Thymine glycol lesions terminate chain elongation by DNA polymerase I *in vitro*

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ABSTRACT

Single-strand circular DNA from bacteriophage M13mp9 was chemically modified with osmium tetroxide to introduce specifically *cis*-thymine glycol lesions, a major type of DNA damage produced by ionizing radiation. An oligonucleotide primer was extended on damaged and undamaged templates using either the large fragment of *E. coli* pol I or T4 DNA polymerase. The reaction products were analysed by electrophoresis alongside a DNA sequence ladder. Synthesis on the damaged templates terminated at positions opposite thymine bases in the template. These results indicate that *cis*-thymine glycol lesions in single-strand DNA constitute blocks to synthesis by DNA polymerases *in vitro*. Surprisingly, replication halts after the correct nucleotide, dAMP, is inserted opposite the lesion. These results imply that the primary effect of the thymine glycol lesion is suppression of DNA synthesis and that the lesion is not a potent mutagen.

INTRODUCTION

Ionizing radiation produces drastic and permanent effects on living cells by damaging DNA. The thymine bases in DNA are particularly susceptible to radiation damage, and undergo an oxidative conversion to thymine 5,6-glycol as one major DNA damage process (1,2). At the molecular level, the precise functional consequences of the introduction of oxidized thymine bases into DNA are unknown. Nevertheless, detailed understanding of the effect of these lesions on processes such as DNA replication is critical to eventual understanding of the mechanisms of the profound effects of ionizing radiation on living organisms.

Enzyme-mediated repair of radiation-induced thymine base damage *in vivo* has been reported (3-5), and enzymes have been isolated from both procaryotic and eucaryotic sources that specifically recognize and remove oxidized thymine bases,

including thymine glycol, from DNA in vitro (6). The existence of repair systems that operate specifically on oxidized thymine bases implies that this type of radiation damage plays a significant role in determining the biological consequences resulting from exposure to ionizing radiation.

A number of studies using a single-strand DNA template/primer system have shown that DNA polymerases replicating a template that contains lesions induced by ultraviolet light or a variety of chemical carcinogens terminate DNA synthesis at or before the nucleotide positions corresponding to potential target sites for the damaging agents (7-11). We used osmium tetroxide (12) to specifically introduce cis-thymine glycol lesions into single-strand DNA and a similar in vitro polymerization assay to assess the effects of this lesion on DNA synthesis.

As a consequence of its non-planar, non-aromatic structure, thymine glycol (cis-5,6-dihydroxy 5,6-dihydrothymine,) is sterically larger than the natural base thymine and lacks all potential for stacking interactions with adjacent bases. Although it should retain the capacity to form normal hydrogen bonds in a base pair with adenine, thymine glycol also has the potential for formation of two additional, abnormal hydrogen bonds. Thymine glycol thus represents a major deviation from the natural structure of DNA; one whose physicochemical, and therefore biological consequences are likely to be significant.

MATERIALS AND METHODS

Reagents

Osmium tetroxide was obtained from Sigma. All DNA polymerases were obtained from New England Biolabs as was a 17 nucleotide primer that anneals with its 3' terminus immediately adjacent to the EcoRI site of M13mp9 DNA or 3 nucleotides upstream of the HindIII site of M13mpl8 DNA.

Template preparation

M13mp9 and M13mpl8 DNAs were isolated from phage by polyethylene glycol precipitation and phenol extraction (13) followed by ethanol precipitation. Oxidized DNA was prepared by incubating 30 ul (6 ug) of DNA in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) with an equal volume of a 4% solution of osmium

tetroxide (80 mM final concentration) at 55⁰ for 15 or 30 min. The sample was extracted 3 times with 5 volumes of ice-cold ether to remove unreacted osmium tetroxide and precipitated with ethanol at -20⁰. This treatment results in 15-30% of the thymine bases being converted to the cis-glycol (5,12,14). Oxidized DNA, resuspended in distilled, deionized water and stored at -20⁰, was routinely used within one week after preparation. Agarose gel electrophoresis of the chemically modified circular DNA showed it to be homogeneous with no evidence of strand scission.

