Functional Effects of *cis*-Thymine Glycol Lesions on DNA Synthesis in Vitro†

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ABSTRACT: Thymine glycol, a DNA lesion produced by ionizing radiation, has been introduced site specifically at high frequency into a synthetic oligonucleotide by chemical oxidation of the single thymine residue within the sequence. The lesion-containing template was then annealed to a complementary synthetic primer and used to study the effects of *cis*-thymine glycol lesions on DNA polymerase function in vitro. Synthesis by polymerase I (Klenow fragment), T4 DNA polymerase, and polymerase α_2 was arrested quantitatively at the site of the lesion. AMV reverse transcriptase was less inhibited and was able to synthesize past a significant fraction of the lesions. Changing the template base immediately 5′ to thymine glycol from A to C did not significantly alter the pattern of synthesis arrest for any of the polymerases. The correct nucleotide, dAMP, was inserted opposite the lesion more than 90% of the time by all four polymerases, suggesting that thymine glycol forms a reasonably stable base pair with adenine. However, the 3′–5′ exonuclease activity of polymerase I removed a 3′-terminal dAMP residue more rapidly from an A·thymine glycol base pair than from an A·T base pair. These results suggest that increased nucleotide turnover at the site of the lesion contributes to the inhibitory effects of thymine glycol lesions on DNA synthesis in vitro, at least for polymerases such as polymerase I that have intrinsic or associated editing exonuclease functions.

Ionizing radiation produces significant deleterious biological effects in vivo including mutagenesis, carcinogenesis, and cell death. The molecular bases for these effects are not well understood at present although DNA appears to be the principal cellular target. Ionizing radiation produces a variety of structural abnormalities in DNA including strand breaks, DNA–protein cross-links, and damage to purine and pyrimidine bases [reviewed in Hutterman et al. (1978)]. The multiplicity of lesions produced has made it difficult to evaluate the relative contributions of specific lesions to overall biological end points such as mutagenesis. This evaluation is rendered even more difficult by the existence of repair pathways that may remove some or all of the damaged products.

Thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) is the major species found in aerated irradiated aqueous solutions of thymine (Têoule & Cadet, 1978), and its presence in DNA irradiated in vitro has been detected by enzymatic methods (Breimar & Lindahl, 1985) and by radioimmunoassay (West et al., 1982). Thymine glycol has also been found in the DNA of irradiated human cells (Frenkel et al., 1981; Breimer & Lindahl, 1985). The lesion can occur in four possible diastereomeric forms in DNA, two cis isomers and two trans isomers; all four isomers have been detected in γ-irradiated solutions of thymidine (Cadet et al., 1981). Enzymes have been isolated from both procaryotic and eucaryotic sources that specifically remove damaged thymine residues including thymine glycol from chemically oxidized or γ-irradiated DNA in vitro (Lindahl, 1982). Enzymatic removal of thymine glycol from the DNA of bacteria (Hariharan & Cerutti, 1972) and mammalian cells (Mattern et al., 1975; Leadon & Hanawalt, 1983) has also been reported.

In earlier studies, we found that *cis*-thymine glycol lesions in template DNA inhibited primer elongation and that synthesis arrest sites corresponded predominantly to presumptive loci of the lesions (Clark & Beardsley, 1986). Similar findings have been reported by others (Rouet & Essigmann, 1985; Hayes & LeClerc, 1986; Ide et al., 1985). These studies utilized single-stranded phage DNA in which a relatively small proportion of the thymine bases had been chemically converted to *cis*-thymine glycol in a quasi-random distribution. Since the lesion frequency at each site was not known, quantitation of the relative degree of synthesis arrest vs. lesion bypass was not possible. Thus, whether the block to DNA replication produced by thymine glycol is complete or only partial could not be determined. In addition, Hayes and LeClerc (1986) and Clark and Beardsley (1986) noted certain lesion sites at which no synthesis arrest was detected, suggesting a sequence context dependency for lesion bypass.

We have now developed techniques for the quantitative introduction of a single *cis*-thymine glycol lesion at a specific site in DNA oligomers. Using this system has allowed us to address the above quantitation and sequence context issues. Furthermore, we have been able to extend our investigations to additional polymerases, including human DNA polymerase α_2, and also study the role of 3′–5′-exonuclease activity in producing the arrest of chain elongation.

