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Thymine glycols and urea residues in M13 DNA constitute replicative blocks *in vitro*

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ABSTRACT

Thymine glycols were produced in M13 DNA in a concentration dependent manner by treating the DNA with osmium tetroxide (OsO_4). For the formation of urea-containing M13 DNA, OsO_4-oxidized DNA was hydrolyzed in alkali (pH 12) to convert the thymine glycols to urea residues. With both thymine glycol- and urea-containing M13 DNA, DNA synthesis catalyzed by Escherichia coli DNA polymerase I Klenow fragment was decreased in proportion to the number of damages present in the template DNA. Sequencing gel analysis of the products synthesized by E. coli DNA polymerase I and T4 DNA polymerase showed that DNA synthesis terminated opposite the putative thymine glycol site and at one nucleotide before the putative urea site. Substitution of manganese for magnesium in the reaction mix resulted in increased processivity of DNA synthesis so that a base was incorporated opposite urea. With thymine glycol-containing DNA, processivity in the presence of manganese was strongly dependent on the presence of a pyrimidine 5' to the thymine glycol in the template.

INTRODUCTION

Thymine glycols are the major stable thymine radiolysis products produced in DNA by ionizing radiation (1). Thymine glycols are also thought to be formed in DNA as a consequence of oxidative stress (2). Urea residues, degradation products of unstable thymine hydroperoxides, are also produced in DNA by ionizing radiation (1) and hydrogen peroxide (3).

It has recently been shown that thymine glycols inactivate single stranded ΦX174 transfecting DNA (4), duplex PM2 transfecting DNA (5), and ΦX174 RF transfecting DNA (Laspia, Kow and Wallace, in preparation). In the studies with PM2 DNA, it was shown that thymine glycols have the same inactivation efficiency as apurinic sites, that is, it takes between 7 and 8 of either of these damages to constitute a single inactivating event (5). Further, urea residues also appear to have an inactivation efficiency similar to that of thymine glycols in ΦXRF transfecting DNA (Laspia et al., in preparation).

Since the measurement of the inactivation efficiency of a particular modified base in a repair proficient host includes both the ability of the lesion in question to consititute a replicative block or some other inactivating event and the efficiency of repair of that lesion, it seemed appropriate to ask whether, like apurinic sites (6), thymine glycols and urea residues were replicative blocks *in vitro*. This question seemed especially interesting in light of the observation that thymine glycols, urea residues and apurinic sites inactivate transfecting DNA to the same extent, yet, apurinic sites and urea residues are noninstructive while thymine glycols presumably would retain some coding specificity.

In this study we report that thymine glycols and urea consititue replicative blocks in an *in vitro* DNA synthesizing system employing either *Escherichia coli* DNA polymerase I or bacteriophage T4 DNA polymerase.

MATERIALS AND METHODS

Chemicals

[Methyl-^{3}H]-thymidine (72 Ci/mmol), [2-^{14}C]-TMP (58 mCi/mmol) were obtained from ICN and [α-^{32}P]dATP (3000 Ci/ mmol) was from Amersham. Unlabeled deoxyribonucleoside monophosphates and triphosphates were obtained from P-L Biochemicals. Dideoxyribonucleoside triphosphates and synthetic M13 primer pentadecamer were purchased from BioLabs. Osmium tetroxide (OsO_4) was obtained from Polyscience.

TLC plates of polyethyleneimine (CEL 300PEI/UV_{254}), cellulose (CEL 300/UV_{254}), and silica gel (SIL G/UV_{254}) were products of Merchery-Nagel. The following systems were routinely used as TLC developing solvents: 5% methanol was added to the lower phase of chloroform/methanol/water (4/2/1) (solvent I); ethylacetate/2-propanol/water (75/16/9) (solvent II); 2-propanol/5% ammonium acetate (pH 3.5) (60/25) (solvent III); and 1 M acetic acid/3 M lithium chloride (9/1) (solvent IV). The spots of urea and compounds bearing urea residue were located by p-dimethylaminobenzaldehyde (PDAB) reagent (1,7). The characteristic yellow color developed immediately after spraying. Compounds having deoxyribose and phospate group were visualized by cysteine (8) and molybdate reagents (9), respectively.

