Differences in replication of a DNA template containing an ethyl phosphotriester by T4 DNA polymerase and *Escherichia coli* DNA polymerase I

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ABSTRACT

A DNA template containing a single ethyl phosphotriester was replicated *in vitro* by the bacteriophage T4 DNA polymerase and by *Escherichia coli* DNA polymerase I (DNA pol I). *Escherichia coli* DNA pol I bypassed the lesion efficiently, but partial inhibition was observed for T4 DNA polymerase. The replication block produced by the ethyl phosphotriester was increased at low dNTP concentrations and for a mutant T4 DNA polymerase with an antimutator phenotype, increased proofreading activity, and reduced ability to bind DNA in the polymerase active center. These observations support a model in which an ethyl phosphotriester impedes primer elongation by T4 DNA polymerase by decreasing formation of the ternary DNA polymerase–DNA–dNTP complex. When primer elongation is not possible, proofreading becomes the favored reaction. Apparent futile cycles of nucleotide incorporation and proofreading, the idling reaction, were observed at the site of the lesion. The replication block was overcome by higher dNTP concentrations. Thus, ethyl phosphotriesters may be tolerated *in vivo* by the up-regulation of dNTP biosynthesis that occurs during the cellular checkpoint response to blocked DNA replication forks.

INTRODUCTION

A variety of adducts are produced in DNA by exposure to endogenous and exogenous alkylating agents. Some of the most abundant lesions formed are 7-alkylguanine, O^6-alkylguanine, 3-alkyladenine and alkyl phosphotriesters. While 7-methylguanine and 3-methyladenine block DNA replication, O^6-methylguanine is replicated, but more slowly than unmodified guanine and bypass results in frequent misincorporation of dTMP (1–4). Methyl and ethyl phosphotriesters, on the other hand, do not appear to be major blocks to replication; however, this conclusion is based on a single report, which measured the ability of *Escherichia coli* DNA polymerase I (DNA pol I) to fully replicate a short template strand containing a single ethyl phosphotriester (5). The apparent relative insensitivity of *E.coli* DNA pol I to ethyl phosphotriester modification of the DNA backbone is in marked contrast to several other DNA binding proteins, which are more severely inhibited by ethyl phosphotriesters. DNA binding proteins sensitive to methylation or ethylation of internucleotide phosphate groups include T4 polynucleotide kinase (6), MutS (7), MutY (8), RNA polymerase (9), human AP endonuclease (10) and all exo- and endonucleases (11,12).

One reason that effects of ethyl phosphotriesters on DNA replication have not been examined further is that many cells replicate their DNA in the presence of these DNA backbone modifications. Even though more than 50% of the DNA damage produced by treatment with ethylnitrosourea is ethyl phosphotriester adducts (13,14), ethylnitrosourea treatment of rats (15) or human cells in culture (16) produces long-lived ethyl phosphotriesters that are detected after several days and several cycles of cell growth and division. There is no known mechanism for the repair of alkyl phosphotriesters in human or rodent cells. Even in *E.coli*, the O^6-methylguanine–DNA methyl transferase removes only the Sp but not the Rp isomer of the methyl phosphotriester (17–19); a related mechanism has been reported for *Aspergillus nidulans* (20). Since DNA replication continues in the presence of ethyl phosphotriesters, these lesions must either not impede DNA replication significantly or there is an effective tolerance mechanism.

In order to learn more about the molecular basis for the partial inhibition of replication produced by an ethyl phosphotriester for *E.coli* DNA pol I and to determine the sensitivity of other DNA polymerases, we carried out a series of *in vitro* reactions with a DNA template containing a single ethyl phosphotriester. *Escherichia coli* DNA pol I was re-examined and the bacteriophage T4 DNA polymerase was tested for the first time. *Escherichia coli* DNA pol I is a member of a sequence-related family of DNA polymerases called Family A DNA polymerases (21) while T4 DNA polymerase is a member of another group of sequence-related DNA polymerases called Family B (21) or alpha-like DNA polymerases (22), which includes the eukaryotic DNA polymerases alpha, delta and epsilon that are essential for chromosome replication. Although all DNA polymerases share similarities in the polymerase active center and in binding DNA at the primer terminus, there are also differences, which may be exacerbated by an ethyl phosphotriester.