Experimental Procedures

Reagents. DNA oligonucleotides, the sequences of which are given below, were synthesized on an Applied Biosystems automated DNA synthesis machine; phosphoramidite chemistry was used. The protecting groups were removed by overnight treatment with concentrated ammonium hydroxide at 55 °C. All oligonucleotides were purified by denaturing, polyacrylamide gel electrophoresis and checked for purity by electrophoresis after 5′ end labeling with T4 polynucleotide kinase. The dinucleotide d(GpA) was obtained from Pharmacia. Deoxynucleoside triphosphates and osmium tetraoxide were obtained from Sigma. Piperidine was purchased from Aldrich. DNA polymerase I [Klenow fragment (KF)[1]] and

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[1] Abbreviations: KF, Klenow fragment; BSA, bovine serum albumin; pol I, polymerase I; pol α_2, polymerase α_2; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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AMV reverse transcriptase were obtained from International Biotechnologies, Inc., T4 DNA polymerase was purchased from New England Biolabs, and HeLa cell DNA polymerase α_2 was provided by Dr. Earl Baril (Worcester Foundation for Experimental Biology, Shrewsbury, MA).

Preparation and Characterization of Template/Primers. Oligonucleotide templates containing a single thymine glycol lesion were prepared by oxidation of 18-mers containing a single thymine base with osmium tetraoxide (Beer et al., 1966) in the presence of aqueous pyridine (Friedmann & Brown, 1978). Each reaction contained approximately 0.5 μg of the oligonucleotide, 2% (79 mM) osmium tetraoxide, and 5% (0.6 M) pyridine in a final volume of 40 μL. Oxidation was carried out at room temperature for 30 min. The samples were then diluted to 80 μL with distilled, deionized water and centrifuged through a Sephadex G-25 column equilibrated against water (Maniatis et al., 1982) to remove unreacted osmium tetraoxide. Control DNA samples were treated in an identical manner except that osmium tetraoxide was omitted from the mock oxidation reaction. All DNA samples were stored in water at −20 °C; oxidized samples were routinely used within 1 week after preparation. Two such 18-mer template sequences were utilized:

3′ GGAAAAGCAGCCGGGTAG 5′ (18A)

and

3′ GGAAAAGCAGCCGGGTCG 5′ (18C)

These sequences differ only in the base immediately to the 5′ side of the single thymine base and are designated 18A or 18C to denote the oligonucleotide length plus the base that differs between the two sequences.

The 14-mer primer, complementary to both of the above 18-mers, had the sequence

5′ CCTTTTCGTCGGCC 3′

and was labeled with ^{32}P at the 5′ end according to standard methods (Maxam & Gilbert, 1980). Polynucleotide kinase and residual [γ-^{32}P]ATP were removed by extraction with phenol–chloroform–isoamyl alcohol (1:0.96:0.04) followed by the spun column procedure described above. Primer/template substrates were prepared by annealing approximately 10 ng of labeled primer to 20–40 ng of template (oxidized or control) in a reaction containing 25 mM Tris-HCl, pH 8.1, and 10 mM $MgCl_2$ in 10-μL final volume. Annealing was carried out as described (Clark & Beardsley, 1986). As a control for strand breakage under the conditions used for annealing, ^{32}P-labeled, oxidized template DNA was incubated under annealing conditions and analyzed by electrophoresis. No detectable strand scission was observed.

Piperidine cleavage reactions were carried out as described (Maxam & Gilbert, 1980), using 5′-end-labeled, oxidized or undamaged oligonucleotides. 3′-Terminal phosphates were removed from 5′-labeled fragments by using the 3′-phosphatase activity of wild-type polynucleotide kinase (Cameron & Uhlenbeck, 1977). The phosphatase reactions were carried out for 30 min at 37 °C in 50 mM Tris-HCl, pH 6.0, 10 mM $MgCl_2$, and 5 mM dithiothreitol in the absence of ATP.