Thymine glycol and thymidine glycol were synthesized by permanganate oxidation of thymine and thymidine, respectively, and purified by cellulose column chromatography (10,11). Thymidine glycol monophosphate (TMP-glycol) was prepared as described by Rajagopalan *et al.* (12). The relative Rf ratio of TMP-glycol to TMP was 0.59 in the TLC analysis with cellulose plate and

solvent III. 2-Deoxyribosylurea phosphate (DRUP, N-(2-deoxy-β-D-erythro-pentofuranosyl) urea-5'-monophospate), a thymine ring fragmented product of TMP, was prepared by alkali hydrolysis of TMP-glycol at pH 12 (see also Fig. 4). DRUP gave single spot in the TLC analysis with PEI plate and solvent IV. The relative Rf ratio of DRUP to TMP-glycol was 0.83. The presence of a urea residue and a phosphate group in DRUP was confirmed by positive reactions of the spot with PDAB and molybdate reagents. Acid hydrolysis of DRUP in 0.5 N HCl at 100°C also yielded urea. The methods for the preparation of [2-^{14}C] TMP-glycol and [carbonyl-^{14}C] DRUP were essentially the same as those for unlabeled compounds. The labeled compounds were purified with TLC as described above using authentic markers.

Enzymes and DNA

E. coli DNA polymerase I Klenow fragment (hereafter referred to as Pol I) and bacteriophage T4 DNA polymerase were obtained from PL-Biochemicals. Single-stranded circular DNA of M13 phage (M13mp11) was purified from a culture medium of phage-transfected E. coli K12 JM101 (13). [^{3}H]-labeled M13mp11 DNA (10^{5} cpm/μg) was obtained by the same method as unlabeled DNA except that the culture medium contained [methyl-^{3}H] thymidine (17 μCi/ml).

For preparation of M13 DNA containing thymine glycol, M13 DNA (300 μg/ml) in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA was incubated with OsO_4 (0.08-0.4%) at 37°C for 0-40 min. OsO_4 reacts almost exclusively with thymine in DNA to oxidize thymine to thymine glycol (14,15,16). The reaction was terminated by removing OsO_4 from the reaction mixture by spun-column procedure (3, 17). The column was packed with Sephadex G-75 equilibrated with 10 mM Tris.HCl, pH 7.5, 1 mM EDTA. The number of thymine glycols introduced into DNA by OsO_4 treatment was evaluated by the alkali degradation assay of Hariharan (18) using [^{3}H]-labeled M13 DNA. For preparation of M13 DNA containing urea residue, thymine glycol-containing M13 DNA was subjected to alkali hydrolysis at room temperature by dialyzing overnight against 40 mM phosphate buffer (pH 12.0), 2 mM EDTA (3). The hydrolyzed DNA was dialyzed against 10 mM Tris.HCl, pH 7.5, 1 mM EDTA. Thymine glycols in DNA were quantitatively converted to urea residues by this procedure, as is discussed below. Apurinic (AP) DNA was prepared by incubating M13 DNA for 17 min in 30 mM KCl, 10 mM sodium citrate at 70°C and pH 5.0. These conditions introduced about 4 AP sites per M13 molecule (19).

Agarose gel electrophoresis

[^{3}H]-labeled M13 DNA, with or without modifications, was loaded onto

a 1.0% agarose gel and electrophoresed at 100 V for 4 h. After electrophoresis, the gel was cut into small pieces, and dissolved in 0.2 ml 1 N HCl. The radioactivity of each piece was counted in a scintillation counter.

DNA synthesis

Control, thymine glycol-, and urea-containing M13 DNA templates (0.5 µg), primed with synthetic pentadecamer, were incubated with E. coli Pol I (1 unit) at 22°C. The reaction mixture (20 µl) contained polymerization buffer (50 mM Tris.HCl, pH 8.0, 5 mM dithiothreitol, and 8 mM $MgCl_2$ (0.5 mM $MnCl_2$ when substituted for Mg^{++}), and was 50 mM for dATP, dCTP, dGTP and [methyl-^{3}H]dTTP (1660 cpm/pmol). The reaction was terminated by spotting aliquots (2 µl) on Whatman glass fiber filters (GF/A), washing with ice-cold 5% TCA-1% sodium pyrophospate, then ethanol. All filters were dried and assayed for radioactivity.

DNA sequencing

Reaction mixes (10 µl) contained primed template (0.5 µg), 50 µM each dCTP, dTTP, dGTP, and 5-10 µCi of [α-^{32}P] dATP (1.2-2.4 µM) and Pol I (1 or 2 units) or T4 DNA polymerase (1 unit) in polymerization buffer. Incubations were carried out for 15 min at 30°C for Pol I or 37°C for T4 DNA polymerase. At the end of 15 min incubation, the concentration of dATP was adjusted to 50 µM. Incubation was carried out for another 15 min. The reaction was then terminated by adding 20 µl of deionized formamide containing 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.37% EDTA. After boiling for 5 min, samples were electrophoresed at 1500 V on an 8% denaturing polyacrylamide gel. Dideoxy sequencing reactions were also carried out according to the method of Sanger et al. (20). The gels were autoradiographed using Kodak XAR-5 film at -70°C overnight.