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Table 1. Ethyl phosphotriester and control DNA substrates

Ethyl phosphotriester[a] decamer	5′-TTT<Et>TCTATTT
Ethyl phosphotriester template	5′-TTT<Et>TCTATTTCCCGTCCACTTCGCCp[b]
Control template	5′-TTTTCTATTTCCCGTCCACTTCGCCp[b]
Primer[c]	5′-^{32}P-GGCGAAGTGGACGGG
Ethyl phosphotriester DNA substrate (PTE)[d]	5′-^{32}P-GGCGAAGTGGACGGG
	3′- pCCGCTTCACCTGCCCT$_1$T$_2$T$_3$A$_4$T$_5$C$_6$T$_7$<Et>T$_8$T$_9$T$_{10}$
Control (C) DNA substrate	5′-^{32}P-GGCGAAGTGGACGGG
	3′- pCCGCTTCACCTGCCCT$_1$T$_2$T$_3$A$_4$T$_5$C$_6$T$_7$T$_8$T$_9$T$_{10}$

[a]The <Et> symbol indicates the position of the ethyl phosphotriester. There is an approximate 50:50 mixture of Rp and Sp ethyl phosphotriesters (6).
[b]A phosphate at the 3′ end protects the DNA from digestion by the exonuclease activity of T4 DNA polymerase.
[c]The primer was labeled on the 5′ end with ^{32}P by T4 polynucleotide kinase.
[d]The template nucleotides are numbered from one to 10 beginning with the first template nucleotide replicated. Full-length primer extension requires the incorporation of 10 nt. If the ethyl phosphotriester impedes DNA replication, replication will pause or stop after incorporation of 7 nt.

Structural studies indicate that Family A and B DNA polymerases make contacts to several oxygen atoms in the phosphodiester backbone (23–26). Some of these interactions are expected to be perturbed by alkylation, particularly contacts with the DNA backbone in the vicinity of the nucleotide insertion site where the template strand is forced into an abrupt turn (23–26). Since DNA polymerases translocate with respect to the template strand for each nucleotide incorporated, protein–DNA contacts must be continuously made and broken. Conformational changes in the template strand have been observed in real time for the Klenow fragment of *E.coli* DNA pol I and for T4 DNA polymerase by using the fluorescence of the base analog 2-aminopurine (27–29). The fluorescence of 2-aminopurine within a DNA strand is quenched by base stacking interactions (30,31), but protein interactions that disrupt base stacking with 2-aminopurine can produce a large increase in fluorescence intensity. Base unstacking and then restoration of base stacking in the template strand were observed during incorporation of two consecutive nucleotides by the T4 DNA polymerase (29). If an ethyl phosphotriester interferes with these conformational changes, then replication will likely be impaired.

We report that an ethyl phosphotriester impedes replication by wild-type T4 DNA polymerase when located on the internucleotide phosphate group between the newly replicated and the next templating base. Additional insights into the mechanism were learned from studies of two mutant T4 DNA polymerases: an exonuclease-deficient T4 DNA polymerase (32) and a mutant DNA polymerase with increased DNA polymerase accuracy, the antimutator I417V–DNA polymerase (33). Translesion replication (TLR) by the wild-type and mutant T4 DNA polymerases was also highly sensitive to nucleotide concentration. In contrast, *E.coli* DNA pol I efficiently bypassed the lesion. Based on these results, we propose a model from these studies that may explain how ethyl phosphotriester adducts in the DNA backbone are tolerated.

MATERIALS AND METHODS

DNA polymerases

Purification and characterization of the wild-type and mutant T4 DNA polymerases, the exonuclease-deficient D112A/

E114A–DNA polymerase and the I417V–DNA polymerase, have been described (32,33). *Escherichia coli* DNA pol I was purchased from Gibco-BRL.

DNA substrates

The ethyl phosphotriester decamer oligonucleotide, 5′-TTT-<Et>TCTATTT (Table 1) was synthesized as a mixture of approximately 50:50 Rp and Sp isomers (6). The <Et> symbol indicates the position of the ethyl phosphotriester. The synthesis of the oligonucleotide and its characterization by HPLC, two-dimensional homochromatography and digestion by snake venom phosphodiesterase, have been previously described (6). The presence of the ethyl phosphotriester was further confirmed by digesting the decamer with the 3′→5′ exonuclease activity of T4 DNA polymerase. Since the ethyl phosphotriester modification protects DNA from nuclease digestion, only DNA that has lost the ethyl phosphotriester will be digested fully to the dinucleotide while the limit digest for the ethyl phosphotriester DNA will be the tetranucleotide. Less than 10% of the decamer was digested to the dinucleotide by the exonuclease activity of the T4 DNA polymerase (34), which indicates the presence of the ethyl phosphotriester in >90% of the DNA substrates.

