Single-Stranded Breaks in DNA but Not Oxidative DNA Base Damages Block Transcriptional Elongation by RNA Polymerase II in HeLa Cell Nuclear Extracts*

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Transcription and repair of many DNA helix-distorting lesions such as cyclobutane pyrimidine dimers have been shown to be coupled in cells across phyla from bacteria to humans. The signal for transcription-coupled repair appears to be a stalled transcription complex at the lesion site. To determine whether oxidative DNA lesions can block correctly initiated human RNA polymerase II, we examined the effect of site-specifically introduced oxidative damages on transcription in HeLa cell nuclear extracts. We found that transcription was blocked by single-stranded breaks, common oxidative DNA lesions, when present in the transcribed strand of the transcription template. Cyclobutane pyrimidine dimers, which have been previously shown to block transcription both in vitro and in vivo, also blocked transcription in the HeLa cell nuclear transcription assay. In contrast, the oxidative DNA base lesions, 8-oxoguanine, 5-hydroxycytosine, and thymine glycol did not inhibit transcription, although pausing was observed with the thymine glycol lesion. Thus, DNA strand breaks but not oxidative DNA base damages blocked transcription by RNA polymerase II.

Transcription-coupled repair (TCR) is a specialized form of DNA repair where damages are repaired preferentially in the transcribed strand of actively transcribed genes (for reviews, see Refs. 1 and 2). TCR was originally believed to be a subpathway of nucleotide excision repair; however, ionizing radiation damage (3), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol; Tg) (4, 5), and 7,8-dihydro-8-oxoguanine (8-oxoG) (5) are removed in a TCR-dependent manner from human cells that lack nucleotide excision repair. Since Tg and 8-oxoG are small non-bulky lesions that are repaired primarily by the base excision repair (BER) pathway (for reviews, see Refs. 6–8), TCR of Tg and 8-oxoG in cells that lack nucleotide excision repair (5) links TCR to BER. TCR of 8-oxoG has also been shown to occur in nonreplicating Escherichia coli cells (9). TCR of oxidative damage does not appear to be universal, since in Chinese hamster ovary cells, TCR of oxidative damage produced by photosensitization and oxidizing agents is not observed in the Dhfr and cFos genes (10–12). Furthermore, DNA strand breaks, oxidative lesions also repaired by BER, do not appear to be repaired by TCR in the Dhfr gene from Chinese hamster ovary cells (13) or human colon cancer cells (14). Interestingly, recent measurements of TCR of cyclobutane pyrimidine dimers in the Hprt gene, integrated at different sites in Chinese hamster ovary cell chromosomes, have suggested that preferential repair of actively transcribed genes, as well as preferential repair of damages in the transcribed strand, is significantly affected by genomic context (15).

The proposed signal for TCR is an RNA polymerase transcription complex stalled at a lesion, which recruits the repair proteins to the damage site (16–18); the ability of a lesion on the transcribed strand to block the RNA polymerase transcription complex has been assumed to be crucial for TCR. In addition to bulky lesions such as cyclobutane pyrimidine dimers, DNA polymerases are blocked by a number of oxidative lesions including sites of base loss (AP sites), single-stranded breaks (SSBs), and Tg, which are thus potentially lethal lesions (for reviews, see Refs. 19–21). DNA polymerases bypass 5,6-dihydrothymine, 5-hydroxycytosine (5-OHC), 5-hydroxyuracil, 5,6-dihydroxy-5,6-dihydrouracil, dihydrouracil, 7,8-dihydro-8-oxoadenine, and 8-oxoG. The oxidized cytosine lesions and 8-oxoG are potentially mutagenic, since they can mispair (for reviews, see Refs. 19–22). In many cases, the effects of oxidative DNA damages on transcription by RNA polymerases differ from their effects on DNA polymerases. Tg is a block to T7 RNA polymerase (23–25). However, RNA polymerase II, partially purified from rat liver, completely bypassed Tg lesions in the transcribed strand located downstream from the adenovirus major late promoter, and the addition of fractions containing transcription factor II D and transcription factor II H (TFIIH) did not have any measurable effect (24). As with DNA polymerases, 8-oxoG does not block T7 (23, 25) or E. coli RNA polymerase in vitro (26) or in cells (9) and at best stalls RNA polymerase II in vitro with a template containing a poly(C) tail (27). In contrast to DNA polymerases, abasic sites in a poly(C)-tailed template are easily bypassed by RNA polymerase II (27); abasic sites are also bypassed by T7 RNA polymerase (19, 25), SP6 (28), and E. coli (28) RNA polymerase. A single-stranded break is a common oxidative lesion produced directly by free radicals and as BER processing intermediates. Depending on
the 5'- and 3'-end chemistries at the strand break site, single-stranded breaks in the transcribed strand can block transcription by SP6, Escherichia coli, and T7 RNA polymerase. T7 RNA polymerase bypasses an SSB with an intact deoxyribose at the 3' terminus and a hydroxyl group on the 5' terminus (28, 29), whereas a single nucleotide gap with terminal 3'- and 5'-phosphoryl groups blocks transcription by SP6, E. coli, and T7 RNA polymerase (28, 29). When gaps in the transcribed strand are bypassed by prokaryotic RNA polymerases, the corresponding transcripts are shortened by the length of the gap (30, 31).

Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are two rare human hereditary disorders that are caused by defective DNA repair (for a review, see Ref. 32). Xeroderma pigmentosum patients lack nucleotide excision repair and are cancer-prone, whereas Cockayne syndrome patients retain nucleotide excision repair and are not cancer-prone (32). Patients with Cockayne syndrome can be divided into two complementation groups, CS-A or CS-B (33, 34). The gene products associated with these complementation groups are required for TCR (3, 35). Although CSA and CSB do not form stable complexes (36), they interact with each other and the human RNA polymerase II transcription factor TFIIH (37) and function in the TCR process. Cockayne syndrome cells have defects in the TCR of oxidative lesions (3–5). Some xeroderma pigmentosum patients from the XP-B, XP-D, and XP-G complementation groups also exhibit the neurological and developmental deficiencies associated with Cockayne syndrome compounded by the sun sensitivity and skin cancer susceptibility associated with xeroderma pigmentosum. The XBP and XPD gene products are helicase components of TFIIH and interact with three other TFIIH subunits, p62, p44, and p34 (38). XPG interacts with multiple subunits of TFIIH and CSB (38) and is required for TCR as well as global genomic repair of Tg (4) and 8-oxoG (5).

TCR of oxidative damages in mammals is paradoxical, since the proposed signal for TCR is a stalled RNA polymerase complex, but most oxidative DNA damages do not appear to block purified RNA polymerases. In this study, we asked whether oxidative DNA lesions block transcription by correctly initiated RNA polymerase II and thus serve as the signal for TCR. To address this, we carried out in vitro transcription assays with HeLa cell nuclear extracts, commonly used for transcription studies, and DNA templates containing oxidative damages placed downstream from the HIV-1 promoter and determined whether the damaged templates blocked transcription in a nuclear run-off assay. We also assumed that any constitutive activities essential to block transcription and signal TCR would be present in the extracts. In order to determine whether oxidative damages could block RNA polymerase II, specific oxidative lesions and a cyclobutane pyrimidine dimer known to block transcription (39) were inserted into the transcribed strand of the template at a defined distance from the transcription start site. A thymine-thymine dimer was used as the representative cyclobutane pyrimidine dimer lesion in our study. We found that correctly initiated RNA polymerase II from HeLa cell nuclear extracts was blocked by site-specifically introduced single-stranded breaks. As expected, a site-specific cyclobutane pyrimidine dimer also blocked transcription. In contrast, the oxidized base lesions tested, 8-oxoG, 5-OHC, and Tg, did not block RNA polymerase II. Some of the 5-OHC and Tg lesions were, however, converted to single-stranded breaks during the transcription assay, which then blocked transcription at these sites. In addition, RNA polymerase II initially paused at Tg, but eventually most of the transcription complexes bypassed this damage.
0.5% SDS, 2 mM EDTA, and 3 μg/ml tRNA. The terminated transcription reactions were extracted with an equal volume of phenol/chloroform, mixed on a vortexer at maximum speed for 10 s, and centrifuged for 5 min at 12,000 × g. The aqueous phase was transferred to a new tube containing 15 μg of glycoBlue (Ambion), and 500 μl of ethanol was added. The samples were incubated for at least 30 min at −20 °C and then centrifuged at 12,000 × g for 10 min. The supernatant was decanted, and the pellets were air-dried and suspended in 4 μl of nucleoside-free water followed by the addition of an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). The samples were heated in a boiling water bath for 5–10 min, separated on a 6% (w/v) polyacrylamide sequencing gel, and visualized by autoradiography and/or analysis with an isotope imaging system (Molecular Imaging FX System, Bio-Rad).

**Determination of BER Activities in HeLa Cell Nuclear Extracts**—Oligonucleotides containing a furan, dihydrouracil (DHU), and 8-oxoG, 30 min in the case of the Tg-containing template. The nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation. The nucleic acids were suspended in distilled H2O, and the template was restricted with EcoRI in 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT (pH 7.9 at 25 °C) and purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) to remove the restriction enzyme and exchange the buffer to 10 mM Tris, pH 8.0.

To assay for the presence of the lesion, 2.5 fmol of template/substrate was incubated with 60 fmol of the appropriate enzyme for 60 min at 37 °C with 5% (v/v) glycerol (Ambion). The oligonucleotide containing the CPD dimer was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and annealed to its complementary strand. The double-stranded CPD dimer substrate was incubated with T4 endonuclease V ( Trevigen, Gaithersburg, MD) at 37 °C in 25 mM NaPO4 (pH 6.8), 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.1 mg/ml bovine serum albumin for 60 min. All reactions were terminated by the addition of an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). All samples, except the AP site-containing DNA, were heated in a boiling water bath for 5 min before subsequent separation on a 12% (w/v) polyacrylamide, 7 M urea sequencing gel. The results were visualized and quantified by autoradiography and/or analysis on an isotope imaging system (Molecular Imaging FX System, Bio-Rad).