RESULTS

Formation of thymine glycol by OsO_4 treatment

Thymine glycols were introduced into single-stranded M13 DNA by treating with OsO_4 either with different concentrations or by incubating for various times at 37°C. The evaluation of the number of thymine glycols introduced was based on the method of Hariharan (18). As shown in Figure 1, the formation of thymine glycol in M13 DNA was a linear function of either incubation time with OsO_4 or concentration of OsO_4. On the average, 2.1, 5.5 and 11.5 thymine glycols were produced per DNA molecule by a 10 min treatment with 0.08, 0.2 and 0.4% OsO_4, respectively.

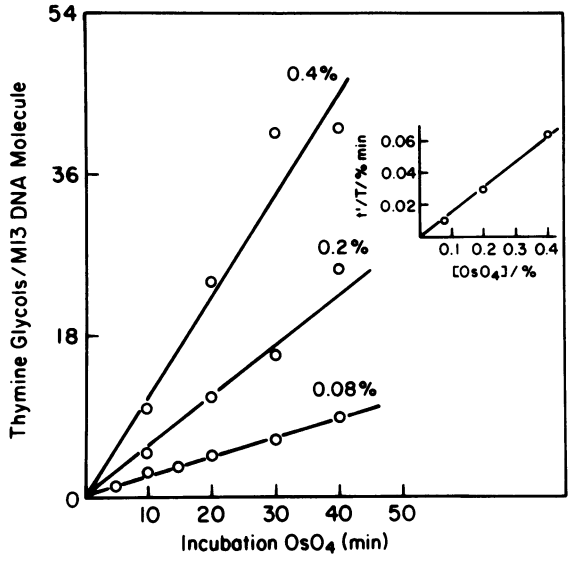

Figure 1. Production of thymine glycols in M13 DNA. DNA was treated with the indicated concentration of OsO_4 and thymine glycols were measured by the acetol fragment assay as described in Materials and Methods. The insert shows the relationship between the rate of thymine glycol formation and OsO_4 concentration. t'/T indicates the ratio of the number of thymine glycols (t')[4] to that of total thymine (T) in the M13 DNA molecule.

Conversion of thymine glycol to urea residue by alkali hydrolysis

The possibility that thymine glycols are converted to urea residues by alkali hydrolysis has been previously suggested (21,22). Recently, a quantitative study on the conversion of thymine glycol to its alkali-hydrolyzed product(s) on PM2 phage DNA was performed using enzyme assay with endonuclease III and exonuclease III from E. coli (3). In order to obtain more defined chemical evidence for the quantitative conversion of thymine glycol to urea, we performed alkali hydrolysis of [2-^{14}C] TMP-glycol. [2-^{14}C] TMP-glycol (10^5 cpm) was incubated in 20 µl of phosphate buffer at pH 12 at room temperature. At appropriate reaction times, 2 µl of the reaction mixture was removed and analyzed by PEI chromatography. Authentic markers were used to locate the glycol and urea derivatives of TMP. Figure 2 shows the rate of production of [carbonyl-^{14}C] DRUP from [2-^{14}C] TMP-glycol. After 2 hours of incubation, TMP-glycol was almost completely hydrolyzed. Further, the amount of TMP-glycol hydrolyzed was equal to that of DRUP formed.

We also attempted the alkali hydrolysis of unlabeled thymine glycol and thymidine glycol. The products were analyzed by TLC (Silica plate,

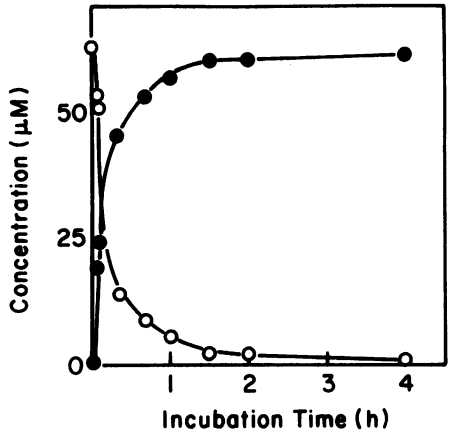

Figure 2. Conversion of thymidine glycol monophosphate (O) to deoxyribosylurea monophosphate (●) (DRUP) by treatment with alkali at pH 12. Both nucleotides were quantitated by PEI chromatography as described in Materials and Methods.

solvent I and II). We found that hydrolysis of thymine glycol gave urea. Interestingly, however, hydrolysis of thymidine glycol yielded a mixture containing at least three components. The Rf ratios of the products to thymidine glycol were 0.48, 0.40 and 0.30 with solvent II. These products showed positive reaction with both PDAB and cysteine reagents, indicating the presence of both urea residue and deoxyribose moiety. It is likely that 2-deoxy-β-D-ribofuranosylurea, formed by alkali hydrolysis of thymidine glycol, isomerized to pyranosides since 2-deoxyribosylurea is present in an equibrium containing 2-deoxy-α- and β-ribopyranosylureas and possibly 2-deoxy-β-D-ribofuranosylurea (23). It is noteworthy that with DRUP, isomerization to pyranoside is impossible because 5-OH is substituted by a phosphate group (Fig. 3).

