In Vitro Effects of a C4′-Oxidized Abasic Site on DNA Polymerases†

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ABSTRACT: Oxidative damage to DNA produces abasic sites resulting from the formal hydrolysis of the nucleotides' glycosidic bonds, along with a variety of oxidized abasic sites. The C4′-oxidized abasic site (C4-AP) is produced by several DNA-damaging agents. This lesion accounts for ∼40% of the DNA damage produced by bleomycin. The effect of a C4′-oxidized abasic site incorporated at a defined site in a template was examined on Klenow fragments with and without 3′ → 5′ exonuclease activity. Both enzymes preferentially incorporated dA > dG ≫ dC, T opposite C4-AP. Neither enzyme is able to extend the primer past the lesion. Experiments with regular AP sites in an otherwise identical template indicate that Klenow does not differentiate between these two disparate abasic sites. Extension of the primer by alternative polymerases pol II, pol II exo⁻, pol IV, and pol V was examined. Pol II exo⁻ was most efficient. Qualitative translesion synthesis experiments showed that pol II exo⁻ preferentially incorporates T opposite C4-AP, followed in order by dG, dA, and dC. Thymidine incorporation opposite C4′-AP is distinct from the pol II exonuclease interaction with a regular AP site in an otherwise identical template. These in vitro experiments suggest that bypass polymerases may play a crucial role in survival of cells in which C4-AP is produced, and unlike a typical AP site, the C4-AP lesion may not follow the "A-rule". The interaction between bypass polymerases and a C4-AP lesion could explain the high levels of G:C → T:A transversions in cells treated with bleomycin.

Exposure of DNA to various forms of oxidative stress produces a variety of lesions that can be cytotoxic and/or mutagenic (*1*−*3*). One of these lesions, the C4′-oxidized abasic site (C4-AP[1]), results from formal hydrogen atom abstraction from the C4′-position. The C4-AP site is one of a group of abasic sites (Figure 1) that are produced in DNA by a variety of chemical pathways (*4*). The C4′-oxidized abasic site accounts for approximately 40% of the products formed following reaction of bleomycin with DNA, and is formed preferentially by oxidation of the pyrimidine in 5′-G-Pyr sequences (*5*). Other antitumor antibiotics also produce the C4-AP lesion, but in smaller amounts (*6*). The propensity for minor-groove-binding DNA-damaging molecules to react at the C4′-position is partially ascribable to the proximity of this atom to the bound oxidizing agents. Its position at the outer edge of the minor groove makes the C4′-hydrogen atom accessible to diffusible species (*7*). Consequently, diffusible species (e.g., hydroxyl radical) produce the C4-AP lesion via scission of the relatively weak C4′-carbon−hydrogen bond (*4*, *7*−*9*). Model studies imply that the C4-AP lesion may also result indirectly from initial oxidation of the phosphate group, as is postulated to occur as a result of the direct effects of γ-radiolysis (*10*). Despite the common occurrence of the C4-AP lesion, very little is known regarding its effects on polymerase enzymes. Here we report the characterization of the C4-AP lesion's effects on the activity of a variety of polymerase enzymes. These in vitro experiments provide insight into the possible effects of the C4-AP site in cells.

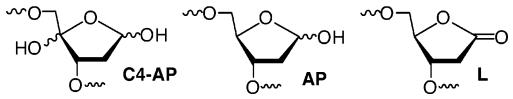

FIGURE 1: Structures of abasic lesions: C4′-oxidized abasic site (C4-AP), "regular" abasic lesions (AP), and 2-deoxyribonolactone (L).

The C4-AP site may exist as an acyclic form in equilibrium with up to four diastereomeric cyclic isomers (Scheme 1). Although the distribution of isomers that make up this equilibrium is unknown, NMR studies on the related nonoxidized "regular" abasic site (AP) suggest that the acyclic (1,4-dicarbonyl) form will be present in very small amounts (*11*). One could also speculate that diastereomers containing the 4*S*-configuration are less favored in duplex DNA because they will distort the backbone more than those containing the 4*R*-configuration. These assumptions aside, the C4-AP site may present a variety of structures to polymerase and repair enzymes that are distinct from previously characterized abasic sites (e.g., AP, L).

