Effects of Endogenous DNA Base Lesions on Transcription Elongation by Mammalian RNA Polymerase II

IMPLICATIONS FOR TRANSCRIPTION-COUPLED DNA REPAIR AND TRANSCRIPTIONAL MUTAGENESIS*

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The blockage of transcription elongation by RNA polymerase II (pol II) is thought to be a trigger for transcription-coupled repair in the pathway of nucleotide excision repair. Purified pol II and oligo(dC)-tailed templates containing a single non-bulky DNA lesion on the transcribed strand such as an apurinic/apyrimidinic (AP) site, uracil, or 8-oxoguanine (8-oxoG) were used for transcription elongation assays. In this system pol II could bypass both the AP site and uracil without pausing and insert cytosine opposite the AP site and either guanine or adenine opposite to uracil. Thus, the AP site on the DNA templates could lead to correct transcription only if depurination at guanine occurred, whereas uracil generated either the correct transcriptional product or an incorrect one with a G:C to A:T transition. In the case of 8-oxoG, pol II stalled at the lesion, but sometimes bypassed it and inserted a cytosine residue or the incorrect adenine residue leading to a G:C to T:A transversion. These findings indicate that 8-oxoG lesions caused a blockage of transcription elongation and/or the misincorporation of a ribonucleotide by pol II, implying the initiation of transcription-coupled repair of 8-oxoG and/or transcriptional mutagenesis.

The genome as a carrier of genetic information in living cells is vulnerable to DNA-damaging agents of both endogenous and environmental origins. Once the DNA has lesions, essential DNA-dependent processes are interfered with and mutation or cell death can occur. Thus, DNA repair removes lesions from DNA to maintain genomic integrity. To remove DNA lesions, cells have two major repair pathways: nucleotide excision repair (NER)³ operating primarily on bulky helix-distorting damage caused by environmental mutagens and base excision repair (BER) for non-bulky and non-helix-distorting DNA modifications caused by endogenous and some chemical carcinogen-induced damage (1–3). When RNA polymerase II in the elongation phase encounters DNA damage that blocks transcription, transcription-coupled repair (TCR), a specialized pathway that efficiently removes lesions on the transcribed strand, operates to counteract the immediate and cytotoxic response of the interference (4–6). The transcribed strand is repaired much faster by TCR than is the non-transcribed strand or inactive regions by global genome repair (GGR) (7). TCR is found not only in eukaryotes but also in prokaryotes (5). Its importance in humans has been suggested by studies of autosomal recessive human inherited diseases: xeroderma pigmentosum (XP) and Cockayne’s syndrome (CS) (8). NER-deficient XP is classified into seven genetic complementation groups (XP-A to XP-G). Both the TCR and GGR subpathways of NER are defective in all these groups except for XP-C and XP-E, in which only GGR is impaired. CS is mostly classified into two genetic complementation groups (CS-A and CS-B). XP-B patients and certain individuals with XP-D or XP-G show features of CS in addition to XP symptoms (XP-B/CS, XP-D/CS, and XP-G/CS). CS-A and CS-B cells are deficient in TCR but proficient in GGR.

Although TCR was first thought to be a specific subpathway of NER, it was recently reported that 8-oxoguanine (8-oxoG) and thymine glycol are also repaired by a TCR subpathway of BER (TCR of oxidative damage), and this subpathway is specifically deficient in CS-A, CS-B, XP-B/CS, XP-D/CS, and XP-G/CS but proficient in XP-A. These results suggest that the symptoms of CS, such as postnatal growth failure and neurological complications, result from a defect in the TCR of oxidative damage, the repair requiring XPG and TFIH as well as CSA and CSB (9, 10).