In order to characterize thymine glycol- and urea-containing M13 DNAs, [^{3}H]-labeled DNAs were electrophoresed in a 1% agarose gel using apurinic DNA as a reference substrate. Both thymine glycol-containing DNA and its hydrolyzed product (urea-containing DNA) migrated in a similar manner to that of the control while alkali treatment of apurinic DNA resulted in strand breakage (data not shown). These results indicate that the urea site, produced by alkali hydrolysis of the thymine glycol residue, was resistant to subsequent hydrolysis under the present conditions (pH 12 and room temperature).

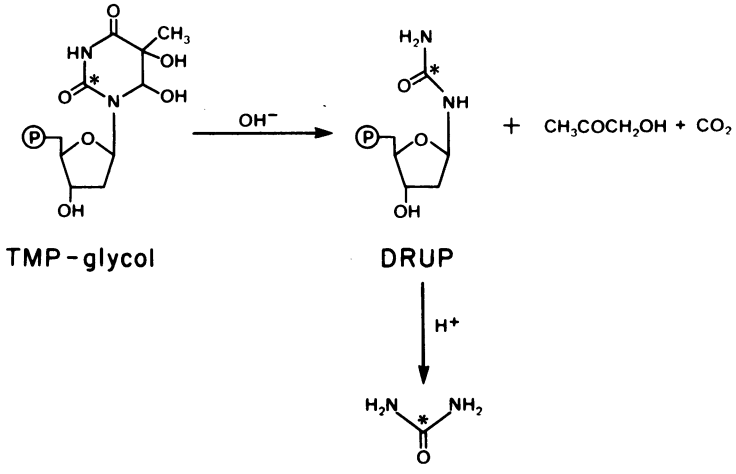

Figure 3. The production of deoxyribosylurea monophosphate (DRUP) by alkali digestion of thymidine glycol monophosphate (TMP-glycol). The labeled carbon (^{14}C) is designated by *.

Inhibition of DNA synthesis on M13 DNA templates containing thymine glycols or urea residues

In order to examine the ability of M13 DNA containing thymine glycol or urea residues to serve as a template for DNA synthesis, single-stranded M13 DNA, with or without the modifications, was primed and replicated by E. coli Pol I. The DNA templates used for replication contained on the average 2.2 or 11 thymine glycols or urea residues per molecule.

The polymerization reaction catalyzed by Pol I was significantly inhibited by the introduction of thymine glycols or urea residues into the template (Fig. 4). The extent of DNA synthesis decreased with increasing numbers of thymine glycols or urea residues in the template. Thus, thymine glycols and urea residues constitute replication blocks for in vitro DNA synthesis.

Analysis of the reaction products on DNA templates containing thymine glycols or urea residues

Although it appeared that both thymine glycols and urea residues consitituted replicative blocks to DNA synthesis, the position of termination was not clear. We therefore analyzed the reaction products formed by Pol I using sequencing gel electrophoresis. The exact position of the termination of synthesis can be determined by a dideoxy sequencing ladder. Termination at putative thymine glycol or urea sites would appear