In order to place the ethyl phosphotriester in a longer DNA strand, the phosphotriester-containing decamer was annealed with the 15mer, 5′-pCCCGTCCACTTCGCCp (synthesized with phosphates on the 3′ and 5′ ends) to the complementary 25mer, 5′-GGCGAAGTGGACGGGAAATAGAAAA. The annealing reaction brought together the decamer and 15mer in position for ligation, which was accomplished by T4 DNA ligase under standard conditions. The complementary 25mer was then degraded by the exonuclease activity of T4 DNA polymerase while the ethyl phosphotriester 25mer was protected from nuclease degradation by the 3′ phosphate. The ethyl phosphotriester 25mer was further purified through a C-18 Sep-pak cartridge (Waters). A 25mer oligomer identical to the phosphotriester-containing 25mer, but without the ethyl phosphotriester, was synthesized by standard procedures as was the primer, 5′-GGCGAAGTGGACGGG (Table 1).

The primer strand was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase under standard conditions. DNA substrates were made by annealing either the template strand

with the ethyl phosphotriester or an identical template strand, except lacking the ethyl phosphotriester, to the ^{32}P-labeled primer (Table 1). The template strand was in 1.5-fold excess.

Primer extension assay

Reactions containing either 1 nM unmodified or ethyl phosphotriester DNA substrate, 5 or 100 μM dNTPs, 1 mM dithiothreitol, 70 mM Tris–HCl (pH 8.0), 18 mM $(NH_4)_2SO_4$, 200 μg/ml bovine serum albumin and 7 mM $MgCl_2$ were pre-incubated at 37°C for 1 min. Reactions were initiated by the addition of 10 nM DNA polymerase and stopped by the addition of an equal volume of loading dye (40% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 mM NaOH and 1 mM EDTA). Reaction products were separated by electrophoresis on denaturing 20% polyacrylamide gels. The gels were dried and exposed to PhosphorImager screens (Molecular Dynamics). ImageQuant software (Molecular Dynamics) was used to calculate the band densities.

RESULTS

Replication of the ethyl phosphotriester-containing DNA template by the wild-type bacteriophage T4 DNA polymerase

The substrates used in this study are described in Table 1. Full-length primer extension required the incorporation of 10 nt. If the ethyl phosphotriester impeded DNA replication, primer extension paused or stopped after the incorporation of 7 nt.

In reactions with 100 μM dNTPs and either unmodified, control (C) template DNA or ethyl phosphotriester-containing DNA (PTE) (Fig. 1), primer extension to produce full-length product was completed in 20 s. Faint bands corresponding to products shorter than full-length were detected due to the high sensitivity of the PhosphorImager screens. These bands may be due to low levels of various base modifications introduced during oligonucleotide synthesis that block DNA replication. The bands were present in both the C and PTE DNA substrates and remained throughout the course of the reaction. The background bands were used to size the primer extension products.

In reactions with the C DNA substrate and 5 μM dNTPs (Fig. 1), full-length primer extension was also observed in 20 s, but a product 1 nt short of full-length (+9) was also detected. The amount of the shorter +9 product was constant over 2 min, which suggests that an idling reaction due to repeated cycles of exonuclease degradation and nucleotide incorporation was taking place at the primer end. Support for this proposal was seen in reactions with the exonuclease-deficient T4 DNA polymerase, which cannot perform the idling reaction; only full-length +10 product was observed with the C DNA substrate (Fig. 2).

In reactions with the wild-type T4 DNA polymerase, the PTE DNA substrate and 5 μM dNTPs (Fig. 1), inhibition of primer extension was detected after incorporation of the first 7 nt, just before TLR of the ethyl phosphotriester. The apparent maximum level of TLR with 5 μM dNTPs was reached by 2 min when ~75% of the primer extension products reached the + 9 and +10 positions. The combination of low dNTPs (5 μM) and the ethyl phosphotriester also increased the idling reaction at the +9 position compared with reactions with
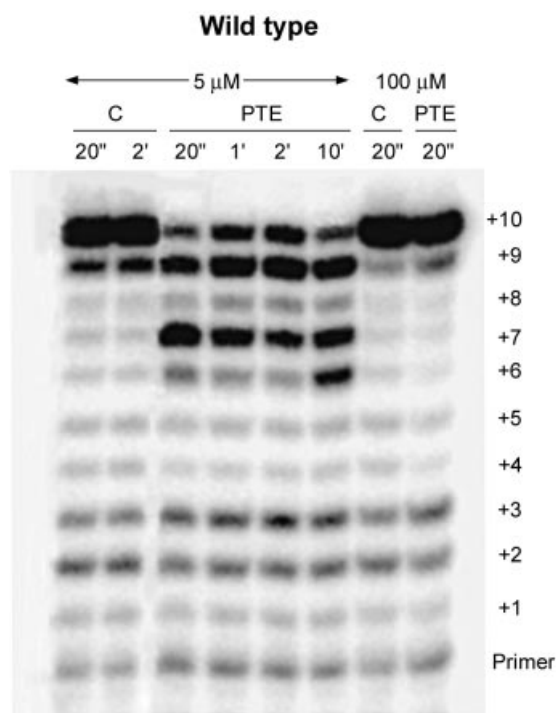