DNA Synthesis Reactions. Each primer extension reaction contained 1–2 μL of the annealed template/^{32}P-primer substrate and 100 μM each dATP, dCTP, dGTP, and dTTP. In some experiments dATP was omitted from the reaction. Each assay contained the appropriate buffer for the particular polymerase as described by the supplier for the Klenow fragment (with the addition of 150 mM KCl) and by Maniatis et al. (1982) for the T4 enzyme and AMV reverse transcriptase. The reaction buffer for pol α_2 contained 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mg/mL BSA (fatty acid free). Klenow fragment and reverse transcriptase assays contained 0.5 unit of enzyme, T4 assays contained 0.3 unit, and pol α_2 assays contained 0.04 unit (all units are those of the supplier). The reactions (5-μL final volume) were carried out for 30 min at room temperature (Klenow fragment and T4 polymerase) or 37 °C (reverse transcriptase and pol α_2). Synthesis was terminated by the addition of an equal volume of a dye/formamide loading solution (Maxam & Gilbert, 1980), and the samples were denatured by heating at 95 °C for 3 min immediately prior to electrophoresis.

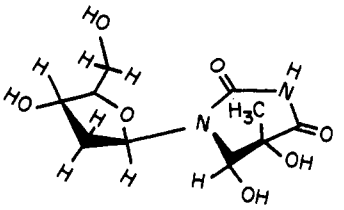

FIGURE 1: Structure of one of the two cis diastereomers of thymidine glycol: *cis*-5(*R*),6(*S*)-dihydroxy-5,6-dihydrothymidine. The other cis isomer has the hydroxyl groups in an inverted configuration with respect to the plane defined by atoms N1, C2, N3, and C4 of the thymine base.

3′–5′-Exonuclease Assay. The substrate for the exonuclease assay was prepared by carrying out a primer extension assay with the Klenow fragment as described above but providing only dCTPαS and dATP (50 μM each) as nucleotide precursors. The reaction was stopped by the addition of EDTA to 20 mM and the volume adjusted to 50 μL with water. Samples were extracted with phenol–chloroform–isoamyl alcohol, and unincorporated precursors were removed as described above with two cycles of exclusion chromatography through Sephadex G-50. The samples were then concentrated in a SpeedVac (Savant Instruments, Inc.). Aliquots were removed and reincubated with varying amounts of Klenow fragment in the absence of nucleotide precursors. Exonucleolytic removal of the 3′-terminal dAMP residue was monitored by electrophoretic analysis of the reaction products.

Electrophoresis and Autoradiography. Electrophoresis was carried out essentially as described (Clark & Beardsley, 1986) except that 20% gels were used and the gels were not dried prior to autoradiography. Autoradiography was done at room temperature or −20 °C for 0.5–2 h.

RESULTS

Quantitation of Lesion Frequency. Treatment of DNA with osmium tetraoxide results in the preferential oxidation of thymine bases at the C5–C6 double bond to form *cis*-thymine glycols (Beer et al., 1966), of which there are two possible diastereomers in DNA (Figure 1). Addition of pyridine to the reaction accelerates the rate of formation of cis glycols by osmium tetraoxide by several orders of magnitude (Schröder, 1980) without significantly altering the specificity of the reagent for thymine bases in DNA. The lesion frequency in the DNA templates was determined by cleaving the 5′-end-labeled, oxidized 18-mers with piperidine and analyzing the products by electrophoresis through high-resolution, denaturing polyacrylamide gels. An example of this is shown in Figure 2 for the 18A oligomer. Piperidine cleaved the oxidized 18A oligomer quantitatively to yield the expected dinucleotide product, 3′-pdApdGp-5′. No significant cleavage of undamaged oligonucleotides was observed. This assay would not detect any modifications introduced at sites 3′ to the thymine base except in molecules that remain unmodified at thymine.