8-oxoG is an important premutagenic lesion due to its potential to mispair with adenine, thus generating G:C to T:A transversions (3). Its biological significance is revealed by the existence of a three-tiered defense system composed of the proteins MutT (8-oxodGTPase), Fpg (DNA glycosylase/AP lyase), and MutY (DNA glycosylase) in Escherichia coli. Inactivation of any of the genes of these three generates a mutator phenotype attributed to the persistence of 8-oxoG in DNA or in the pool of deoxynucleoside-triphosphates (11, 12). In human cells, the hOGG1 gene encodes a DNA glycosylase/AP lyase that catalyzes the removal of 8-oxoG and incises DNA at the resulting AP site. However, it was reported that the TCR of 8-oxoG was proficient in mOGG1 (−/−) cells, suggesting that the TCR of oxidative damage occurs independent of OGG1 (13).
Although the mechanism of TCR is unknown, the process is thought to be initiated by a blockage of transcription elongation by RNA polymerase II at DNA lesions (6, 23). Considering that 8-oxoG is neither a bulky nor a helix-distorting lesion (3), it is unlikely that it blocks RNA polymerase II. Moreover, it was reported that DNA polymerases preferentially insert an adenine residue opposite 8-oxoG, leading to A:8-oxoG mispairs (14, 15) and that E. coli or T7 RNA polymerase efficiently bypasses such a lesion without pausing or arresting during elongation by inserting either an adenine or a cytosine residue (16, 17). To assess the contribution of RNA polymerase II to the TCR of 8-oxoG in mammalian cells, we investigated whether the purified RNA polymerase II stalls at the AP site, uracil, and 8-oxoG on the transcribed strand during the elongation reaction.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Chemicals**—RNA polymerase II (pol II) was prepared from HeLa nuclear pellets as described (18). E. coli formamidopyrimidine-DNA glycosylase (Fpg) and human apurinic/apyrimidinic endonuclease (AP endonuclease) were purchased from Trevigen. Uracil DNA glycosylase (UDG) was obtained from USB. NTPs were from Amersham Biosciences, and restriction enzymes were from New England Biolabs and TOYOBO.

**Construction of DNA Templates Containing a Site-specific DNA Lesion**—The 24-mer oligodeoxyribonucleotides containing an AP site analogue (3-hydroxy-2-(hydroxymethyl)-tetrahydrofuran) (dSpacer), uracil DNA glycosylase (UDG) was obtained from USB. NTPs were from Amersham Biosciences, and restriction enzymes were from New England Biolabs and TOYOBO.

To generate a substrate suitable for transcription elongation by mammalian RNA pol II on a lesion, oligonucleotides containing a lesion were 5'-phosphorylated by T4 polynucleotide kinase using ATP and incorporated into covalently closed circular DNA as described (19). The plasmid pBlueScript II KS-GTG (pBSII KS-GTG) was constructed by replacing the Apol-Kpol fragment of pBlueScript II KS- with the synthetic 99-bp DNA duplex containing the damage site (Fig. 1A). The purity of each DNA substrate was assessed by agarose gel electrophoresis. To confirm the proper insertion of a lesion into pBSII KS-GTG, each DNA substrate was incubated with the indicated enzyme and analyzed by 1% agarose gel electrophoresis. For the elongation reaction by pol II, an oligo(dC)-tailed template was prepared as described (20) from the covalently closed circular DNA containing a site-specific DNA lesion. The pBSII KS-GTG containing a lesion at a specific site was digested with PolI, and poly(dC) tails of 35–40 nucleotides were added to the 3' ends by terminal deoxynucleotidyl transferase using dCTP. After digestion of the DNA with SmaI to generate two fragments, the DNA substrate fragment with a lesion was purified on an agarose gel and used as a template.

**RNA Polymerase II Elongation Reactions**—For the transcription elongation assay (21), 20 μl of reaction mixtures containing 50 ng of dC-tailed template and 0.5 μl of pol II in a buffer (10 mM HEPES (pH 7.9), 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 3.2% polyethylene glycol, 0.25 mM dithiothreitol, 6 mM MgCl₂, and 8 units RNase inhibitor (Promega)) were preincubated for 30 min at 30 °C. Elongation was started by adding 5 μl of NTP mixture (30 μM each of ATP, CTP, and GTP, 10 μM UTP, and 1 μCi [α-32P]UTP) and terminated by adding 100 μl of Stop buffer (7 M urea, 0.35 M NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 1% SDS) at the times indicated. The purified transcripts were resuspended in formamide loading dye (98% deionized formamide, 25 mM Tris borate-EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol) and separated on a denatured 6% polyacrylamide gel. The dried gels were analyzed using a FUJIFILM BAS 2500 bio-image analyzer.