Figure 1. Replication of an ethyl phosphotriester lesion by wild-type T4 DNA polymerase. Control (C) or ethyl phosphotriester (PTE) DNA substrates (Table 1) were replicated by the wild-type T4 DNA polymerase in reactions with either 5 or 100 μM dNTPs. The primer was labeled with ^{32}P as described in the Material and Methods. Primer extension reactions were run from 20 s to 10 min as indicated. Denaturing gel electrophoresis was used to separate the products. If the ethyl phosphotriester blocked replication, a build-up of +7 product was produced.
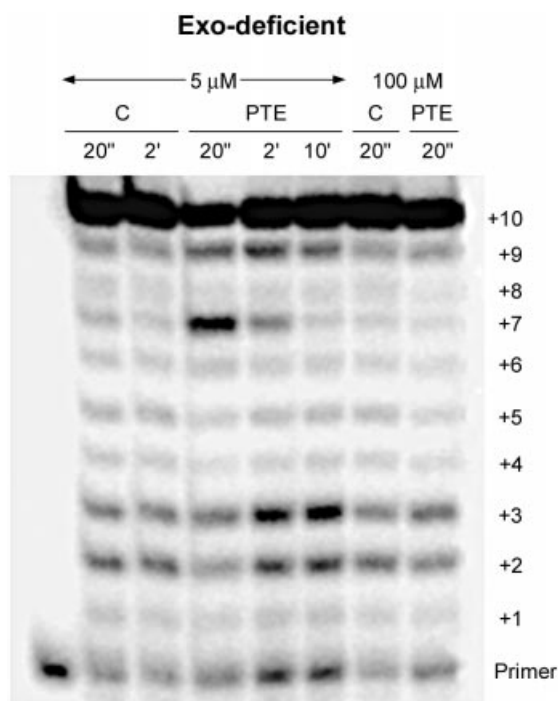

Figure 2. Replication of an ethyl phosphotriester lesion by exonuclease-deficient T4 DNA polymerase. The reaction conditions were the same as described for the wild-type T4 DNA polymerase (Fig. 1).

the C DNA substrate. More +9 product than full-length (+10) product was observed with the PTE DNA substrate while more +10 product was observed with the C DNA substrate. After 10 min of reaction, increased exonuclease degradation was observed as the amount of +10 product decreased and the amount of +6 product increased (Fig. 1). Increased degradation is likely to be caused by the idling reaction at the +7 and +9 positions with the PTE substrate. As the dATP pool is depleted, there is a further decrease in nucleotide incorporation and an increase in proofreading (35).

Replication of the ethyl phosphotriester-containing DNA template by the T4 exonuclease-deficient D112A/E114A–DNA polymerase

Full-length (+10) product was observed with the C or PTE DNA substrates and 5 or 100 µM dNTPs in 20 s (Fig. 2); however, primer extension was inhibited transiently by the ethyl phosphotriester in reactions with 5 µM dNTPs. A band corresponding to incorporation of 7 nt was present at 20 s, but unlike the reactions with the wild-type T4 DNA polymerase, nearly complete TLR was observed in 2 min. A minor +9 product was also detected with the PTE substrate.

Replication of the ethyl phosphotriester-containing DNA template by the T4 I417V–DNA polymerase

The T4 I417V–DNA polymerase has a valine substitution for isoleucine 417 in the conserved Motif A sequence in the polymerase active center (33). The I417V substitution decreases the ability of the mutant DNA polymerase to bind the primer terminus in the polymerase active center, which increases exonucleolytic proofreading and produces an antimutator phenotype *in vivo* (29,33). Decreased DNA binding can also explain the reduced ability of the I417V–DNA polymerase to replicate the C and PTE DNA substrates (Fig. 3). While full-length primer extension was observed with the C DNA substrate within the first 20 s of reaction with 100 µM dNTPs, replication was inhibited severely when the dNTP pools were reduced to 5 µM (Fig. 3). Major replication pause bands were seen after 20 s of reaction at +7 and at +9 with the C DNA. Since the I417V–DNA polymerase displayed difficulty in replicating past the +7 position at low dNTP concentrations in the absence of the ethyl phosphotriester modification, the DNA sequence must present an obstacle to DNA replication for the mutant DNA polymerase that was not detected for the wild-type T4 DNA polymerase.