**RESULTS**

**RNA Polymerase II Transcription Is Blocked by Site-specific Single-stranded Breaks in the Template Strand**—To determine what effect SSBs had on transcription by RNA polymerase II, we carried out run-off transcription assays with HeLa cell nuclear extracts on templates containing SSBs with different end chemistries located on the transcribed strand downstream from the HIV-1 promoter. For all transcription reactions, the extent of lesion bypass was calculated as the amount of full-length transcript divided by the amount of full-length transcript plus blocked transcript and quantified using an isotope imaging system. The number of radioactive nucleotides incorporated into the transcript was taken into account, since the difference in the number of radioactive nucleotides incorporated between the run-off transcript and a transcript blocked at the lesion was significant (~7% in the case of the 54-mer damage-containing oligonucleotides and ~9% in the case of the 71-mer damage-containing oligonucleotides). All SSBs tested inhibited transcription by RNA polymerase II (Fig. 2). It should be noted that all the SSBs examined here are actually single base gaps where the gaps have the end chemistries described and are intermediates in the BER process. An SSB with a 3'-unsaturated aldehyde and a 5'-phosphate caused almost 90% blockage between 2 and 10 min from transcript initiation (Fig. 2, lanes 2–4, and Table II). An SSB with a 3'-hydroxyl and a 5'-deoxyribose demonstrated ~90% blockage between 2 and 10 min (Fig. 2, lanes 6–8, and Table II), whereas an SSB with a 3'-phosphate and a 5'-phosphate showed about 85% blockage between 2 and 10 min (Fig. 2, lanes 10–12, and Table II). Finally, an SSB with a 3'-hydroxyl and a 5'-phosphate demonstrated just over 75% blockage at 2 min that decreased to ~65% at 10 min (Fig. 2, lanes 14–16, and Table II). The majority of...
transcripts had a length of ~364 nucleotides (nt), which corresponds to the position of the SSB. The transcription template containing an SSB with a 3'-hydroxyl and 5'-deoxyribose produced an additional transcript at ~344 nt (Fig. 2, lanes 6–8). Some transcripts corresponding to full length were produced from all of the SSB-containing transcription templates, due to a small fraction of the template that did not contain a SSB as well as possible repair of the SSB by the nuclear extract. In fact, there were 10–20% fewer SSB in the transcription templates after the assay (see Table I), suggesting that some repair did occur (see below). Some full-length transcripts may reflect lesion bypass and presumably would be 1 nt shorter than a full-length transcript. The limited resolution of the 6% polyacrylamide gel used in these assays did not allow us to discern whether the longest transcripts are actually full-length or 1 nt short of full-length as a consequence of single nucleotide gap bypass in the transcribed strand. At 10 min, the SSB with a 3'-unsaturated aldehyde and a 5'-phosphate showed little bypass (Fig. 2, lane 4, and Table II), whereas the SSBs with a 3'-hydroxyl and 5'-deoxyribose (Fig. 2, lane 8) or a 3'-phosphate and a 5'-phosphate (Fig. 2, lane 12) showed about 20% bypass (Table II). Only the SSB with a 3'-hydroxyl and a 5'-phosphate showed significant bypass, about 35% at 10 min (Fig. 2, lane 16, and Table II). In summary, the SSBs with different end chemistries were strong blocks to transcriptional elongation, although some bypass occurred with an SSB containing a 3'-OH and 5'-P. Also, analysis of the template after transcription (see below and Table I) suggests that some SSB repair was taking place during the transcription reaction.

Base Excision Repair in HeLa Cell Nuclear Extracts—Because the oligonucleotides containing strand breaks (and sites of base loss (AP sites)) were prepared by enzymatic treatment of a site-specific uracil and uracil is a nondistorting base, the uracil-containing oligonucleotide was initially used as a transcription template control. However, a uracil in the transcribed strand caused significant termination of the transcripts at the position of the uracil (Fig. 3A, lane 5), whereas thymine at the same position did not result in termination of transcription (Fig. 3A, lane 1). We hypothesized that in the nuclear extracts uracil was converted to an AP site by uracil DNA glycosylase and subsequently to a transcription-blocking SSB by an AP endonuclease. To test this idea, different concentrations of uracil DNA glycosylase inhibitor (UGI; New England Biolabs) were added to the transcription reactions containing either uracil (Fig. 3A, lanes 6–8) or thymine in the transcribed strand (Fig. 3A, lanes 2–4). The addition of UGI to the uracil-containing template obviated the block at the uracil site and resulted in transcriptional bypass (Fig. 3A, lanes 6–8). An AP site, which can form spontaneously or as a result of a DNA glycosylase activity, was also tested in the transcription reaction. AP sites also resulted in transcription termination at the lesion site (Fig. 3B, lanes 3–5), presumably because it was converted to a strand break (see Table I) by an AP endonuclease in the extract (see below).