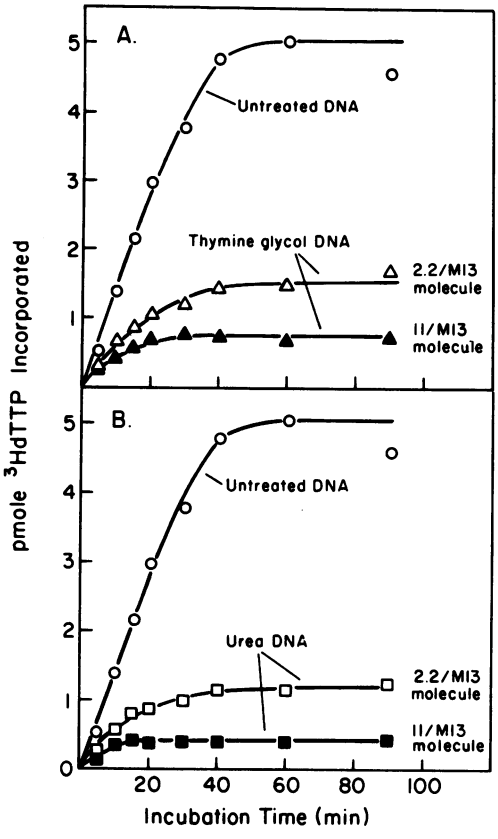

Figure 4. Inhibition of E. coli DNA polymerase I catalyzed DNA synthesis by an M13 template containing thymine glycols (A) or urea residues (B). Reaction conditions were described in Materials and Methods. Template DNA contained either no damages (O), or 2.2 (△, □), or 11 (▲, ■) thymine glycols or urea residues per M13 DNA molecule.

as a band opposite to dideoxy A in the sequencing ladder. The modified templates used contained about 11 thymine glycols or urea residues per M13 DNA molecule.

As shown in Figure 5, all termination bands occurred opposite to putative thymine glycol sites or at one nucleotide before putative urea sites. By adjusting the electrophoresis time, the termination ladders were read up to about 200 bases from the primer terminus and the relative intensities of the bands were scored (Fig. 6).

For comparison, DNA synthesis was also carried out with T4 DNA polymerase. With DNAs containing thymine glycol or urea, the patterns of

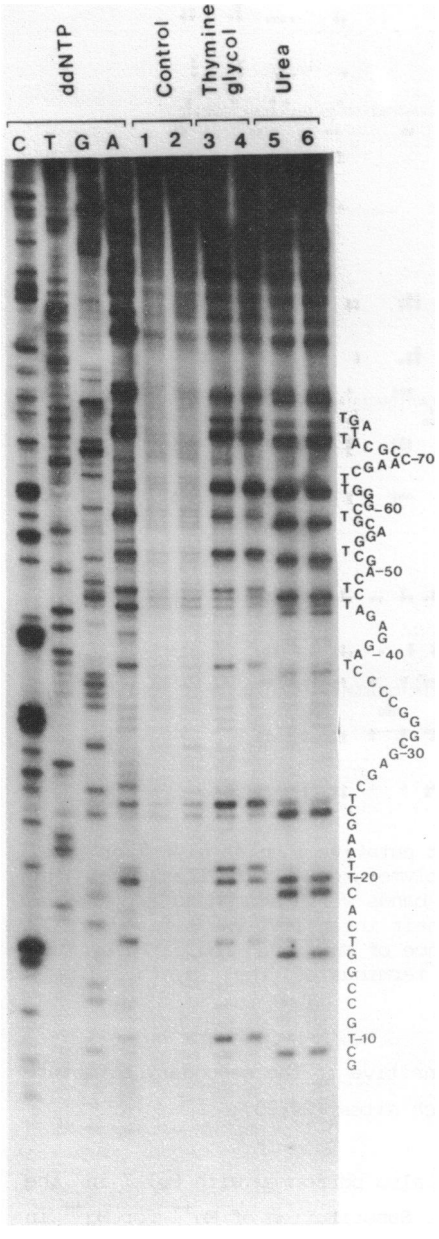

Figure 5. Polyacrylamide gel analysis of the products synthesized by E. coli DNA polymerase I Klenow fragment (Pol I) on control M13 DNA template and thymine glycol- or urea-containing templates. DNA synthesis was perfomed with 1 unit (lanes 1, 3, 5) or 2 units (lanes 2, 4, 6) of Pol I. Lanes C, T, G, A are sequence standards synthesized with Pol I and dideoxynucleotides on a control template. Numbering is from the position of the terminus of the synthetic primer (15-mer).

termination of DNA synthesis by T4 polymerase were essentially the same as those observed using Pol I (Fig. 7). The termination bands observed with the untreated DNA template are presumably due to hairpin barriers in the