The ethyl phosphotriester further inhibited replication by the I417V–DNA polymerase. Replication pause bands were detected at +7 and +9 in reactions with 100 µM dNTPs, but little TLR was detected when the dNTP pools were reduced to 5 µM with major bands at +6 and +7 (Fig. 3). It is likely that the trace amounts of +9 and +10 products detected with the PTE DNA at 5 µM are due to small amounts of substrate in which the ethyl phosphotriester has been lost. While the +6 product may indicate a direct block of replication by the ethyl phosphotriester, it is more likely that excessive idling at the +7 position rapidly depleted the dATP pool, and as a consequence, the +6 product is the extent of the reaction in the absence or near absence of dATP. This proposal is supported by the observation that +6 product was also detected for the wild-type T4 DNA polymerase in 5 µM reactions, but not until after 10 min of reaction when idling at the +7 and +9 positions
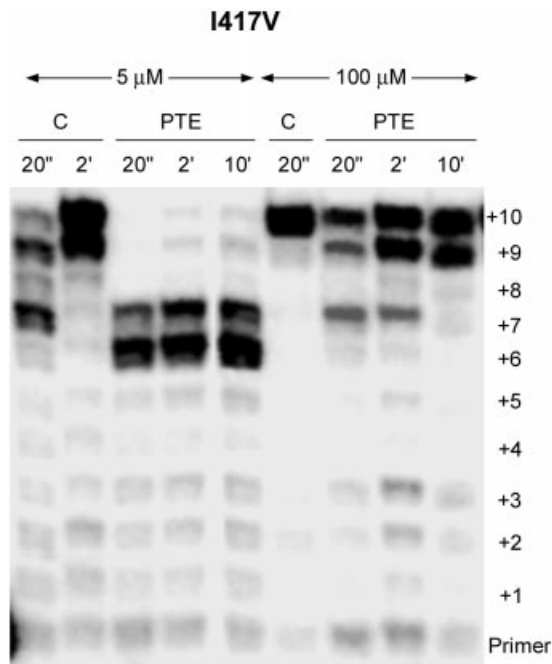

Figure 3. Replication of an ethyl phosphotriester lesion by the T4 I417V–DNA polymerase. The reaction conditions were the same as described for the wild-type T4 DNA polymerase (Fig. 1).

had reduced the concentration of dATP (Fig. 1). Furthermore, no +6 product was detected in the presence of high (100 µM) concentrations of dNTPs for the wild-type (Fig. 1) or I417V–DNA polymerase (Fig. 3), which supports the proposal that the ethyl phosphotriester blocks primer extension at the +7 position and the +6 product is produced secondarily when the dATP pool is depleted.

Replication of the ethyl phosphotriester-containing DNA template by *E.coli* DNA pol I

Unlike reactions with the wild-type and mutant T4 DNA polymerases, no replication pause bands due to the ethyl phosphotriester were detected at the +7 position for *E.coli* DNA pol I (Fig. 4). However, the ethyl phosphotriester inhibited full-length product formation at low (5 µM) and high (100 µM) dNTP concentrations.

The observation that an ethyl phosphotriester impairs full-length replication by *E.coli* DNA pol I is important in interpreting the previous experiments reported by Miller *et al.* (5). Their studies with *E.coli* DNA pol I were not designed to detect primer extension pausing at an ethyl phosphotriester; instead, only full-length primer extension was measured by the incorporation of radioactive dGMP, which occurred only if the primer was extended past the ethyl phosphotriester and as far as the last two terminal dC nucleotides in the template strand. [The ethyl phosphotriester was 4 nt from the 5′ end of the template strand in the previous studies (5) and 3 nt from the 5′ end in the DNA substrates used here (Table 1)]. Miller *et al.* (5) reported that full-length primer extension was reduced by as much as 25–50% in reactions with 33 µM dNTPs. Since no +7 product was observed in our reactions
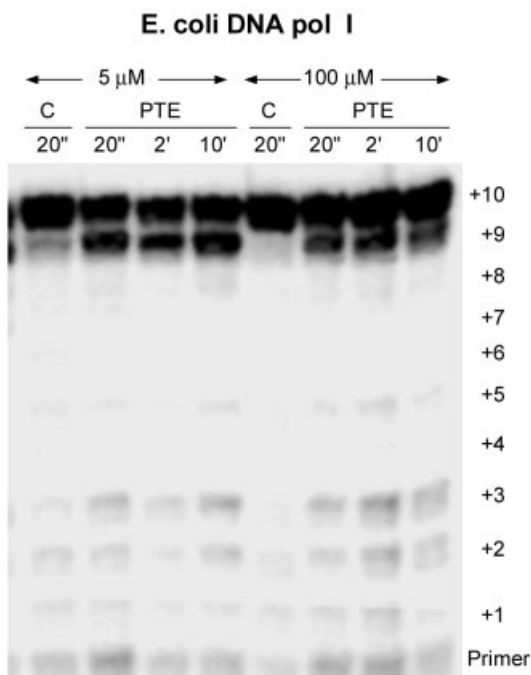
E. coli DNA pol I

Figure 4. Replication of an ethyl phosphotriester lesion by *E.coli* DNA pol I. The reaction conditions were the same as described for the wild-type T4 DNA polymerase (Fig. 1).