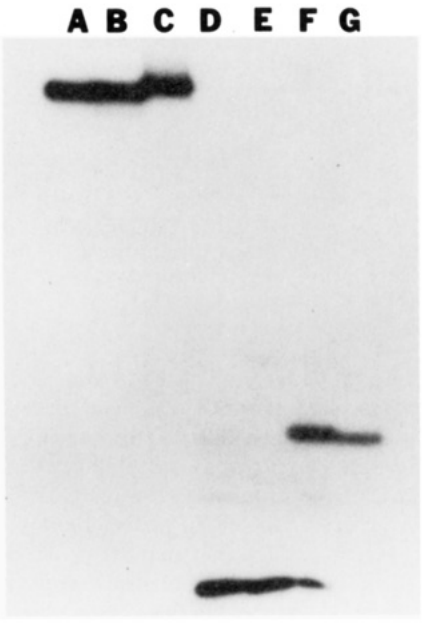

FIGURE 2: Determination of thymine glycol lesion frequency. 5′-End-labeled control (A, B) and oxidized (C, D) oligonucleotides (18A) were analyzed by denaturing, polyacrylamide gel electrophoresis before (A, C) and after (B, D) cleavage with piperidine. The identity of the dinucleotide cleavage product was confirmed by incubating piperidine-cleaved, oxidized DNA in buffer alone (E) or with a 3′-phosphatase (wild-type polynucleotide kinase) in the absence of ATP (F) to remove the 3′-phosphate group from the cleavage product. Lane G is authentic 5′-^{32}P-labeled, 3′-dApdGp-5′.

Overexposure of the autoradiogram does reveal a small (<5%) proportion of piperidine cleavage fragments larger than the expected dinucleotide; these presumably are derived from molecules that have been modified at sites other than thymine. Thus the lesion frequency at the position of thymine in the template strand was greater than 95%. A small proportion of lesions at other sites should not affect the primer elongation reaction, although they may inhibit annealing of the primer to some fraction of the template molecules.

It is of interest that the 18-mer template that contains thymine glycol migrates at a slightly slower rate than the undamaged template (Figure 2, lane C). This presumably reflects the slight decrease in charge to mass ratio of the thymine glycol containing oligonucleotide compared to the undamaged control. The altered mobility is not due to the formation of a DNA–osmate–pyridine complex (Subbaraman et al., 1971) since treatment with sodium bisulfite, which reductively cleaves such esters (Baran, 1960), did not change the relative mobility of the oxidized oligonucleotide (data not shown).

Determination of Synthesis Arrest Patterns. The ^{32}-P-labeled 14-mer primer was annealed to control or oxidized 18A oligomer templates to form a partial duplex with a four nucleotide extension

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5′ 32P-CCTTTTCGTCGGCC      3′
3′     GGAAAAGCAGCCGGGTAG 5′
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where T is either thymine or thymine glycol. The annealed primers were then extended by DNA polymerases in the presence of dNTP precursors (Figure 3). Since the primer is 5′-end-labeled, the band intensities on the autoradiogram are directly proportional to the number of molecules that terminate synthesis at any given position along the template and thus to the frequency of arrest. Furthermore, these experiments were carried out with equimolar concentrations of all four dNTPs to avoid any effects of nucleotide pool imbalance on the pattern of synthesis arrest (but see below). Synthesis on undamaged templates showed essentially complete elongation of the primer to the full length of the template strand (Figure 3, lanes A, C, E, G). Pol I (KF) and T4 DNA polymerase both terminated chain elongation at the site corresponding to a putative thymine glycol lesion (Figure 3, lanes B and D). Arrest of synthesis by polymerase α_2 occurred either at the lesion site or, to a lesser extent, one nucleotide before the lesion site. No significant degree of extension beyond the lesion site can be detected with these three polymerases when the oxidized 18A template was used. These results also provide independent evidence that the lesion frequency in the template strands is nearly 100%. However, the pattern of synthesis arrest obtained with AMV reverse transcriptase was more complex. Strong bands occurred one nucleotide before, and at both positions after, the lesion site (Figure 3, lane H). A weaker band was also detectable at the position corresponding to the thymine glycol site. A small amount of unextended primer was also observed in all cases and, for the procaryotic enzymes, some partially degraded fragments as well.

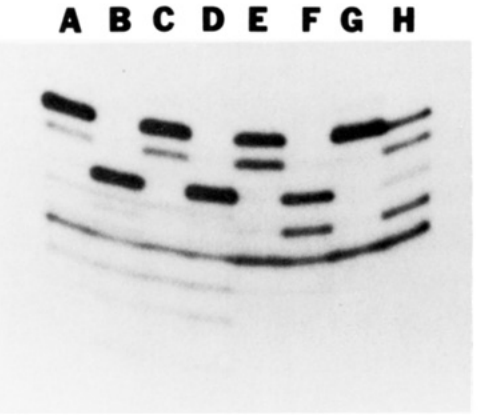

FIGURE 3: Patterns of synthesis arrest on the 18A oligonucleotide template. Primer extension experiments were carried out as described under Experimental procedures for the Klenow fragment (A, B), T4 DNA polymerase (C, D), pol α_2 (E, F), and AMV reverse transcriptase (G, H). Lanes A, C, E, and G show synthesis on undamaged templates; lanes B, D, F, and H show synthesis on templates containing a single *cis*-thymine glycol lesion. The band of moderate intensity that is present in all eight lanes represents the unextended primer population and serves as an internal size marker (14-mer).