**RT-PCR and RNA Sequencing**—RNA transcripts for sequence analysis were extracted from the mixture of elongation reactions in the presence of a cold NTP mixture (50 μM each of ATP, CTP, GTP, and UTP) with an RNA purification kit (Qiagen) and then treated with RNase-free DNase I (TAKARA) as recommended by the supplier. Purified RNAs were employed to synthesize PCR products with a One-step RT-PCR kit (Qiagen) or a cMaster RT-PCR system and RT kit (Eppendorf) and primers (751–770) 5'-GCCCTGTGGCCATGCGGG-3' and (1003–984) 5'-ACTCATTAGCCACACCCGAC-3'. The PCR products (253 bp) were purified with a PCR purification kit (Qiagen), analyzed by electrophoresis on a 2% agarose gel or on a 6% non-denatured polyacrylamide gel. The dried gels were analyzed using a FUJIFILM BAS 2500 bio-image analyzer.

**RESULTS**

**DNA Templates Containing DNA Lesions at Specific Sites**—To analyze the transcription elongation at DNA lesions (AP site, uracil, and 8-oxoG) by human RNA polymerase II, oligo(dC)-tailed templates were generated from purified closed circular duplex DNA substrates containing a lesion at a specific site (Fig. 1A). A 3054-bp substrate, designated pBSII KS-GTG, contains these DNA lesions within the recognition sequence (5'-GTGCAC-3') of the restriction enzyme AluI (Fig. 1A).
When control DNA substrate (with no damage) was digested with Alw44I, three DNA fragments (1246 bp, 1078 bp, and 730 bp) were observed (Fig. 1B, lanes 2). DNA substrates containing either an AP site or uracil were also completely digested by Alw44I (Fig. 1B, lanes 4 and 6), while DNA substrate with 8-oxoG was completely resistant to the cleavage (Fig. 1B, lane 8), indicating that ~100% of the substrate contained 8-oxoG lesions. When closed circular DNA substrates containing either uracil or 8-oxoG were incubated with DNA repair enzymes (uracil DNA glycosylase and HAP endonuclease, or Fpg glycosylase), those with the lesion were cleaved to an open circular form but the control substrate was not. The partial digestion of the template with DNA repair enzymes was due to an insufficient concentration of enzymes available from the supplier (Fig. 1, C and D). The AP site was not tested because the dSpacer, a (3-hydroxy-2-(hydroxymethyl)-tetrahydrofuran) that was used as an analogue of the AP site in the experiments, is known to be resistant to the nicking activity of AP lyase. These results, as well as those from the sequence analysis of RT-PCR products (Fig. 3C), indicate the presence of site-specific DNA damage in the DNA substrate.

RNA Polymerase II Elongation Reaction—Transcription elongation by pol II was carried out with oligo(dC)-tailed templates containing no damage (control template), AP site, uracil, or 8-oxoG. (The lesions were built on the template strand.) pol II could synthesize transcripts using the control template as expected (Fig. 2B, lanes 1 and 2). Using control template digested with either Alw44I or N.BstNBI, which partially generated double or single strand breaks under the conditions, pol II produced 129 nt (from the PstI site to the Alw44I site) and 101 nt (from the PstI site to the N.BstNBI site) transcripts (Fig. 2, A and B, lanes 3–6). When the template containing either the AP site or uracil was used, pol II could catalyze the synthesis of the transcript (Fig. 2B, lanes 7–10). On the other hand, using the template with 8-oxoG, pol II synthesized 128-nt transcripts from the PstI site to 8-oxoG (Fig. 2B, lanes 11 and 12, arrow) and bypassed the lesion as well (Fig. 2B, bracket), suggesting that some pol II stalled at the 8-oxoG site while some bypassed the lesion. When these templates containing a base lesion were incubated with T7 RNA polymerase (T7 RNA pol), instead of human pol II, T7 RNA Pol did not stall at the lesion (data not shown).