The above results demonstrated that at least two base excision repair enzymes in the HeLa cell nuclear extracts were capable of interacting with the transcription template containing DNA damages. Since we were trying to determine the effect of specific lesions on transcription by RNA polymerase II, it was important to know if the lesions under investigation were processed by the HeLa cell nuclear extracts during the transcription assay. To assess BER activity in the extracts, incision of three representative lesion-containing DNA substrates was measured under conditions optimized for cleavage (Fig. 4, lanes 1–12). The production of a strand break at the site of the lesion by an oxidative DNA glycosylase or an AP endonuclease is the first step in BER. Furan, an AP site analog, is a substrate for hAPE1; DHU is a substrate for hNTH1, hNEIL1, and hNEIL2; and 8-oxoguanine is a substrate for hOGG1 (for reviews, see Refs. 6, 7, and 45). Significant activity was detected with each of the representative substrates determined under optimal conditions for cleavage (Fig. 4, lanes 1–12). Fifty-fold more protein (1.82 μg) was needed to cleave the DHU-containing substrate, and 100-fold more protein (3.64 μg) was needed to cleave the 8-oxoG-containing substrate compared with the amount of protein (36.4 ng) used with the furan-containing substrate. The same assay was performed using the buffer conditions for transcription (Fig. 4, lanes 16–27). Compared with optimal cleavage conditions, incision activity on the 35-base pair substrates under transcription conditions was much lower. Activity on a furan-containing substrate was significantly less (Fig. 4, compare lanes 16–19 with lanes 1–4), substantially reduced with a DHU-containing substrate (Fig. 4, compare lanes 20–23 with lanes 5–8), and barely detectable with the 8-oxoG-containing substrate (Fig. 4, compare lanes 24–27 with lanes 9–12). Furthermore, most lesion-containing templates were exposed to the HeLa cell nuclear extract during the transcription assay for only 15 min. At 15 min, there was some activity against the furan-containing substrate (Fig. 4, lane 18); however, the activity against the DHU-containing substrate was very low (Fig. 4, lane 22) and activity on the 8-oxoG-containing substrate (Fig. 4, lane 26) was negligible.

The results shown in Fig. 4, lanes 1–12 and 16–27, are representative of the initial steps in global base excision repair, since the DNA damages were on substrates that were not undergoing transcription. The DNA templates used in our transcription assays were undergoing transcription, and thus it was possible that the lesions tested could be repaired in a transcription-coupled manner. Since we showed that BER enzymes are present in the extracts used for transcription, the templates containing the oxidative lesions were examined before and after the transcription assay to determine whether the damages were present after transcription (i.e. were the lesions still there, or had they been repaired?). Although all templates used in this study were examined, only the results from an AP site, SSBs, 8-oxoG, and thymine glycol (Fig. 5, A–D) are shown, since these lesions are representative DNA damages and are good substrates for BER enzymes. To quantify the amount of the lesion in the transcription templates, they were 5'-end-labeled with γ-32P by T4 polynucleotide kinase and restricted with EcoRI so that the 65-nt end-labeled lesion-containing fragment (substrate) could be separated by polyacrylamide gel...
Electrophoresis from the remaining ~935-base pair fragment containing the HIV-1 promoter. The restricted transcription templates were then used as substrates in vitro for enzymes that cleaved DNA at the site of the lesion. There appeared to be an enzymatic activity present in the HeLa cell nuclear extracts that removed the radioactive phosphate label from the 5′-end of the substrate as indicated by a lower radioactivity signal in the samples that were isolated after incubation in the nuclear extracts (Fig. 5A, compare lanes 1 and 2, lanes 3 and 4; Fig. 5B, compare lanes 1 and 2, lanes 3 and 4, lanes 5 and 6, and lanes 7 and 8; Fig. 5C, compare lanes 1 and 2, lanes 3 and 4; Fig. 5D, compare lanes 1 and 2 and lane 3 with lanes 4–6).

Templates containing AP sites were relatively intact before the transcription assay (Fig. 5A, lane 1), and virtually all the template contained AP sites as evidenced by its nearly complete conversion by endonuclease III (Fig. 5A, lane 3). The small amount of cleavage observed in the untreated sample (Fig. 5A, lane 1) probably occurred during sample preparation and/or electrophoresis. Nearly all of the AP sites were converted to SSBs by the end of the 10-min transcription reaction with the nuclear extract, presumably by the activity of hAPE1 in the extract (Fig. 5A, lane 2, Table I). The smallest bands observed in lanes 2–4 may be the result of a small fraction of the original uracil-containing oligonucleotide that was 70 nucleotides (n – 1) instead of 71 nucleotides in length. To assess whether the AP site was repaired by the nuclear extract during transcription, the transcription template was isolated after transcription, and the fraction of template containing an AP site was quantified by its susceptibility to cleavage (Fig. 5A, lane 4). Nearly the same amount of cleavage was observed after transcription as before transcription (Fig. 5A, compare lanes 3 and 4) showing that the AP site-containing template was not repaired after the cleavage step during transcription (see Table I).

The single-stranded break-containing templates were also evaluated in a similar manner. Before transcription, about 90% of all the templates containing SSBs with different end chemistries contained the SSB in question (Fig. 5B, lanes 1, 3, 5, and

| Lesion                  | Lesion in template before assay | Lesion in template after assay | Intact transcription template before assay | Intact transcription template after assay |
|-------------------------|---------------------------------|---------------------------------|-------------------------------------------|------------------------------------------|
| Abasic site             | 95                              | 13                              | 95                                        | 14                                       |
| SSB; 3′-sugar, 5′-P     | 90                              | 80                              |                                           |                                          |
| SSB; 3′-OH, 5′-sugar    | 90                              | 75                              |                                           |                                          |
| SSB; 3′-P, 5′-P         | 90                              | 70                              |                                           |                                          |
| SSB; 3′-OH, 5′-P        | 90                              | 70                              |                                           |                                          |
| TT dimer                | 83a                            |                                 |                                           |                                          |
| 8-oxoG                  | 92                              | 91                              | 96                                        | 95                                       |
| 5-OHC                   | 91                              | 95                              | 97                                        | 75                                       |
| Tg                      | 91                              | 82                              | 98                                        | 81                                       |
| Tg, α-amanitin          | 91                              | 76                              | 91                                        | 61                                       |

* a 83% in oligonucleotide; the percentage of TT dimer in template after the assay was not quantified.