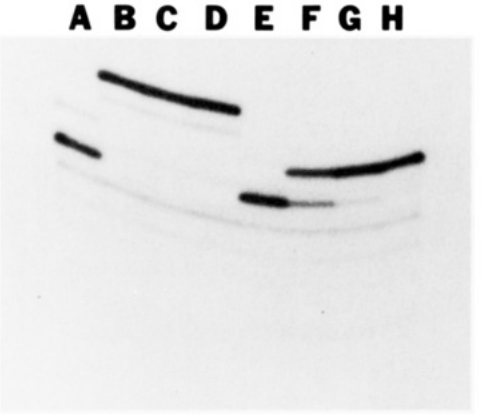

FIGURE 4: Primer extension by pol I (KF) on the 18A template as a function of dATP concentration. 5′-End-labeled primers were extended on control (A–D) or oxidized (E–H) templates in the absence of dATP (A, E) or in the presence of 2.5 (B, F), 10 (C, G), or 50 μM (D, H) dATP.

Specificity of Nucleotide Insertion Opposite the Lesion. The identity of the nucleotide inserted opposite thymine glycol was determined by carrying out primer extension experiments in the presence or absence of dATP. The results obtained with

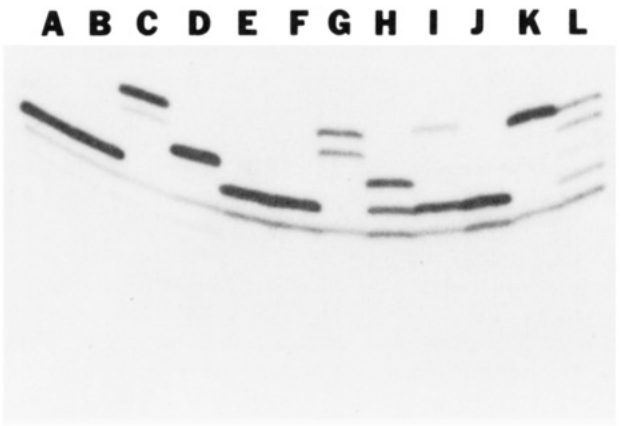

FIGURE 5: Primer extension by T4 DNA polymerase (A–D), pol α_2 (E–H), and reverse transcriptase (I–L) on the 18A template in the presence or absence of dATP. The first two lanes for each polymerase group are reactions done without dATP on control (A, E, I) or oxidized (B, F, J) templates. The remaining two lanes for each group are reactions done with 100 μM dATP on control (C, G, K) or oxidized (D, H, L) templates.

the Klenow fragment are shown in Figure 4. In the absence of dATP, synthesis on both control and damaged templates stops one nucleotide before the position corresponding to thymine (control DNA) or thymine glycol (oxidized DNA) in the template. Addition of dATP restores the synthesis arrest pattern seen previously (Figure 2). Misincorporation of nucleotides other than dAMP opposite thymine glycol was not observed at a detectable frequency (Figure 4, lane E). Similar results were obtained with T4 DNA polymerase, polymerase α_2, and reverse transcriptase (Figure 5). Thus all four polymerases primarily inserted dAMP opposite thymine glycol. It is of interest that reverse transcriptase was able to fully extend some of the primers even in the absence of dATP; however, this occurred only on undamaged templates (Figure 5, lanes I and J).

Role of the 3′–5′-Exonuclease Activity. The susceptibility of the adenine·thymine glycol base pair to hydrolysis by the 3′–5′-exonuclease activity of the Klenow fragment was tested by using partially extended primer/template substrates that contained a phosphorothioate diester linkage at the position immediately preceding the 3′-terminal dAMP residue in the primer strand (see Experimental Procedures). Incubation of these substrates with Klenow fragment in the absence of dNTPs should release only the 3′-terminal dAMP since phosphorothioate linkages are resistant to exonucleolytic cleavage by this enzyme (Kunkel et al., 1981). Since the primer was labeled at its 5′ end, exonucleolytic removal of the terminal dAMP could be monitored as a shift in electrophoretic mobility of the reaction products. The results of this experiment are shown in Figure 6 for the adenine·thymine base pair (control) and adenine·thymine glycol base pair. A comparison of lanes D (control) and I (oxidized) shows that, at equivalent enzyme:DNA ratios and reaction times, more dAMP was removed from the primer terminus when A was paired with thymine glycol than when it was paired with thymine. Excision was essentially complete, however, with both substrates at higher enzyme concentrations (Figure 6, lanes E and J).

