Cloned DNA damage, where two or more lesions are located proximally to each other, is frequently induced by ionizing radiation. Individual base lesions within a cluster are repaired by base excision repair. In this study we addressed the question of how thymine glycol (Tg) within a cluster would affect the repair of opposing lesions by human cell extracts. We have found that Tg located opposite to an abasic site does not affect cleavage of this site by apurinic/apyrimidinic (AP) endonuclease. However, Tg significantly compromised the next step of the repair. Although purified DNA polymerase β was able to incorporate the correct nucleotide (dAMP) opposite to Tg, the rate of incorporation was reduced by 3-fold. Tg does not affect 5′-sugar phosphate removal by the 2-deoxyribose-5-phosphate (dRP) lyase activity of DNA polymerase β, but further processing of the strand break by purified DNA ligase III was slightly diminished. In agreement with these findings, although an AP site located opposite to Tg was efficiently incised in human cell extract, only a limited amount of fully repaired product was observed, suggesting that such clustered DNA lesions may have a significantly increased lifetime in human cells compared with similar single-standing lesions.

Numerous cytotoxic agents exert their deleterious effects via the formation of lesions and adducts in DNA; these effects may include oxidized purine and pyrimidine residues, abasic sites, and single and double strand breaks. Ionizing radiation induces damage in DNA by direct ionization and through the generation of hydroxyl radicals that attack DNA, resulting in single strand breaks and oxidative damage to sugar and base residues (1). Two or more DNA lesions of the same or different nature may be produced proximal to each other on the same or opposite DNA strands, generally within two helical turns of the DNA. These various types of DNA damage, known as “clustered DNA lesions,” may include strand breaks containing damaged DNA termini accompanied by multiple base lesions of varying complexity (2, 3).

Approximately 10–20% of the damage to DNA induced by ionizing radiation is the result of thymine base oxidation and fragmentation (4). Thymine is an easily oxidized base and is frequently found as a component of clustered lesions (2, 3). Individual base damages within a cluster are repaired by base excision repair (BER). 1 BER is a multiprotein pathway with a broad substrate specificity that is determined by the damage-specific glycosylases. DNA glycosylases initiate BER by recognizing damaged or abnormal bases and cleaving the glycosyl bond linking the base to the sugar phosphate backbone (5). The majority of the apurinic/apyrimidinic sites (AP sites) formed are further processed by the so-called “short-patch” BER pathway (6). In human cells this pathway is activated by an AP endonuclease (APE1) that introduces a DNA strand break 5′ to the AP site (7). This strand break cannot be ligated directly; therefore, DNA polymerase β (Pol β) first adds one nucleotide to the 3′-end of the nicked AP site, and then the dRP lyase activity of Pol β catalyzes β-elimination of the 5′-sugar phosphate residue (8). This creates a nick containing a 3′-OH and a 5′-phosphate end that can then be sealed by the DNA ligase III-XRCC1 (x-ray cross complementing factor 1) complex (9). These repair events result in a single nucleotide repair patch, and this is therefore known as short-patch BER (6).

Several groups (reviewed in Refs. 10 and 11) have studied the effects of opposing or multiple tandem lesions on DNA glycosylases and AP endonucleases. Studies with oligonucleotides containing synthetic damage clusters on opposing strands and purified glycosylases/lyases indicate that both the identity of the component lesions and their relative spacing determine the repairability of the clustered DNA lesion (12, 13). Studies have shown that DNA glycosylases can efficiently remove one of two closely opposed base lesions generating an abasic site. Cleavage of the AP site by AP endonuclease produces a nick close to the remaining lesion on the opposite strand. The removal of the remaining base lesion is thereby inhibited (reviewed in Refs. 10 and 11). Therefore, in the course of repair, clustered lesions containing a thymine glycol opposed by damaged adenosine or an abasic site may be converted into a lesion consisting of Tg opposite to a 5′-sugar phosphate-containing single strand break. Tg blocks replication by the major replicative DNA polymerases δ and ε (14, 15); however the effect of Tg on APE1, Pol β, and DNA ligase III, key enzymes in the major base excision repair pathway, is not known.

In this study we have used oligonucleotide duplexes containing Tg located directly opposite to an AP site or an AP site preincised with APE1. Using these substrates and purified human BER proteins or human cell extracts, we characterized the effect of Tg on the repair of clustered lesions.

EXPERIMENTAL PROCEDURES

Materials—Synthetic oligodeoxyribonucleotides, purified by high performance liquid chromatography, were obtained from MWG-Biotech. [α-32P]dATP and [γ-32P]ATP (3000 Ci/mmol) were purchased from

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† To whom correspondence should be addressed. Tel.: 44-1235-841-134; Fax. 44-1235-841-200; E-mail: g.dianov@har.mrc.ac.uk.