**Table II**

Quantification of transcripts paused or blocked at specific lesions at 2 and 10 min after transcription initiation corrected for length of run-off transcript versus truncated transcript.

| Lesion                  | Transcripts initially paused or blocked at lesion (2 min) | Transcripts paused or blocked at lesion at the end of assay (10 min) |
|-------------------------|---------------------------------------------------------|------------------------------------------------------------------|
|                         | %            | %                      | %                        | %                      |
| SSB; 3′-sugar, 5′-P     | 88           | 90                     | 88                       | 100                    |
| SSB; 3′-OH, 5′-sugar    | 81           | 81                     | 81                       | 81                     |
| SSB; 3′-P, 5′-P         | 83           | 85                     | 83                       | 85                     |
| SSB; 3′-OH, 5′-P        | 76           | 65                     | 76                       | 65                     |
| TT dimer                | 100          | 100                    | 100                      | 100                    |
| 8-oxoG                  | 11           | 6*                     | 11                       | 6*                     |
| 5-OHC                   | 31           | 2*                     | 31                       | 2*                     |
| Tg                      | 76           | 9*                     | 76                       | 9*                     |

* a Normalized to percentage of lesion in template before transcription.

* Taking into account single-stranded break formation during assay.

* d 83% in oligonucleotide; transcription template not assayed.
Fig. 4. Enzymatic activity of HeLa cell nuclear extracts on substrates containing a furan (F), DHU, and 8-oxoG. Cleavage of oligonucleotides containing representative oxidative lesions by the nuclear extract without transcription are shown under conditions optimized for glycosylase activity (lanes 1–12) and under in vitro transcription conditions (lanes 16–27). Lane 13, C:F incubated with endonuclease IV; lane 14, G:DHU incubated with endonuclease III; lane 15, C:8-oxoG incubated with hOGG1. The locations of the substrate (35 nt) and glycosylase cleavage products DHU (19 nt), 8-oxoG (17 nt), and furan (13 nt) are indicated.

7, and Table I). After the transcription assay, there was an apparent decrease in the level of SSBs to about 80% in the case of an SSB with a 3'-unsaturated aldehyde and a 5'-phosphate (Fig. 5B, lane 2, and Table I), 75% in the case of an SSB with a 3'-hydroxyl and 5'-deoxyribose (Fig. 5B, lane 4, and Table I), and 70% in the cases of an SSB with a 3'-phosphate and 5'-phosphate (Fig. 5B, lane 6, and Table I) and an SSB with a 3'-hydroxyl and 5'-phosphate (Fig. 5B, lane 8, and Table I).

There are two product bands present after glycosylase treatment, because the original uracil substrate contained the full-length 71-mer contaminated with some 70-mer. In summary, there were 10–20% fewer SSBs in the transcription templates after transcription than before (Table I). Thus, a small fraction of the SSBs with different end chemistries may have been repaired by the nuclear extracts during the 15 min that the transcription template was in contact with HeLa cell nuclear extract and could account for at least some of the bypass observed during the transcription reaction (Fig. 2).

The 8-oxoG-containing template was also assayed in the same way as the AP site-containing transcription templates. This transcription template contained greater than 90% 8-oxoG before transcription (Fig. 5C, lane 1, and Table I), and nearly the same amount remained intact after the transcription assay (Fig. 5C, lane 2, and Table I), indicating that 8-oxoG was not converted to an SSB during transcription. Incubation of the template before and after transcription with formamidopyrimidine DNA glycosylase, a glycosylase that recognizes and cleaves DNA at a C:8-oxoG base pair, showed that more than 90% of the template contained 8-oxoG before (Fig. 5C, lane 3, and Table I) and after the transcription reaction (Fig. 5C, lane 4, and Table I). Thus, 8-oxoG was not cleaved by the extract and was not repaired during transcription (Table I).

Comparison of the Tg-containing template before (Fig. 5D, lane 1) and after (Fig. 5D, lane 2) the transcription assay shows that about 20% of the Tg was converted to an SSB by the nuclear extract by the end of the 10-min transcription reaction (Table I). Similar amounts of SSB formation were observed with the 5-hydroxycytosine template (Table I). Incubation of the thymine glycol-containing template with endonuclease III before the transcription assay showed that about 90% of the template was cleaved and thus contained Tg (Fig. 5D, lane 3, and Table I). After the transcription assay, ~80% of the Tg-containing template was cleaved by endonuclease III (Fig. 5D, lane 4, and Table I), implying that some of the Tg-containing template may have been repaired by the nuclear extract during transcription. Interestingly, when α-amanitin was added to the transcription reaction to prevent transcription, almost 40% of the Tg was converted to a SSB (Fig. 5D, lane 5, and Table I) by the end of the 10-min transcription assay. Endonuclease III digestion of the Tg-containing template after transcription in the presence of α-amanitin showed nearly the same amount of cleavage as observed under transcription conditions (see Table I), suggesting that if repair was occurring, it was not likely to be transcription-coupled. In fact, fewer strand breaks were observed in the presence of transcription (Table I), perhaps because the template was protected from cleavage by the RNA polymerase complex.