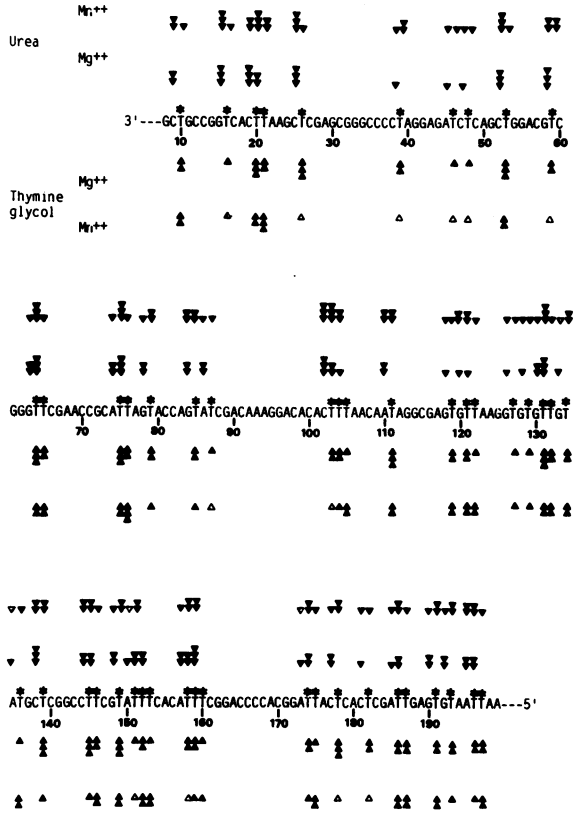

Figure 6. Termination of DNA synthesis at putative thymine glycol or urea sites (indicated by *) by E. coli DNA polymerase I Klenow fragment in the presence of Mg^{++} or Mn^{++}. The termination bands were scored as ▼▼ (strong), ▼ (medium), or ▼ (weak) according to their intensity. The sites where DNA synthesis passed through in the presence of Mn^{++} are indicated by ∇. Numbering is from the position of the terminus of the synthetic primer (15-mer).

template, since T4 DNA polymerase is sensitive to the secondary structure of the template and synthesis stops at such sites (24,25).

Effect of substitution of Mn^{++} for Mg^{++}

The DNA polymerization reaction was also performed with Pol I in the presence of Mn^{++} instead of Mg^{++} (Fig. 8). Substitution of Mn^{++} for Mg^{++} in the reaction mix resulted in increased procession of synthesis with both thymine glycol- and urea-containing DNAs. With thymine glycol-containing DNA, synthesis passed through the glycol site almost completely in several cases (positions 16, 26, 46, 48, 50, 59, 87, 103, 151, 158, 178, 182). It

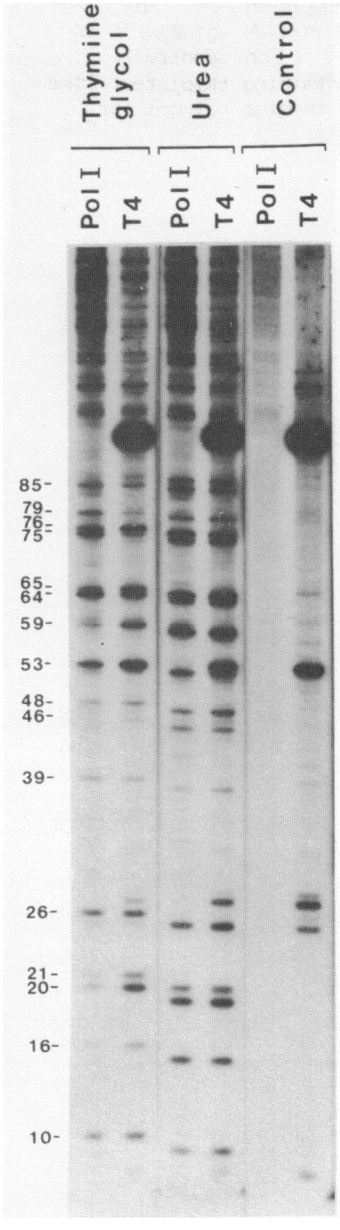

Figure 7. Polyacrylamide gel analysis of the products synthesized by E. coli DNA polymerase I Klenow fragment (Pol I) or phage T4 DNA polymerase (T4). The numbers mark the putative thymine glycol or urea sites.

appears that, under conditions of relaxed specificity, DNA synthesis processed through the thymine glycol site most efficiently if the glycol was followed by cytosine or thymine in 5' direction in the the template (positions 16, 26, 46, 48, 59, 87, 103, 151, 158, 178, 182). With urea-

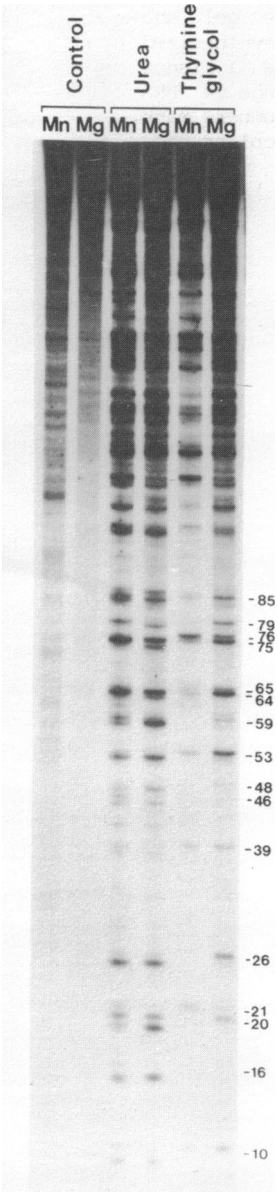

Figure 8. Effect of substitution of Mn^{++} for Mg^{++} on the processivity of DNA synthesis by E. coli DNA polymerase I on control, thymine-glycol- or urea-containing templates. The numbers mark the putative thymine glycol or urea sites.