Effect of Sequence Context on Synthesis Arrest. The effect of altering the sequence of the DNA template adjacent to the thymine glycol was investigated by using a different 18-mer template (18C) in which the adenine base immediately 5′ to the lesion site was replaced by cytosine:

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5′ 32P-CCTTTTCGTCGGCC      3′
3′     GGAAAAGCAGCCGGGTCG 5′
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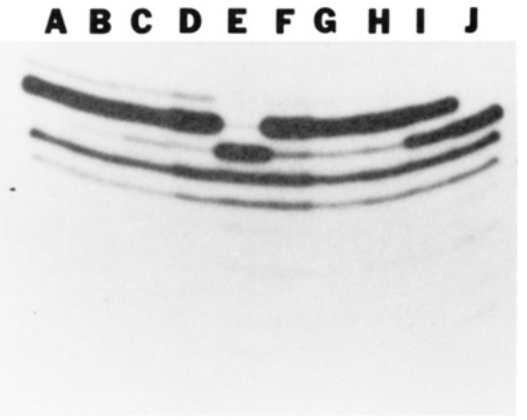

FIGURE 6: Exonucleolytic removal of a 3′-terminal dAMP residue base paired with thymine (A–E) or thymine glycol (F–J). The DNA substrates for this assay were prepared as described under Experimental procedures and had the sequence shown below, where the penultimate

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5′ CCTTTTCGTCGGCCSCA   3′
3′ GGAAAAGCAGCCGG GTAG 5′
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residue of the primer strand (dCMP) is joined to the preceding nucleotide via a phosphorothioate linkage (denoted by s). T represents either thymine or thymine glycol. These substrates were incubated without KF for 20 min (A, F) or with 0.06 unit of enzyme for 1 (B, G), 5 (C, H), or 20 min (D, I) at room temperature. Lanes E (control templates) and J (oxidized templates) show DNA substrates that were incubated with 0.6 unit of KF for 20 min at room temperature.

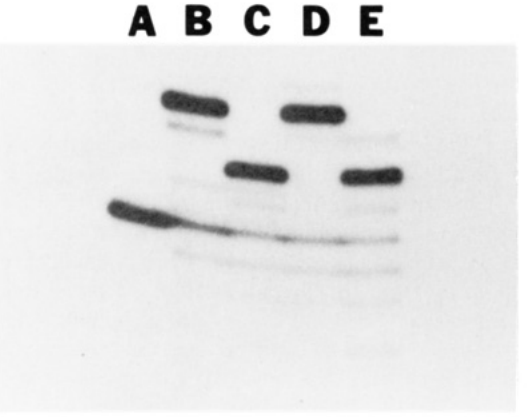

FIGURE 7: Comparison of primer extension by the Klenow fragment on the 18A and 18C templates. DNA synthesis reactions were carried out on undamaged 18A (B) or 18C (D) templates and on oxidized 18A (C) or 18C (E) templates. Lane A is the unextended primer.

This particular sequence context was chosen because of the observation that some sequences in M13 DNA of the form 3′-(Pu)TC-5′ do not represent arrest sites for the Klenow fragment (Hayes & LeClerc, 1986; Clark & Beardsley, 1986). The lesion frequency at the position corresponding to thymine in the oxidized 18-mer template was again determined by piperidine cleavage to be >95% (data not shown). The results of primer extension experiments using the Klenow fragment with both the 18A and 18C templates are shown in Figure 7. In both cases synthesis on the undamaged templates proceeds predominantly to the end of the molecule (Figure 7, lanes B and D). However, synthesis on the oxidized templates is almost completely blocked at the site of the lesion for both the 18A and 18C templates. There is, in addition, a weak band present at the position one nucleotide beyond the lesion site for the 18C template, which is absent in the corresponding 18A lane (Figure 7, compare lanes C and E).