Cyclobutane Pyrimidine Dimers Block RNA Polymerase II in HeLa Cell Nuclear Extracts—Cyclobutane pyrimidine dimers are a common ultraviolet light-induced DNA lesion that are repaired by the nucleotide excision repair system and are a known block to RNA polymerase II in vitro and in cells (39). Fig. 6A shows that, as expected, the thymine-thymine dimer is a blocking lesion to transcription in HeLa cell nuclear extracts. The small fraction of transcripts that bypass the lesion, less than 20% (Fig. 6A, lanes 4 and 5), is directly correlated with the fraction of template that does not contain the lesion as determined by T4 endonuclease V treatment (see Table I).

Oxidized DNA Bases Do Not Block RNA Polymerase II in HeLa Cell Nuclear Extracts—8-Oxoguanine is a common oxidative DNA lesion that does not block DNA polymerases or prokaryotic RNA polymerases (19, 21, 23, 25, 26). 8-Oxoguanine is a premutagenic lesion due to its ability to pair with an adenine during replication, which results in a G → T transversion (for reviews see Refs. 19–22). Fig. 6B shows that the transcript pattern observed with 8-oxoG is similar to that of the control C:G pair, with a vast majority of the product being run-off transcripts. The 8-oxoG was not converted to a SSB or repaired by the HeLa cell nuclear extract during transcription (Fig. 5C, Table I). The results of the transcription assay taken together with the glycosylase data show that 8-oxoG is not a transcription-blocking lesion to correctly initiated RNA polymerase II in HeLa cell nuclear extracts (Table II).

Oxidized cytosine lesions do not distort the DNA helix and do not block DNA polymerases, T7 RNA polymerase (19, 20, 23), or in the case of dihydrouracil E. coli RNA polymerase (46). Damages derived from cytosine are highly mutagenic, giving rise to C → T transitions (19–22). 5-Hydroxycytosine was chosen to represent the nonhelix distorting pyrimidine lesions in the transcription assay. The majority of the transcripts produced with the 5-OHC-containing template were full-length; however, some shorter transcripts (364 nt), which correspond to the site of the lesion, were also produced (Fig. 6C). The amount of the shorter transcripts correlates with the fraction of 5-OHC converted to an SSB by the HeLa cell nuclear extract during transcription (Table I). Thus, 5-OHC, a non-distorting pyrimidine lesion, does not block transcriptional elongation by RNA polymerase II.

Thymine glycol, like 5-OHC, is a common oxidized DNA base.
In contrast to 5-OHC, Tg distorts the DNA helix and blocks both DNA polymerases and T7 RNA polymerase (19, 21, 23). However, Tg does not block purified rat liver RNA polymerase II (24). Fig. 6D shows that Tg in the transcribed strand initially blocked transcription or caused the RNA transcription complex to pause (Fig. 6D, lane 3), but after 10 min of transcription the majority of the transcripts produced were full-length (Fig. 6D, lane 5). Additional time points at 20 and 30 min after transcription initiation did not show an increase in the fraction of run-off transcripts compared with the 10-min time point (data not shown). Interestingly, under in vitro transcription conditions, where transcription was prevented by the addition of α-amanitin, approximately twice the amount of the Tg template was converted to an SSB at Tg as compared to transcription conditions (Fig. 5D, compare lanes 2 and 5, Table I). Also, as shown in Fig. 5D, some repair of Tg may be taking place over...
FIG. 6. A, CPD dimer in the transcribed strand blocks transcription by RNA polymerase II from HeLa cell nuclear extracts. Lane 1, A:T control; lanes 2–5, template containing CPD dimer (1-, 2-, 5-, and 10-min transcription). The locations of the run-off transcript and lesion site are indicated.

B, 8-oxoG in the transcribed strand does not block transcription by RNA polymerase II from HeLa cell nuclear extracts. Lane 1, C:G control; lanes 2–5, template containing C:8-oxoG (1-, 2-, 5-, and 10-min transcription). The locations of the run-off transcript and lesion site are indicated.

C, 5-OHC in the transcribed strand does not block transcription by RNA polymerase II from HeLa cell nuclear extracts. Lane 1, G:C control; lanes 2–5, template containing G:5-OHC (1-, 2-, 5-, and 10-min transcription). The locations of the run-off transcript and lesion site are indicated.

D, Tg in the transcribed strand causes pausing but does not block transcription by RNA polymerase II from HeLa cell nuclear extracts. Lane 1, A:T control; lanes 2–5, template containing A:Tg (1-, 2-, 5-, and 10-min transcription, respectively). The locations of the run-off transcript and lesion site are indicated.

the course of the reaction, but this did not appear to be coupled to transcription, since approximately the same amount of endonuclease III-induced cleavage of the template occurred in the presence and absence of transcription (Fig. 5D, compare lanes 4 and 6, Table I). Taken together, the analysis of correctly initiated RNA polymerase transcription past a Tg lesion suggests that the RNA polymerase initially stalls at the lesion site but eventually bypasses the Tg lesion (Table II); some of this bypass may be accounted for by repair of the lesion.