containing DNA, the termination bands occurred not only at one nucleotide before the putative urea site but also opposite to the site. The appearance of termination bands opposite the putative urea site shows that under conditions of relaxed specificity, one nucleotide can be incorporated

opposite the urea site. However, complete loss of pairing at the primer terminus did not permit further elongation of the strand by Pol I. In the presence of Mn^{++}, the incorporation of one nucleotide opposite the damage residue has been reported for both AP DNA and UV-damaged DNA templates using Pol I (6,26,27).

DISCUSSION

Thymine glycol and fragmented thymine residues such as urea and N-substituted ureas are produced by ionizing radiation (1) and certain chemical reagents (3,11,12,14,15,16,22,28). Although the damaged thymine residues may be removed by base excision-repair, such lesions may be encountered by the replication fork before they are repaired. In the present work we have demonstrated that thymine glycols and urea residues, a model for fragmented thymine residues, constitute replicative blocks of DNA synthesis catalyzed by E. coli DNA polymerase I Klenow fragment and T4 DNA polymerase. Furthermore, sequencing gel analysis revealed that in the presence of Mg^{++}, DNA synthesis terminated opposite the putative thymine glycol site or at one nucleotide before the putative urea site. Similar results have been observed by Hayes and LeClerc (personal communication). Incorporation of a nucleotide opposite the thymine glycol suggests that although the pyrimidine ring structure is distorted due to saturation of C5-C6 double bond, the thymine glycol is still instructive, that is, it possesses some information for base pairing. In contrast, termination at one nucleotide before the urea site indicates that like AP sites (6,29,30), urea residues are noninstructive, that is devoid of information for base pairing. We are currently assuming that dAMP was incorporated opposite the thymine glycol site since, as in the case for intact TA pair, the 3-amino and 4-carbonyl groups in thymine glycol could form hydrogen bonds with the 2-nitrogen and 6-amino groups of adenine, respectively. These hydrogen bonds would be expected to be weaker than those of the intact TA pair due to ring distortion. Further, if dGMP, dTMP or dCMP had been incorporated opposite thymine glycol, the mismatched nucleotides should have been efficiently excised efficiently by the 3'→5' exonuclease activity associated with DNA polymerase I and T4 DNA polymerase. At this stage, the possibility of hydrogen bonding between the amino group of urea and the 2-nitrogen of adenine can not be fully ruled out.

The incorporation of the correct base opposite thymine glycol might inhibit the 3'→5' exonuclease activity associated with the polymerase used,

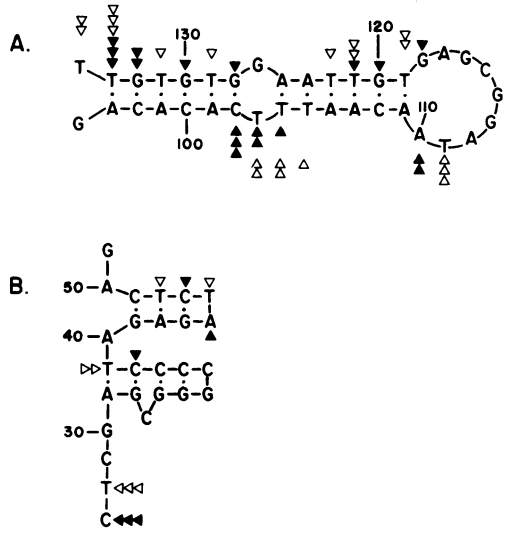

Figure 9. Potential secondary structures in M13mp11 template and the relative intensity of the termination bands of DNA synthesis by E. coli DNA polymerase I on thymine glycol (▽) and urea (▼) containing templates.

but the weak stacking and pairing interactions might prevent proper binding of the polymerases to the primer terminus site and subsequent incorporation of nucleotide. Incorporation of a nucleotide opposite a damaged site using DNA polymerase I has been shown with a DNA template containing 4-hydroxyaminoquinoline-1-oxide adducts of guanine (31). With DNA containing N-acetyl-2-aminofluorine adducts of guanine (AAF-dG), incorporation catalyzed by Pol I stops immediately preceding the lesion, while with reverse transcriptase, the correct nucleotide (dCMP) is incorporated opposite certain lesions (32). The incorporation of dCMP seems to reflect the normal base pairing of C with AAF-dG in the anti structure in two possible conformations. Thus, it appears that the nucleotide incorporated opposite a damaged lesion, such as thymine glycol or AAF-dG in anti structure, will not be excised by the 3'→5' exonuclease activity since proper base pairs can be formed. However, incorporation of a nucleotide opposite lesions such as apurinic sites (6,29,30), pyrimidine dimers (6,27), and urea (this work) leads to loss of pairing at the primer terminus and apparently results in excision by 3'→5' exonuclease activity.