To confirm the pausing of 8-oxoG on transcription elongation by pol II at 8-oxoG, time course experiments were carried out (Fig. 2C), and the transcripts (shown by brackets in Fig. 2C) were quantified (Fig. 2D). In the case of the templates contain-
ing no damage, AP site, or uracil, pol II generated similar amounts of transcripts beyond the lesion in a time-dependent manner. In contrast, the amount of bypassed transcripts that was synthesized for 12 min by pol II using the oligo(dC) template containing 8-oxoG, was less than one-third that of the others. These results indicate that 8-oxoG lesions partly inhibited the transcription elongation by pol II.

**RT-PCR and RNA Sequencing**—To examine the nucleotide preference for incorporation opposite a lesion, we next investigated the sequences of DNA fragments produced by RT-PCR using bypassed transcription products. The RT-PCR products (253 bp) were observed only when a purified RNA fraction was incubated with RT (Fig. 3A, upper panel). Incubation of the RNA fraction with RNase I generated no RT-PCR products (Fig. 3A, lower panel). These results indicated that under our experimental conditions, the PCR product was generated by the transcripts but not by the template DNA. The RT-PCR products were digested with Alw44I. If there are mutations at a restriction site (GTGCAC) containing a lesion, PCR products from the transcripts should be resistant to cleavage. The results (Fig. 3B) showed that the PCR products from the uracil template were partially resistant, but the products derived from templates with no damage, AP site, or 8-oxoG were not resistant. Sequence analyses (Fig. 3C) indicated that in the transcripts from the template with no damage or AP site, no change of sequence was observed. In contrast, the uracil lesion partially generated G:C to A:T transitions. The results are consistent with the finding that UDG-deficient *E. coli* cells have more G:C to A:T transitions in DNA and mRNA (3, 17, 22). As for 8-oxoG, pol II inserted not only cytosine but also adenine opposite the lesion, although less adenine was incorporated than cytosine.

**Effects of Adding Nucleotides on pol II-stalled Transcription at 8-oxoG**—It is known that the 8-oxoG lesion is mutagenic because of its ambiguous pairing with cytosine and adenine (15, 16). To examine which pairing causes pol II to stall, the product of transcription stalled at the 8-oxoG site was quantified at different concentrations of CTP, ATP, or GTP. As the CTP concentration increased, the amount of transcript at pausing sites increased (Fig. 4B, lanes 1–3 and C, circle), whereas as the ATP concentration increased the amount decreased (Fig. 4B, lanes 5–7 and C, square). When the concentration of GTP or UTP was increased, no effect was observed (Fig. 4A, lanes 1 and 4, and data not shown). In addition, sequence analyses of the bypassed transcription products (Fig. 4D) revealed that G:C to T:A transversions were increased at high ATP concentrations, but decreased at high CTP concentrations (Fig. 4E and see the panel for 8-oxoG in Fig. 3C). Considering that the flanking sequence of the lesion is very rich in TC, high levels of ATP may indirectly improve read-through by facilitating elongation around the lesion. However, this is not the case because high levels of GTP did not affect the read-through. Although we did not directly examine the inserted nucleotide of the paused transcripts, our results indicate that pol II stalled at 8-oxoG lesions due to the insertion of a cytosine, while it bypassed the lesions by inserting adenine.

**DISCUSSION**

In this study, we found that elongating pol II can efficiently bypass AP sites by inserting cytosine residues and bypass uracil lesions by inserting either adenine or guanine residues. On the other hand, a significant fraction of pol II stalled at 8-oxoG lesions by inserting the correct base, while the rest bypassed the site using either correct or incorrect base insertions. The results are summarized in Table I.