Undamaged single-stranded DNA (2.5 ug) was annealed to 5 ng of primer in a total volume of 10 ul containing 15 mM Tris-HCl, pH 8.5, 15 mM MgCl₂ at 55⁰ for 15 min followed by slow cooling to room temperature. Approximately 0.6 ug of the oxidized template was annealed to 1.2 ng of the primer under similar conditions in a final volume of 10 ul.

Enzyme Reactions

For DNA synthesis reactions on damaged templates, a 2.5 ul aliquot of the annealed, oxidized template/primer was incubated with 1 unit (1 ul) of the Klenow fragment or polymerase I in a reaction mix containing 36 uM each of dGTP, dCTP, and dTTP plus 10 uCi (17 pmol) of [α -³²P]dATP (ICN, >600 Ci/mmol) in a final volume of 5.5 ul. After 15-30 min (see Figure legends) at room temperature, unlabeled dATP was added to a final concentration of 100 uM and the incubation continued for another 15-30 minutes. In the control reaction 1 ul of a 1:20 dilution of the annealed, undamaged template/primer was incubated with 1 unit of the Klenow fragment as described above except that unlabeled triphosphates were present at 50 uM in a reaction volume of 4 ul. The reactions with T4 DNA polymerase were similar to those with the Klenow fragment except that the buffer contained 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and unlabeled dNTPs at a concentration of 40 uM.

In some experiments dATP was either present at 38 uM or omitted. These reaction mixtures, which were otherwise identical, contained 30 uM each of dCTP and dTTP plus 20 uCi (50 pmol) of [α -³²P]dGTP (Amersham, >400 Ci/mmol) in a final volume of 8 ul. The reactions were incubated at room temperature for 30

min. The dideoxy sequencing reactions were carried out as described (15,16) using [α - ^{32} P]dATP as the label. Samples of 2 μ l were removed from each reaction and prepared for electrophoresis by the addition of 2 μ l dye/formamide solution (17). All samples were heated at 95 $^{\circ}$ for 3 min and loaded immediately onto the gel.

Electrophoresis

Products from the in vitro DNA synthesis reactions were analyzed by electrophoresis through thin (0.4 mm) 8% polyacrylamide (19:1 w/w acrylamide:N,N'-methylene-bis-acrylamide) gels containing TBE buffer (100 mM Tris, pH 8.3, 100 mM borate, 2 mM EDTA, 8.3 M urea) at 1500 V (20-25 mA) until the bromophenol blue dye marker was 2-3 cm from the bottom of the gel (approximately 2 hours). The gel was fixed in 2 liters of 10% methanol:10% acetic acid in water for 20 minutes, transferred to Whatman 3 mm paper and dried under vacuum. Autoradiography was performed overnight at room temperature using Kodak XAR-5 x-ray film.

RESULTS

Mapping of Arrest Sites

We identified sites at which DNA polymerase was arrested when replicating a template DNA containing damage lesions using an approach based upon that of Moore and Strauss (7). Single-strand circular DNA from bacteriophage M13 (M13mp9) was chemically oxidized with osmium tetroxide to selectively convert a fraction of the thymine bases to the cis-thymine glycol derivative. Osmium tetroxide is highly selective for thymine bases in DNA (12) and produces two cis diastereomers. The damaged template DNA was annealed to a primer which was extended using E. coli DNA polymerase I (Klenow fragment). As a control we also carried out the elongation on undamaged DNA templates. Fragments synthesized on the damaged template were dramatically shortened in comparison to controls, and corresponded exactly in length to the positions of thymine residues in the template (see Figure 1). Moreover, the average fragment length synthesized by the polymerase decreased when the template DNA was more extensively modified, but the sequence specificity of the arrest sites was

Figure 1. Autoradiogram of a high resolution denaturing polyacrylamide gel used to map arrest sites in the osmium tetroxide-treated M13mp9 sequence. Lanes A, C, G, T refer to dideoxy sequencing reactions on the M13mp9 DNA template. Lane 1 is the analysis of products synthesized on undamaged templates. Lanes 2 and 3 are the analyses of products synthesized on template DNA treated with osmium tetroxide for 15 min and 30 min respectively. Reactions were carried out at room temperature for 30 min in the presence of labeled dATP, followed by a 30 min chase in the presence of excess unlabeled dATP. Approximately 10-fold less total DNA (μg of template) was loaded in Lane 1 compared to Lanes 2 and 3.