Figure 5. TLR of an ethyl phosphotriester lesion by T4 DNA polymerase. The region of the DNA substrate containing the ethyl phosphotriester is illustrated; the full DNA substrate is described in Table 1. The green circles indicate the phosphodiester linkages. The ethyl phosphotriester adduct is illustrated by an Et, which may be in one of the two positions indicated. (Note that only a single ethyl phosphotriester, the Sp or Rp isomer, was present in individual DNA substrates.) Either idling opposite template T_7 or primer elongation can occur at the lesion. Additional details are supplied in the text.

with either 5 or 100 μM dNTPs (Fig. 4), an ethyl phosphotriester does not appear to block replication as observed for the T4 DNA polymerase; however, the ethyl phosphotriester did reduce the ability of *E.coli* DNA pol I to replicate to the end of the template strand (Fig. 4). Thus, the ethyl phosphotriester does not appear to affect TLR, but does reduce the ability of *E.coli* DNA pol I to incorporate the last nucleotide. Since the ethyl phosphotriester reduces replication at the end of the DNA substrate more than TLR, DNA replication at ends may require formation of an enzyme–DNA complex that differs from the complex formed at other positions. Miller *et al.* (5) proposed that one of the isomers, Sp or Rp, might present more of an obstacle to replication. Our results support this proposal since a relatively constant amount of +9 product was observed at 20 s compared with 10 min in reactions with 5 or 100 μM dNTPs (Fig. 4), which suggests that either the Sp-PTE or the Rp-PTE interferes with full-length replication by *E.coli* DNA pol I. Further analysis of these experiments is presented in the Discussion.

DISCUSSION

The main result of this study is that ethylation of an internucleotide phosphodiester linkage in the template strand hindered replication by the bacteriophage T4 DNA polymerase (Fig. 1) under conditions that did not block replication by *E.coli* DNA pol I (Fig. 4). The ethyl phosphotriester, however, reduced the ability of both DNA polymerases to produce full-length +10 product (Figs 1 and 4). The effects of an ethyl phosphotriester on TLR and for replicating the end of a DNA substrate are discussed separately.
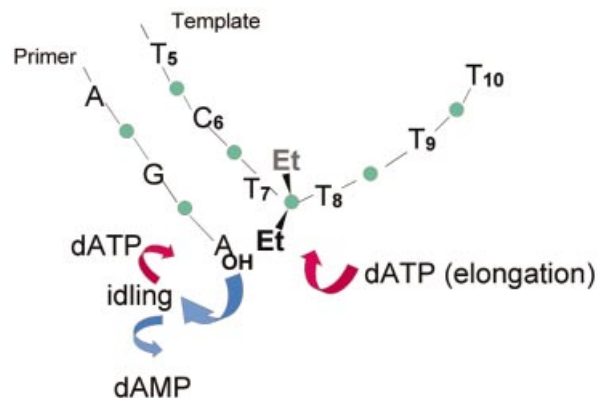
TLR of an ethyl phosphotriester by the T4 DNA polymerase and *E.coli* DNA pol I

Efficient TLR of an ethyl phosphotriester by the wild-type T4 DNA polymerase was observed only in the presence of 100 μM dNTPs (Fig. 1). In contrast, efficient TLR was observed for the exonuclease-deficient T4 DNA polymerase even at low (5 μM) dNTP concentrations (Fig. 2), but almost no bypass replication was detected for the excessive-proofreading I417V–DNA polymerase at 5 μM dNTPs (Fig. 3) and some inhibition was even detected at 100 μM dNTPs (Fig. 3). Since detection of the inhibitory effect of an ethyl phosphotriester on DNA replication is maximized under conditions where dNTP concentrations are low and exonucleolytic proofreading is permitted, we propose that the combination of relatively low dNTP concentrations plus the ethyl phosphotriester, converts the T4 DNA polymerase from primarily a replicative DNA polymerase to more of an exonuclease, which results in arrest of primer elongation at the +7 position (Fig. 1).

Nucleotide incorporation rather than proofreading is the preferred reaction catalyzed by DNA polymerases unless primer elongation is slowed by an incorrect nucleotide at the primer terminus (36,37), by reduced dNTPs (35) or by an obstruction (38). Mutant DNA polymerases with reduced ability to carry out any aspect of the nucleotide incorporation reaction also have increased proofreading activity and these mutants frequently remove correct as well as incorrect nucleotides at the primer terminus (33,36,39,40). Thus, whenever primer elongation is slowed, exonucleolytic proofreading becomes more likely (40).