Figure 8 shows the results of primer extension experiments on the 18C template using T4 DNA polymerase (lanes B and C), polymerase α_2 (lanes D and E), and AMV reverse tran-

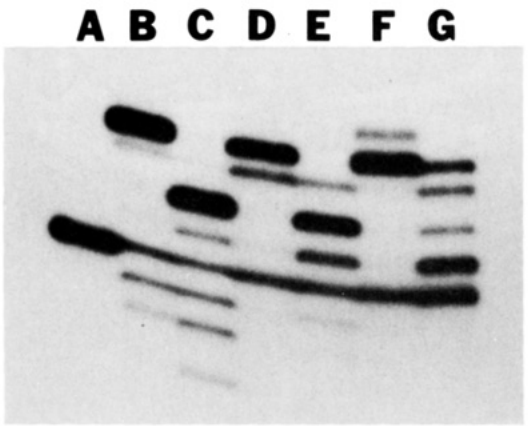

FIGURE 8: Primer extension experiments carried out on the 18C template with T4 DNA polymerase (B, C), pol α_2 (D, E), and AMV reverse transcriptase (F, G). Lanes B, D, and E show the synthesis patterns from undamaged templates; lanes C, E, and G show the results from oxidized templates. Lane A is the unextended primer.

scriptase (lanes F and G). The pattern of synthesis arrest for all three polymerases was very similar to the corresponding patterns obtained with the 18A template (see Figure 3). For pol α_2, however, there is an additional weak band visible one nucleotide beyond the lesion site (Figure 8, lane E), which was not evident when the 18A template was used (Figure 3, lane F).

DISCUSSION

The results presented in this paper confirm our earlier finding that *cis*-thymine glycol lesions in DNA constitute significant blocks to synthesis by DNA polymerases in vitro (Clark & Beardsley, 1986). We have now extended these earlier results considerably through the use of a defined primer/template system that allowed us to assess such effects in a more quantitative manner. This system has also been used to carry out initial studies on the role that DNA sequence plays in modulating replicative bypass of thymine glycol lesions in vitro. Specifically, we find that synthesis by pol I (KF), T4 DNA polymerase, and pol α_2 is arrested quantitatively by the presence of *cis*-thymine glycols in the template strand. For the KF and T4 DNA polymerase, synthesis terminated at the site of the lesion; for pol α_2, synthesis terminated at, or one nucleotide before, the lesion. The difference between pol α_2 and the procaryotic polymerases may reflect less efficient utilization of these small DNA substrates since a substantial fraction of the primer population was not fully extended by pol α_2 even on undamaged templates.

We do not detect a significant degree of bypass by any of the above polymerases for either of the two template sequences that we tested. It is possible that the weak bands that occur one nucleotide beyond the lesion site for the Klenow fragment (Figure 7, lane E) and pol α_2 (Figure 8, lane E) represent a limited amount of bypass on the 18C template. These bands are, however, close to the detection limit, and their significance is therefore not conclusive. The lack of significant bypass on the template that contains a 3′-GTC-5′ sequence (18C) is not consistent with the finding that bypass of thymine glycol lesions can occur for lesions located within this sequence context on M13 DNA templates (Hayes & LeClerc, 1986). The difference between our results using oligonucleotide substrates and the results using the M13 system may, in part, be attributable to the different DNA substrates employed.

Our conclusion that thymine glycol is responsible for the observed effects on DNA synthesis is based principally upon the known specificity of the osmium tetraoxide reagent (Beer et al., 1966). The principal breakdown products of thymine glycol (urea and N-substituted urea derivatives arising from opening of the saturated pyrimidine ring; Breimer & Lindahl, 1984) form most readily at alkaline pH (Iida & Hayatsu, 1970) and under our conditions should not contribute significantly to the effects on DNA synthesis that we observe. Moreover, the pattern of synthesis arrest seen with urea residues in M13 DNA differs from that seen with thymine glycol in the same system; synthesis terminates one nucleotide before the urea lesion rather than at the lesion site as is seen with the glycol (Ide et al., 1985). This difference presumably reflects the fact that urea residues cannot form a proper base pair and are therefore noninstructional lesions whereas thymine glycol apparently retains the ability to base pair properly with adenine (Clark et al., 1987). Since the arrest of synthesis that we observe occurs almost exclusively at the site of the lesion, we conclude that breakdown products of thymine glycol do not contribute significantly to the observed effects. Thymine glycol is therefore most likely to be the lesion responsible for inhibiting DNA synthesis. However, we cannot exclude the formal possibility that other, minor products of the osmium tetraoxide reaction may also contribute to synthesis arrest in vitro.