[illegible][illegible]

unchanged (compare lanes 2 and 3, Figure 1). Thus chain elongation is arrested at sites corresponding to putative thymine glycol lesions in the template, with the insertion of a base at the site of the lesion. Figure 2 shows a portion of the DNA sequences from M13mp9 and M13mpl8 indicating the positions at which the Klenow fragment terminated synthesis when replicating the damaged template. Strong arrest sites occur only at the positions of thymine bases in the template, in keeping with the known specificity of osmium tetroxide.

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Figure 3. Autoradiogram of a gel showing the analysis of products synthesized on oxidized M13mp9 DNA templates by T4 DNA polymerase. Lane A refers to the sequencing reaction using dideoxyATP. Lanes 1-3 show the analysis of products synthesized on undamaged templates by T4 DNA polymerase, Pol I, and Pol I (Klenow fragment) respectively. Lanes 4-6 show the products synthesized on template DNA treated with osmium tetroxide for 15 min by T4 DNA polymerase, pol I, and Klenow fragment respectively. Reactions were carried out at room temperature for 15 min in the presence of labeled dATP, followed by a 15 min chase in the presence of excess unlabeled dATP.

When T4 DNA polymerase was used to replicate damaged templates, we observed a pattern of arrest sites similar to that obtained with the Klenow fragment (Figure 3, Lane 4). The T4 polymerase was also arrested at a number of specific sites on undamaged templates (Figure 3, Lane 1). However these sites did not correlate with the positions of thymine bases in the template and presumably reflect the known sensitivity of this polymerase to secondary structure in the DNA template (18). We also attempted to use intact polymerase I (having 5'-3' exonuclease activity) in this system. Although Pol I was able to synthesize relatively long fragments on undamaged templates (Figure 3, Lane 2), few fragments longer than about 20 nucleotides were detectable when synthesis occurred on damaged templates (Figure 3, Lane 5). This result presumably reflects the strong inhibition of DNA synthesis by the presence of damaged thymine bases in the template, coupled with exonucleolytic degradation from the 5' end of the primer.

Identification of base inserted opposite the lesion

To identify the base inserted opposite the lesion, we carried out primer extension experiments with the Klenow fragment in the presence and absence of dATP. In the absence of dATP, a single major band occurs at the position corresponding to one nucleotide before the first thymine base encountered in the template (Figure 4, Lane 1). A similar result was observed with an undamaged template (not shown). This represents the addition of the first three nucleotides (GCC) to the 3' end of the primer (see Figure 2, M13mp18 DNA). When dATP is present in the reaction, strong arrest sites at positions corresponding to thymines in the template are again observed (Figure 4, Lane 2). In particular, a strong arrest site occurs at the first thymine base encountered in the template (position 21). The band intensity at position 21 is proportional to the number of molecules in which synthesis stopped at this site since only one labeled dGMP residue is incorporated per fragment of this length. A much weaker band is observed at position 21 in the absence of dATP than in the presence of dATP under otherwise identical reaction conditions. Therefore we conclude that dAMP is inserted opposite the lesion at position 21 in molecules that contain an oxidized

Figure 4. Autoradiogram of a gel showing the analysis of products synthesized on thymine glycol-containing templates by the Klenow fragment in the absence or presence of dATP. Lanes A, C, G, and T refer to dideoxy sequence standards. Lanes 1 and 2 are the analyses of products synthesized in the absence or presence of dATP respectively. M13mp18 DNA was treated with osmium tetroxide for 15 min and DNA synthesis was carried out for 30 min at room temperature.

thymine base at this site. However we cannot exclude the possibility that nucleotides other than dAMP may be inserted opposite a lesion at this site at low frequency. Preferential incorporation of dAMP presumably occurs opposite other damaged thymine bases in the template; however our data do not directly demonstrate that this is true.