‡ The abbreviations used are: BER, base excision repair; AP sites, apurinic/apyrimidinic sites, abasic sites; APE1, human AP endonuclease; Pol β, DNA polymerase β, dRP, 2-deoxyribose-5-phosphate; Tg, thymine glycol (5,6-dihydroxy-5,6-dihydrothymidine); DTT, dithiothreitol.
Repair of Clustered DNA Lesions

In human cells after the incision of an AP site by APE1, base excision repair is carried out by Pol β, which incorporates a single nucleotide into the repair gap (6, 19, 20). Using an oligonucleotide duplex substrate containing an AP site or a preincised AP site with a 5′-sugar phosphate moiety opposite Tg on the template strand (Fig. 1A), we first investigated whether Tg located opposite to the damage would affect DNA repair synthesis by Pol β. Surprisingly, we found that purified human Pol β (Fig. 1B) is able to incorporate the first nucleotide opposite to Tg, although at a slower rate than on the undamaged template. Incorporation on a Tg-containing substrate by Pol β is reduced by ~3-fold compared with the control substrate, with 25 fmol of Pol β per reaction (Fig. 1B); however, at higher concentrations Pol β was more than 80% efficient. The important question remains whether the incorporation opposite to Tg is error-free. Using reactions containing only one of four deoxyribonucleotide triphosphates, we next analyzed which nucleotide is incorporated and found that incorporation by Pol β was very specific, with the dAMP residue exclusively incorporated opposite to Tg (Fig. 2).

At the next step of short-patch BER, Pol β catalyzes removal of the 5′-dRP residue. To study the effect of Tg on this reaction, we constructed a 3′-end-labeled substrate containing thymine or Tg opposite to the preincised AP site (Fig. 3A). Removal of the dRP from this substrate will generate a 11-mer-labeled substrate, schematic representation of the [32P]-5′-labeled (1) oligonucleotide substrate used. The substrate was generated by incubation of the AP site-containing duplex with APE1. dRP stands for the 5′-sugar phosphate. B, 1 pmol of the 5′-end-labeled substrate oligonucleotide duplex containing thymine or thymine glycol opposite to the preincised AP site was incubated for 20 min at 37 °C with the indicated amount of Pol β in conditions described under “Experimental Procedures.” After incubation, reactions were stopped by the addition of formamide dye solution and, following incubation at 90 °C for 3 min, the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

RESULTS

In human cells after the incision of an AP site by APE1 base excision repair is carried out by Pol β, which incorporates a single nucleotide into the repair gap (6, 19, 20). Using an oligonucleotide duplex substrate containing an AP site or a preincised AP site with a 5′-sugar phosphate moiety opposite Tg on the template strand (Fig. 1A), we first investigated whether Tg located opposite to the damage would affect DNA repair synthesis by Pol β. Surprisingly, we found that purified human Pol β (Fig. 1B) is able to incorporate the first nucleotide opposite to Tg, although at a slower rate than on the undamaged template. Incorporation on a Tg-containing substrate by Pol β is reduced by ~3-fold compared with the control substrate, with 25 fmol of Pol β per reaction (Fig. 1B); however, at higher concentrations Pol β was more than 80% efficient. The important question remains whether the incorporation opposite to Tg is error-free. Using reactions containing only one of four deoxyribonucleotide triphosphates, we next analyzed which nucleotide is incorporated and found that incorporation by Pol β was very specific, with the dAMP residue exclusively incorporated opposite to Tg (Fig. 2).

At the next step of short-patch BER, Pol β catalyzes removal of the 5′-dRP residue. To study the effect of Tg on this reaction, we constructed a 3′-end-labeled substrate containing thymine or Tg opposite to the preincised AP site (Fig. 3A). Removal of the dRP from this substrate will generate an 11-mer-labeled fragment, whereas a dRP-containing fragment will migrate slightly slower on a gel. After reactions, all samples were treated with sodium borohydride to stabilize the AP sites and prevent their self-degradation during electrophoresis. Under these conditions, we found no inhibitory effect of Tg on dRP removal by Pol β. In fact, removal of the dRP from the Tg-
acrylamide gel. Products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

During the last step of BER, DNA ligase III seals the DNA ends broken during repair. The ability of Tg to affect DNA ligase III was tested with a substrate simulating the last step of BER, i.e. an oligonucleotide duplex containing a single strand break opposite to thymine or Tg (Fig. 4B). After incubation, reactions were further incubated for 10 min on ice with 0.1 M NaBH4. Reactions were stopped by the addition of a formamide dye solution and, following incubation at 90 °C for 3 min, the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