DISCUSSION

When uracil, sites of base loss (AP sites), and single-stranded breaks located at a particular site in the transcribed strand were examined, the uracil and AP sites were quickly converted to SSBs by the extract (Figs. 3A and 5A, respectively). Therefore, we were unable to replicate the recent transcription elongation study with purified RNA polymerase II and oligo(dC)-tailed templates, which showed that purified RNA polymerase II could bypass uracil and AP sites on the transcribed strand (27). However, the SSBs with various physiologically relevant 5′- and 3′-end chemistries did block transcription by RNA polymerase II (Fig. 2 and Table II). This was interesting, since it had been shown that T7 and SP6 RNA polymerase and E. coli RNA polymerase to a lesser extent, were capable of bypassing gaps in the DNA template (28, 30, 31). A number of the defined single base gaps examined (28) did block E. coli RNA polymerase, and SSBs with 3′- and 5′-phosphate groups block even T7 RNA polymerase. Despite the fact that SSBs are effective blocks to transcriptional elongation, repair of SSBs is not coupled to transcription in Chinese hamster ovary cells (13) or in a human colon cancer cell line (14). TCR of DNA from x-irradiated human cells has been demonstrated using an antibody to 5-bromo-2-deoxyuridine in repair patches, and some of these repair patches could have resulted from SSB repair (3–5).

The reason that transcription-coupled repair may not be observed with SSBs although they block transcription may be due to both the abundance of repair proteins that process strand breaks, such as poly(ADP-ribose)/polymerase, XRCC1, and polynucleotide kinase (47, 48), and their demonstrated rapid global repair (49).

Initially, the thymine glycol lesion on the transcribed strand caused the RNA polymerase II transcription complex to stall, but eventually the Tg lesion was bypassed (Fig. 6D, Table II) with the possibility that some repair had taken place (Fig. 5D, Table I). The observation that Tg bypass increased from 25 to 90% over the course of the transcription reaction could be due to the activity of protein factors promoting lesion bypass, such as transcription elongation factor SII. The function of transcription elongation factor SII is to facilitate bypass of potential road blocks to transcription by associating with the transcription complex and activating a cryptic nascent transcript cleavage activity found within the RNA polymerase II transcription complex (for a review, see Ref. 50). Transcription elongation factor SII was shown to induce transcript cleavage by RNA polymerase II arrested at a cyclobutane pyrimidine dimer; the 3′-end of the transcript was cleaved, whereas the transcription complex containing the polymerase, DNA template, and nascent transcript was stable and capable of resuming transcription. However, the cyclobutane pyrimidine dimer was not
bypassed, since, unlike Tg, it is a permanent block to transcription (39). It is possible that transcription elongation factor II functioned in a similar manner at the Tg lesion in our transcription template; however, we did not observe any shortened transcripts. Interestingly, when an inhibitor of RNA polymerase II, α-amanitin, was added to the transcription reaction with the Tg template, we observed approximately twice the amount of SSB formation compared with transcription conditions (Fig. 5D; compare lanes 2 and 5, Table 1). It is possible that during transcription, the RNA polymerase II complex stalled at Tg shields the lesion from the base excision repair enzymes that recognize Tg and convert it to an SSB. It has previously been shown that the conformation of RNA polymerase II arrested at a cyclobutane pyrimidine dimer lesion prevents photolyase from accessing that lesion (39).

In contrast to cyclobutane pyrimidine dimer lesions (Fig. 6A), none of the oxidative base lesions examined, 8-oxoguanine, 5-hydroxycytosine, or thymine glycol, blocked RNA polymerase II (Fig. 6, B-D, respectively), although, as discussed above, Tg caused initial pausing of the RNA polymerase II transcription complex at the lesion. The ability of the RNA polymerase II transcription complex to bypass all of these oxidative lesions in human cell extracts was unexpected, since TCR of Tg and 8-oxoG had been demonstrated in human cells. Furthermore, 8-oxoG in the transcribed strand of a plasmid transfected into a human cell line was shown to block transcription, whereas 8-oxoG in the nontranscribed strand did not (5). An in vitro transcription elongation study with purified RNA polymerase II and oligo(dC)-tailed templates showed that 8-oxoG in the transcribed strand caused RNA polymerase II to pause during transcription (27), and a recent study2 with correctly initiated RNA polymerase II using purified enzyme with accessory factors, HeLa cell extracts, or transfected mouse embryonic fibroblast cells demonstrated 95% or more bypass of 8-oxoG. Partially purified RNA polymerase II also readily bypasses Tg (24). It is difficult to reconcile the in vitro studies with the cellular studies demonstrating TCR of oxidative damage. It is possible that there are technical idiosyncrasies such as the time frame of the measurements, the presence of particular TCR-coupling proteins, or the sequence context of the lesions.