We also note that weak arrest sites were occasionally observed at bases other than thymine. The majority of these weak stops occur either one nucleotide before a strong thymine arrest site or opposite a cytosine in the template (Figure 4). The origin of these weak bands is unknown but they may be the result of cleavage by the 3'-5' exonuclease activity of pol I at sites of strong arrest or a limited degree of modification of other bases, particularly cytosine, by osmium tetroxide.

DISCUSSION

We conclude that thymine glycol in DNA can form a proper base pair with adenine and that it can serve as an appropriate template residue for addition of the correct nucleotide at the site of the lesion. This suggests that this particular radiation damage lesion is not a potent mutagen. The conclusion that thymine glycol lesions are responsible for the effects on DNA synthesis that we observe in vitro is based upon the known specificity of the osmium tetroxide reagent (12,19). Moreover, cis-thymine glycol lesions are stable at neutral and acidic pH although they do break down under alkaline conditions (pH > 11) to form predominantly urea and N-substituted urea derivatives (20,21). Our experimental conditions were designed to minimize the contribution of breakdown products to the analysis by using freshly-prepared, oxidized DNA at a pH near neutrality. We also note that synthesis by pol I or T4 DNA polymerase in the presence of magnesium terminates one nucleotide before putative urea residues rather than at the site of the lesion (22). We observed no significant changes in the pattern of arrest sites for any given template preparation during the time period within which it was used. Thus we consider it unlikely that lesions other than thymine glycol contribute significantly to the pattern of strong arrest sites that we observe. However we cannot

exclude the formal possibility that a minor, unknown (non-urea) reaction product of osmium tetroxide with thymine could contribute to the observed effects on DNA synthesis. It is also possible that some of the minor bands that were observed one nucleotide before a template thymine could represent stops before spontaneously-occurring urea breakdown products.

Our result with thymine glycol is in marked contrast to those obtained in similar experiments with a variety of other DNA lesions, in which synthesis generally terminates one nucleotide before the site of the lesion (7-11). However, the further incorporation of successive nucleotides beyond the lesion site is dramatically inhibited. This unexpected finding indicates that the template/primer containing this modification of the thymine ring is not utilized efficiently by the polymerase beyond the point of the lesion. The distortions of overall geometry as well as disturbances in interactions with flanking and opposing bases which necessarily arise from this structural lesion may underlie this functional inadequacy. Additionally, the possible formation of hydrogen bonds between the extra hydroxyl groups of the glycol and amino acid side chains in the DNA binding site of the polymerase could prevent motion of the DNA relative to the enzyme, thus blocking further chain elongation. The recently reported crystallographic data for the Klenow fragment (23) may allow us to address these questions in greater detail.

Cunningham and Weiss (24) have recently isolated a mutant strain of E. coli that is deficient in endonuclease III and shows no detectable thymine glycol glycosylase activity in crude extracts in vitro. Surprisingly, this strain was not unusually sensitive to agents that produce thymine glycol such as gamma irradiation or hydrogen peroxide. Thus, as these authors suggest, thymine glycol may not represent a particularly lethal lesion in vivo. There is obviously a dichotomy between the dramatic effect on DNA polymerase function that we observe in vitro and the mutant studies. There are several possible explanations that should be considered. First, it is conceivable that, in the absence of the specific glycosylase, thymine glycol lesions could be repaired by an alternative pathway (e.g.

nucleotide excision repair). Alternatively, the effect of thymine glycol lesions on pol III, the major replicative enzyme of *E. coli*, could be qualitatively different from that seen with the Klenow fragment and T4 DNA polymerase. Finally, it is possible that a polymerase can replicate past a thymine glycol lesion when it occurs in the template strand of a duplex DNA but not when it is present in a single-strand template. The latter hypothesis was suggested by the work of Piette and Hearst (25) who showed that psoralen monoadducts in single-strand DNA block synthesis by pol I but do not block synthesis via nick translation on a DNA duplex.

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