When T4 DNA polymerase encounters an ethyl phosphotriester (Fig. 5), primer elongation can either continue or the idling reaction can take place where cycles of dAMP incorporation and removal take place opposite template T_7. If dNTP pools are high, TLR is favored, but if the dNTP pools are low, increased idling produces a build up of +7 product. Thus, the ease in forming the ternary enzyme–DNA–dNTP
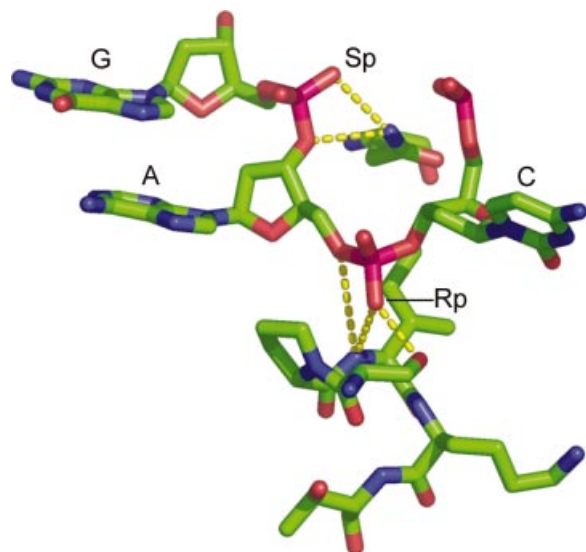
Figure 6. RB69 DNA polymerase contacts with oxygen atoms in phospho-diester linkages in the primer terminal region of the template strand. The primer terminal region of the template strand for the ternary DNA polymerase–DNA–dNTP structure reported by Franklin *et al.* (23) for the RB69 DNA polymerase is shown in the figure. The template strand is forced into an abrupt turn, which is between template bases A and C. Protein contacts are made with the Rp oxygen in the turn region and with the Sp oxygen in the phosphodiester linkage between template bases G and A. Additional details are supplied in the text.

complex appears to determine whether TLR or idling will predominate. Since primer-extension pause bands in 5 μM reactions were seen primarily at the +7 and +9 positions, this indicates that a higher concentration of dATP is required to form the ternary complexes at these sites compared with other positions in the template strand. In other words, the K_m for dATP appears to be higher if the template is modified by an ethyl phosphotriester. For unmodified DNA, the dNTP concentration at which proofreading starts to predominate over primer extension is <1.7 μM (35), but 5 μM appears to be the pivotal concentration in the presence of an ethyl phosphotriester (Fig. 1).

These observations support the proposal that one or more protein contacts with oxygen atoms in the phosphodiester backbone, which can be prevented by ethylation, are required in order for the DNA polymerase–DNA complex to readily bind the next incoming dNTP. Recent crystallographic data obtained with the RB69 DNA polymerase (23), which is a close relative of the T4 DNA polymerase (41), indicate two protein–DNA backbone contacts are made to the template strand in the primer terminal region, as illustrated in Figure 6. The template G shown in Figure 6 is at the primer terminus and is paired with the chain-terminator dideoxy C (data not shown); the adjacent template A is paired with the incoming dTTP (data not shown). There is an abrupt turn in the template strand between template A and the next template base, which is a C. There are two likely protein contacts with oxygen atoms in the phosphodiester linkages: an asparagine (Asn572) is close enough to form a H-bond with the Sp oxygen atom between the template G and A bases and a serine (Ser360) as well as an isoleucine (Ile362) could form H-bonds with the Rp oxygen atom where the template strand is forced into an abrupt

turn. An ethyl group at either the Sp or Rp contact sites would be expected to alter these interactions, which could slow the nucleotide incorporation reaction.

Since the DNA template strand used in our assay is an approximate 50:50 mixture of Sp and Rp ethyl phosphotriester-modified DNA, we cannot determine if one of the isomers is more inhibitory than the other. However, in reactions with the wild-type T4 DNA polymerase and 5 μM dNTPs (Fig. 1), the persistence of a major +7 product throughout the time course of the reaction is consistent with the proposal that one of the isomers is the major block to replication. This hypothesis can be tested in future experiments by preparing DNA substrates with the ethyl phosphotriester in either the Sp or Rp conformation or by treating a mixture of Sp and Rp DNA substrates with the *E.coli* O[6]-methylguanine–DNA methyl transferase, which removes the Sp-ethyl phosphotriester (17–19).