The variation in the intensity of the termination bands (Fig. 6) can be partially explained by non random production by OsO_4 of thymine glycols

in the template (OsO_4 preferentially reacts with thymine in single stranded DNA (14)). For example, a variation in intensities of bands was observed in the region 96-134 which can form a hairpin structure (Fig. 9A). The weak termination bands at 39, 46, 48 can also be accounted for by less efficient formation of thymine glycol due to potential secondary structure (Fig. 9B). The existence of these secondary structures at positions 29-49 and 96-134 has also been observed in studies with T4 DNA polymerase (33), which is sensitive to secondary structure in the template molecule (24,25,33). Termination bands of DNA synthesis with T4 polymerase were observed in M13 DNA at positions around 27 and 94, that is, just before the potential hairpin loops (positions 29-49 and 96-134). In addition to the termination bands around 27 and 94, T4 DNA polymerase gave a band at position 53 (Fig. 7). This result suggests that secondary structure is also possible in the region following position 53. Termination bands opposite thymine glycols were strong in this region however, suggesting that the potential secondary structure may have melted during the OsO_4 treatment at 37°C.

Recently, Refolo *et al.* (34) have shown that the aflatoxin B_1-induced replication blocks occur predominantly at potential hairpin loop regions in single-stranded M13 DNA template. These authors suggested that the reaction of aflatoxin B_1 with the single-stranded M13 DNA is highly sequence specific and that the compound has a preference for potential hairpin regions compared with single-stranded regions. This is in contrast with the reactivity of OsO_4 which prefers single stranded regions.

In several cases, in the presence of Mn^{++}, DNA synthesis catalyzed by Pol I passed through thymine glycol sites almost completely (Figs. 6 and 8). Similar results have been observed with UV-induced lesions (27), AAF lesions (27,32), and AP sites (6,29,30). Manganese is known to be highly mutagenic and to reduce the fidelity of DNA synthesis by DNA polymerases (35). The efficiency of the procession of DNA synthesis beyond the thymine glycol site appears to be dependent on the nucleotide following the thymine glycol. The relative intensity of the termination bands $I(Mn^{++})_{AV}/I(Mg^{++})_{AV}$ in Table I indicates that DNA synthesis passed through the lesion most easily if the putative thymine glycol in the template was followed by a C in the 5' direction. Thymine was slightly less efficient than C, and the purines had almost no effect. If hydrogen bonding determined the ability to process beyond the thymine glycol site, efficient procession should have occurred when the thymine glycol was followed by guanine as well as cytosine. Since pyrimidines were preferred, we suggest that stacking

Table I. The Effect of the 5' Nearest Neighbor Nucleotide on the Procession of DNA Polymerase I Past the Thymine Glycol

Sequence	Number of Sequences	$I(Mg^{++})_{AV}$	$I(Mn^{++})_{AV}$	$I(Mn^{++})_{AV}/I(Mg^{++})_{AV}$
3'..T-C..5'	13	1.8	0.6	0.3
T-G	9	1.7	1.8	1.1
T-A	13	1.9	1.8	1.0
T-T	15	2.3	1.3	0.6

I = average of termination band intensities, based on the scores in Figure 6; 0, no band; 1, weak band; 2, medium band; 3, strong band.

interactions between the nucleotide at the primer terminus and the nucleotide to be inserted could facilitate the elongation of the primer. Since we are currently assuming that dAMP was incorporated opposite the thymine glycol, these interactions could be stacking between A and G, or A and A.

Since both thymine glycols and urea residues are strong replicative blocks to polymerases *in vitro*, these lesions should be potentially lethal *in vivo* as has been observed (4, 5, Lapsia *et al.* in preparation). Urea residues might also be mutagenic since they, like AP sites, appear to be noninstructive. If however, as with AP sites, A is preferentially inserted by the polymerase (29,30) no mutation would result since in the case of the urea residue, incorporation of A would result in a correct base pair. With the thymine glycol, incorporation occurs opposite the damage. Since it is likely that the incorporated base is A, the correct base would be inserted and no mutation would be expected.

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