AMV reverse transcriptase was markedly less sensitive to the presence of thymine glycol lesions in the template strand than were the other polymerases since this enzyme was able to synthesize past a substantial fraction of the lesions. This follows from the fact that a significant fraction of the primer population was extended beyond the lesion site even though the lesion frequency was nearly 100%. The reason for this apparent permissivity is unknown but may be related to the much higher spontaneous error rate in vitro exhibited by this enzyme as compared to those of other polymerases (Loeb & Kunkel, 1982). It is also not clear why reverse transcriptase can only synthesize past a fraction of the lesions. One possibility is that the enzyme is sensitive to only one of the two possible cis diastereomers of thymidine glycol. If this hypothesis were correct, the observed partial bypass would reflect the distribution of cis isomers formed in DNA treated with osmium tetraoxide. In the absence of any data regarding the actual isomer distribution, however, this must be considered purely speculative.

Our experimental data indicate that the perturbations of DNA structure introduced by *cis*-thymine glycol lesions do not significantly impair the ability of DNA polymerases to insert nucleotides at positions preceding the lesion site or opposite the lesion itself. Moreover, all of the polymerases preferentially insert dAMP, the correct nucleotide, opposite thymine glycol. Within the detection limits of the assay (ca. 5–10%), no misincorporation of incorrect nucleotides was observed. The frequency of dAMP insertion (>90%) is higher than would be expected solely on the basis of the tendency of polymerases to insert dAMP opposite noninstructional lesions (Kunkel, 1984; Sagher & Strauss, 1983). Thus we conclude that thymine glycol retains the ability to base pair with adenine and behaves as a coding lesion. Our computer modeling studies support this conclusion (Clark et al., 1987). Thymine glycol is therefore unlikely to be a potent mutagenic lesion in vivo. A recent report that C → T transitions are the predominant class of point mutations produced in M13mp10 phage by ionizing radiation suggests that cytosine, rather than thymine, bases may be the principal targets for radiation-induced point mutations in vivo (Ayaki et al., 1986).

However, with the exception of reverse transcriptase, addition of the next nucleotide beyond the lesion site does not

occur to any appreciable extent with any of the polymerases. For the enzymes that have 3′–5′-exonuclease activity (KF and T4 DNA polymerase), it is possible that an increased rate of turnover of dATP to dAMP ("idling") opposite thymine glycol could account for the inability to add bases beyond the lesion site. The increased susceptibility of the 3′-terminal dAMP residue of an A·thymine glycol base pair to hydrolysis by the 3′–5′-exonuclease activity of the KF is consistent with this hypothesis. Pol α_2 exhibits, in our hands, a weak 3′–5′-exonuclease activity (unpublished data), a finding consistent with the recent report of such an activity associated with a more highly purified preparation of this enzyme (Skarnes et al., 1986). In view of this finding, it is intriguing to speculate that all three of the DNA polymerases that fail to synthesize past thymine glycol lesions do so, at least in part, because of increased nucleotide turnover at the site of the lesion. According to this hypothesis, then, synthesis by reverse transcriptase is less inhibited because it lacks an editing exonuclease function. Since the physiological role of the pol α_2 associated exonuclease activity has yet to be established, its contribution to the inhibitory effects of thymine glycol lesions on synthesis by this enzyme remains hypothetical.

Our modeling studies indicate that the formation of the next base pair beyond the lesion site is energetically unfavorable, primarily as a consequence of the steric overlap of the methyl group of thymine glycol with the 5′ adjacent base in the template. Thus the model is again consistent with the experimental data insofar as it indicates that addition of the first nucleotide beyond the lesion is not an energetically favorable event. It should be noted, however, that the computer model deals only with initial and final states of the DNA molecule itself. The effect that the polymerase has on the DNA structure, i.e., the nature of the transition-state complex, is unknown but is of obvious importance in determining the response of a given polymerase to structural abnormalities in the DNA template.

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Registry No. *cis*-Thymine glycol, 1124-84-1; DNA polymerase, 9012-90-2; reverse transcriptase, 9068-38-6.

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