Although we did not detect any pausing in the primer elongation reaction catalyzed by *E.coli* DNA pol I at the +7 position (Fig. 4), there is a possibility that pausing could be detected with a more sensitive assay since DNA polymerase contacts with the DNA backbone have been observed for Family A DNA polymerases (24–26) as they have for the RB69 DNA polymerase (23). Also, the lower exonuclease activity of *E.coli* DNA pol I (42) compared with the much higher level present in the T4 DNA polymerase may assist TLR; however, some pausing was detected at the ethyl phosphotriester for the exonuclease-deficient T4 DNA polymerase (Fig. 2), which suggests that lower exonuclease activity is not the only reason for more efficient TLR by DNA pol I. Nevertheless, under the experimental conditions reported here and for the experiments reported by Miller *et al.* (5), an ethyl phosphotriester does impede *E.coli* DNA pol I from replicating to the end of the template strand (Fig. 4).

Replication at the end of the template strand; effect of an ethyl phosphotriester

In addition to differences in TLR by the T4 DNA polymerase compared with *E.coli* DNA pol I, differences were also observed for replicating to the end of the template strand in the presence of an ethyl phosphotriester. In reactions with 100 μM dNTPs, full-length replication was nearly complete for the T4 DNA polymerase in 20 s (Fig. 1), but replication was still not complete for *E.coli* DNA pol I even after 10 min (Fig. 4). High dNTP concentrations did not appear to assist *E.coli* DNA pol I in full-length replication; about the same level of inhibition was observed in 5 μM dNTP reactions as observed in reactions with 100 μM dNTPs (Fig. 4). These observations suggest that one of the ethyl phosphotriester isomers is more inhibitory to *E.coli* DNA pol I as was also concluded by Miller *et al.* (5). One interpretation of these experiments is that replication at the end of the template strand is more difficult than replication of internal positions in the template strand and that the ethyl phosphotriester, which is near the end of the template strand, further increases the difficulty for incorporation of the terminal nucleotide.

How are ethyl phosphotriester lesions tolerated?

Since an ethyl phosphotriester in the template strand can partially block DNA replication by the T4 DNA polymerase (Fig. 1), a tolerance mechanism may be needed for efficient

TLR by this and other replicative DNA polymerases. This point is worth considering since ethyl phosphotriesters can persist for several days in cellular DNA, which attests to both their chemical stability and the inability of most organisms to even partially remove this lesion (15,16). Furthermore, dNTP pools in mammalian cells are low, only 5–23 μM (43) and, thus, may be subject to rapid depletion by DNA polymerase idling.

If the T4 DNA polymerase is representative of other Family B DNA polymerases, such as DNA polymerases epsilon and delta that are required for eukaryotic chromosome replication, then these DNA polymerases are expected to pause replication transiently at ethyl phosphotriester adducts and to carry out cycles of idling (Fig. 5). Pausing and some idling are expected even if dNTPs are in good supply, but TLR without much delay will likely predominate. However, if the local concentrations of dNTPs are low, which may arise if there are multiple phosphotriester lesions and considerable idling that depletes the dNTP pools, then an ethyl phosphotriester is expected to present a more severe block to replication fork progression. We have observed (unpublished results) that a low, non-inhibitory concentration of hydroxyurea, which reduces dNTP pools by its action on ribonucleotide reductase, increases the killing action of ethylnitrosourea in yeast; however, it is not clear if the increased sensitivity produced by hydroxyurea is caused by the large concentration of ethyl phosphotriesters or by any of the numerous other types of lesions produced by the alkylating agent. Fortunately, one cellular response to stalled replication forks is to up-regulate ribonucleotide reductase, which increases the pools of dNTPs (44). Thus, any replication fork stalling caused by ethyl phosphotriesters will likely be overcome as soon as the pools of dNTPs are increased, thus providing a tolerance mechanism.

Another explanation for the relative insensitivity of DNA replication to ethyl phosphotriester adducts is that DNA polymerases may encounter ethyl phosphostriesters primarily in a configuration that is not inhibitory. Although we cannot determine from our studies if an ethyl phosphotriester in the Sp or Rp configuration is more likely to impede replication, contacts are made with the Rp oxygen atom at a critical position where the template strand is bent and where dynamic changes in the DNA polymerase–DNA complex take place in order for primer elongation to continue (Fig. 6). Thus, T4 DNA polymerase replication may be hindered primarily because of the Rp ethyl phosphotriester isomer. However, alkylation of DNA may favor production of the Sp isomer, as this position may be more accessible to alkylating agents (45). If formation of the Sp isomer is favored *in vivo*, but if only the Rp isomer is a major block to replication, then DNA replication *in vivo* may not be affected significantly.

ACKNOWLEDGEMENTS

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