A number of cellular factors have been shown to be involved with or required for TCR of oxidative damage. XPG and the Saccharomyces cerevisiae ortholog Rad2 are required for nucleotide excision repair, but XPG also appears to play an important role in TCR of oxidative damage (5), and S. cerevisiae Rad2 mutants exhibit general transcription defects (51). Although XPG protein is a structure-specific endonuclease required for nucleotide excision repair (52), its function in base excision repair-mediated TCR has not been elucidated. If XPG were limiting in the HeLa cell nuclear extracts and required for RNA polymerase blockage, this could account for the lack of transcription blockage at the lesion site. However, this does not appear to be the case, since we have added XPG to our transcription assays with 8-oxoG, 5-OHC, and Tg at 1:1 and 1:10 molar ratios of template to XPG and observed no effect on transcription (data not shown). TFIIH has also been shown to be required for TCR of oxidative damage by the BER pathway (5), but TFIIH is probably not limiting, since transcription was proficient in the HeLa cell nuclear extracts. Another possible candidate is hMSH2, which is required for mismatch repair as well as TCR of Tg; hMLH1 does not appear to be required for oxidative TCR and UV damage (53). In yeast, all mismatch repair proteins participate in TCR of oxidative damage but not UV damage (53, 54). The role of mismatch repair proteins in TCR of oxidative damage is not understood, but it is possible that they could provide strand discrimination and/or act as a general sensor for DNA damage. Again, the mismatch repair proteins should be present in the HeLa cell nuclear extracts used here and in the mouse embryo fibroblast cells used in the Larsen et al. study.2

TCR, including TCR of oxidative DNA damage, requires CSB and is assisted by CSA (5); mutant cells are sensitive to UV light (55) and ionizing radiation (5). Deletion of the yeast homolog of CSB, RAD26, also results in TCR defects (56) and a general transcription defect (51). Interestingly, CSB/RAD26 are members of the SNF2 protein family and contain DNA-dependent ATPase activity (57). Members of the SWI/SNF family can remodel chromatin in addition to recombining CSB protein, which has been shown to remodel chromatin and stimulate transcription enabling RNA polymerase II to add an extra nucleotide when stalled at a transcription-blocked lesion (58, 59). A major difference between the in vitro studies and the cellular studies showing TCR of oxidative damage is that the in vitro studies have used DNA lesions embedded in naked DNA, whereas in some of the cellular studies, TCR of oxidative damage takes place in chromatin. A particularly pleasing model would invoke the SNF2-like ATPase activity of CSB to remodel chromatin to not only allow efficient transcription but provide access for the ubiquitous housekeeping BER proteins, albeit in a strand-specific manner. In this model, the oxidative damages may not need to block transcription elongation for a TCR effect to be observed. Recent observations from Chambon’s laboratory (60) showed that thymine DNA glycosylase, which removes thymine and uracil from G:T and G:U mismatches, associates with transcriptional coactivators CBP/p300 acetylase, resulting in a complex that is competent for both the excision step of BER and histone acetylation. G:T and G:U mismatches are not known to be blocks to transcription. CBP/p300 are believed to activate transcription through chromatin remodeling via their intrinsic histone acetyltransferase activity in addition to their interactions with the basal transcription machinery (61, 62), thus providing a link between chromatin remodeling, transcription, and BER although no basis for strand bias. Chromatin remodeling has also been shown to be required for repair of certain DNA lesions (63). However, chromatin remodeling cannot explain the results in E. coli (9), which demonstrated transcription-coupled repair of 8-oxoG but no transcription blockage.

Finally, it may be possible that a strand break produced by a DNA glycosylase at an oxidative lesion in a transcription bubble would serve as a block to the RNA polymerase apparatus. It has been very recently shown (45) that two of the human oxidative DNA glycosylases, NEIL1 and NEIL2, that have a fairly broad substrate specificity (64), can cleave the lesions they recognize in a DNA bubble structure. In fact, NEIL2 prefers the bubble structure as a substrate. We postulate that the single-stranded break generated by NEIL2 could prevent forward movement of the transcription complex and induce either retrograde movement or dissociation from the template. Clearly, the mechanism of transcription-coupled repair of oxidative DNA damages that do not block RNA polymerase remains to be elucidated.

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2 E. Larsen, K. Kwon, J.-H. Egly, and A. Klungland, submitted for publication.
Single-Stranded Breaks in DNA but Not Oxidative DNA Base Damages Block Transcriptional Elongation by RNA Polymerase II in HeLa Cell Nuclear Extracts
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Additions and Corrections

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Kruppel-like factor 4 (KLF4) represses histidine decarboxylase gene expression through an upstream Sp1 site and downstream gastrin responsive elements.

Wandong Ai, Ying Liu, Michael Langlois, and Timothy C. Wang

Page 8687, Fig. 2: Panel B was duplicated and printed as panel C. The correct Fig. 2C is shown below:

![Image of Fig. 2C]

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Single-stranded breaks in DNA but not oxidative DNA base damages block transcriptional elongation by RNA polymerase II in HeLa cell nuclear extracts.

Scott D. Kathe, Guang-Ping Shen, and Susan S. Wallace

Page 18519: The second sentence in the last paragraph should read: “It has been very recently shown that two of the human oxidative DNA glycosylases, NEIL1 and NEIL2, which have a fairly broad substrate specificity (45), can cleave the lesions they recognize in a DNA bubble structure (64). In fact, these authors (64) show that NEIL2 prefers the bubble structure as a substrate and postulate that the single-stranded break generated by NEIL2 could . . . .”

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