Thus, at certain enzyme concentrations, both purified Pol β and DNA ligase III are able to catalyze repair of an AP site opposite Tg. An important question, however, was whether the concentration of Pol β and DNA ligase III in whole cell extracts is high enough to support efficient repair of clustered lesions containing Tg. To address this question, we compared repair of the oligonucleotide duplexes containing an AP site located opposite to the Tg or opposite to thymine by human cell extracts. Tg located opposite to the AP site did not affect cleavage of the AP site by human AP endonuclease. When incubated with human cell extract, the AP site-containing substrate was cleaved within 30 s, generating the 22-mer 5'-labeled incision product (data not shown). Further repair of the AP site was monitored as restoration of the full-length 45-mer labeled product (Fig. 4A). We found that about 20% of the Tg-containing substrate was repaired within 20 min of incubation with cell extract in comparison to 50–60% for the thymine-containing substrate (Fig. 4B). We thus conclude that, although Tg does not completely block repair, and such lesions are to some degree repairable by BER, the inhibitory effect of Tg may cause substantial delay in the repair of clustered lesions by BER enzymes.

**DISCUSSION**

The existence of complex DNA lesions induced by ionizing radiation has been demonstrated experimentally (2, 3, 21).
Such complex lesions may include different combinations of base lesions and/or single strand breaks. It was previously demonstrated that clustered lesions lead to the formation of mutations, deletions, and chromosome rearrangements (22–25); however, very little is known about the molecular events leading to such dramatic genetic changes. In this study we addressed the repair of clustered lesions containing Tg. During short-patch BER, when a single damaged nucleotide is excised by repair enzymes, the major threat may be simultaneous damage of the complementary base, and the presence of Tg in the repair gap may have a major impact on the quality and the rate of repair. Nevertheless, as we demonstrate in this study, not all BER reactions are affected to the same extent by such damages. Incision of an AP site by APE1 as well as the removal of the 5'-sugar phosphate by the AP lyase activity of Pol β were not affected at all, and Tg had only a moderate (1.5-fold) inhibitory effect on the ligation reaction. However, Tg in a repair gap not affected at all, and Tg had only a moderate (1.5-fold) inhibitory effect on the ligation reaction. However, Tg in a repair gap was reconstituted with purified Pol β and further incorporation was completely blocked in repair reactions reconstituted with purified Pol β (Fig. 1B), as well as in cell extract (Fig. 5B, right panel). This suggests that long-patch repair, which requires incorporation of at least two nucleotides, would not be efficient in the repair of such lesions.

Tg, when present on the DNA template strand, blocks the progression of replication by the major replicative DNA polymerases α and δ, although a limited incorporation opposite Tg is observed (14, 15, 26). We also found that Pol β was able to catalyze limited incorporation opposite to Tg and that Tg does not change the specificity of incorporation (Figs. 1B and 2). In agreement with our findings, early studies indicated that Tg forms a reasonably stable base pair with adenine and that the DNA sequence immediate to Tg does not affect the specificity of incorporation by a Klenow fragment of DNA polymerase I or by DNA polymerase α (14, 26). Theoretically, two major known isomeric forms of Tg (5S and 5R) (27) may affect human DNA polymerases differently. In this study, Tg was generated by direct oxidation of the single thymine in the template DNA using osmium tetroxide. This procedure generates 85% of the 5R isomer (28), suggesting that the reduced rate of incorporation of dAMP opposite Tg observed in our experiments was mainly due to the effect of this isomer. In support of this conclusion, kinetic analyses performed by Hanaoka and co-workers revealed that Pol α incorporates dAMP opposite 5R-Tg about 16-fold less efficiently than on an undamaged template (14).

8-oxoguanine and Tg are the major oxidative lesions induced by indirect effects of ionizing radiation caused by the generation of reactive oxygen species. Individually, those lesions are efficiently repaired by BER (29, 30). Moreover, as we have recently shown, 8-oxoguanine within a repair gap does not inhibit short-patch BER (31). However, as we demonstrate here, Tg is a much more harmful lesion. When located within a cluster, Tg causes a substantial delay in short-patch BER of the opposing lesion. As a result of such a delay, gapped DNA would be exposed for a longer time to the cellular milieu. Delays in the processing of repair intermediates may cause a significant increase in genomic instability and affect cellular resistance to ionizing radiation (32, 33). In summary, our data suggest that clustered lesions containing Tg are repaired slower than a single-standing lesion of a similar type and may be partially responsible for the deleterious effect of ionizing radiation.

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Mode of Inhibition of Short-patch Base Excision Repair by Thymine Glycol within Clustered DNA Lesions
Helen Budworth and Grigory L. Dianov

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