AP sites are the most frequent DNA base lesions in cells and potentially genotoxic and mutagenic (1, 3). These lesions are generated by spontaneous depurination at guanine at an estimated rate of ~9000/day/genome in humans (24). Our results indicate that pol II could insert cytosine residues opposite AP sites without pausing. When depurination occurred at guanine, no mutant proteins were generated through a bypass elongation reaction at the AP site. The mechanism by which pol II inserts cytosine opposite AP sites without any Watson-Crick pairing information is unknown as yet. It is known that *E. coli* RNA or DNA polymerase exhibits the preferential insertion of adenine opposite AP sites (4, 30). Therefore, in mammals, bypass transcription at the AP site might play a role in maintaining genome integrity. However, we cannot rule out the possibility, due to the limitation of the sensitivity of our assay that pol II inserts bases other than cytosine at low frequency.

Uracil in DNA is frequently generated by the deamination of cytosine in a pH- and temperature-dependent manner (3, 24). Uracil can pair with adenine without blocking DNA replication and therefore is very mutagenic. In fact, UDG-deficient *E. coli*
shows a high frequency of spontaneous G:C to A:T transitions. However, the present study indicates that in transcription, pol II inserts guanine and adenine opposite uracil without blocking transcription elongation. *E. coli* RNA polymerase also can insert guanine and adenine opposite lesions in vitro (17). Thus, uracil in DNA would cause transcriptional infidelity and produce mutant proteins. In fact, it has been reported that the transcriptional bypass of uracil by *E. coli* RNA polymerase results in the production of mutant proteins in vivo (22). These results suggest that uracil has to be removed before pol II meets the lesion to avoid the production of a mutant protein. In mammalian cells, four types of uracil DNA glycosylase exist to remove uracil lesions (1). Although the exact role of each of these uracil DNA glycosylases in the cells is not known, it is likely that they remove uracil lesions on transcribed DNA and thus prevent transcriptional mutagenesis.
8-oxoG on the template strand of DNA directs the incorporation of non-cognate dAMP as well as dCMP through DNA replication leading to G:C to T:A transversions (14, 15). It has been shown that CS cells are deficient in the TCR of oxidative damage such as 8-oxoG and thymine glycol as well as TCR in NER. Moreover, the frequency of G:C to T:A transversions at 8-oxoG in CS cells was 30–40%, while that in normal cells was 1–4%. The results indicate that the unrepaired 8-oxoG blocked transcription by pol II in CS/XP-G cells but not in normal cells (9). Their results are consistent with the present findings that pol II stalled at 8-oxoG on the transcribed strand in the in vitro transcription elongation assay using oligo(dC) template. In addition, some pol II stalled at 8-oxoG lesions upon inserting cytosine, while others could bypass the site by inserting cytosine. What mechanisms are involved in the preference for either stalling or bypassing? 8-oxoG forms base pairs with both cytosine and adenine, depending on whether the base adopts a syn or an anti conformation about the glycosidic bond (25, 26). The syn conformations, which 8-oxoG favors, can pair with adenine, while the anti forms can pair with cytosine. It is speculated that when pol II inserts an adenine opposite the syn conformation or cytosine opposite the anti form it might be able to bypass the lesion because of the expected base pairing. However, when pol II inserts a cytosine opposite an 8-oxoG syn form, cytosine can not pair in the proper manner and pol II stalls at the lesion. Our results indicate that higher concentrations of adenine decreased the pausing, while higher concentrations of cytosine led to the stalling of pol II at the lesion, indicating that adenine can properly pair with 8-oxoG but cytosine can not (Fig. 4). These findings would support the idea that the syn conformation of 8-oxoG lesions blocks pol II elongation. However, in regard to the blocking of pol II by 8-oxoG, we cannot rule out the possibility that additional factors may be required to ensure the stalling of pol II at 8-oxoG (27). On the other hand, we obtained mutant products of bypassed transcription from the 8-oxoG templates as well as the uracil templates. It has been reported that MutT-deficient strains of E. coli that lack the sanitization of 8-oxo-dGTP, produced such mutant proteins (28, 29). These results suggest that mutant transcripts generated by bypass transcription produce mutant proteins. Since 8-oxoG is not repaired in CS cells, more mutant proteins might be produced in CS cells, which could contribute to the symptoms of CS.

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