and is subjected to TC-NER in human cells (24). While *N*3-CMdT directs specifically the misinsertion of uridine, *N*3-EtdT was found to display promiscuous miscoding properties during transcription (24). The observed difference may be attributed to the unique chemical properties of these two lesions. In this context, the deprotonated carboxyl group in *N*3-CMdT may foster hydrogen-bonding interaction with the hydrogen atom on *N*3 of uridine, thereby promoting the misincorporation of uridine opposite the lesion, whereas the presence of an alkyl functionality on the *N*3 position of thymine may abolish the Watson–Crick hydrogen-bonding property, and thus the base-pairing capabilities of the nucleobase (24,42,43). On the other hand, both O^4-CMdT and O^4-EtdT lesions were observed to direct exclusively the misincorporation of guanosine opposite the lesion sites during DNA transcription; however, unlike O^4-CMdT, O^4-EtdT is not a strong blockage to DNA transcription machinery and cannot be efficiently repaired by TC-NER in human cells (24).

In summary, we investigated, for the first time, how *N*3-CMdT and O^4-CMdT perturb the efficiency and fidelity of DNA transcription mediated by T7 RNA polymerase *in vitro* or human RNA polymerase II *in vitro* and in human cells. Our results also suggested that these carboxymethylated DNA lesions may constitute efficient substrates for TC-NER in cells. These findings provide important new insights into the biological consequences of DNA damage induced by carboxymethylating agents in living cells. In this vein, it is worth noting that a more complete understanding about the biological relevance of *N*3-CMdT, O^4-CMdT and other carboxymethylated DNA lesions also requires a rigorous assessment about their formation in human cells and tissues. Our laboratory is in the process of developing LC-MS/MS-based methods for examining the occurrence of these lesions in cultured mammalian cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Profs. Timothy R. O'Connor and Karlene A. Cimprich for kindly providing the initial pTGFP-Hha10 vector and human fibroblast cells (XP12RO and GM15876A), respectively.

FUNDING

National Institutes of Health (NIH) [DK082779 to Y.W.]. Funding for open access charge: NIH [R01 DK082779].
Conflict of interest statement. None declared.

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