Characterization of the effects of AP and 2-deoxyribonolactone (L) lesions on polymerase fidelity and proficiency is facilitated by chemical and enzymatic methods for their independent generation at defined sites in oligonucleotides.

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[1] Abbreviations: C4-AP, C4′-oxidized abasic site; AP, abasic site; L, 2-deoxyribonolactone; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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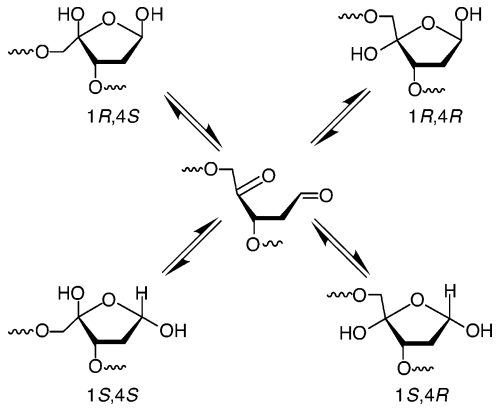
Scheme 1. Equilibration of the C4-AP Lesion



AP sites are efficiently produced by the action of uracil DNA glycosylase (UDG) on oligonucleotides containing a 2′-deoxyuridine (Figure 2) (*12*). They can also be prepared chemically by treatment of an oligonucleotide containing a strategically designed vicinal diol with periodate (*13*). Three different photochemical precursors for 2-deoxyribonolactone (L) have been reported (*14*, *15*). In these experiments we use the ketone precursor (**1**, Figure 2) (*16*, *17*). Specific studies of the effects of C4-AP on polymerase and repair enzymes are lacking due to the absence of a method for generating the lesion at defined sites in DNA. Intracellular studies using random but selective generation of the C4-AP site by bleomycin indicate that the lesion is mutagenic (*18*, *19*). Generation of the C4-AP site in vitro by bleomycin was also used to qualitatively gauge the ability of repair endonucleases to initiate repair of the lesion (*20*, *21*). We recently reported a method for synthesizing oligonucleotides containing a C4-AP site at a defined position from **2** (Figure 2) (*22*). The photochemical transformation utilizes the well-known *o*-nitrobenzyl photoredox reaction, proceeds very efficiently, and produces the C4-AP site in high yield. The photochemical precursor **2** made it possible to carry out the studies described below on the C4-AP lesion.

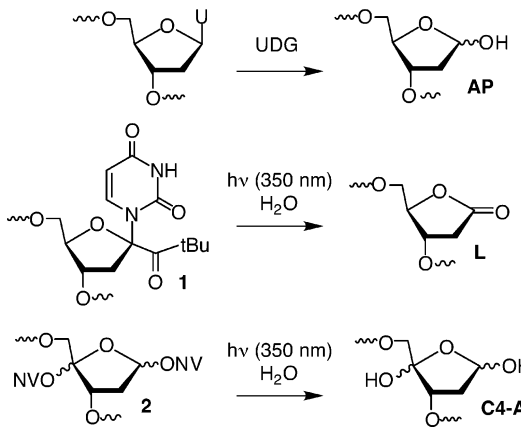


FIGURE 2: Methods for generating the AP, L, and C4-AP lesions.

MATERIAL AND METHODS

General Procedures. Oligonucleotide synthesis was carried out on an Applied Biosystems Inc. 394 DNA synthesizer using standard protocols. Oligonucleotides containing C4-AP or 2-deoxyribonolactone (L) were synthesized as described (*16*, *22*). Oligonucleotides containing AP sites were generated from 2′-deoxyuridine by UDG treatment (*12*). Purified oligonucleotides were characterized by ESI-MS or MALDI-TOF MS. Electrospray and MALDI-TOF mass spectrometry samples were prepared by ethanol precipitating from NH_4OAc. Electrospray mass spectra were obtained on an LCQ-Duo instrument. MALDI-TOF mass spectra were obtained on a Kratos Seq 5 instrument. The oligonucleotides used in these experiments are shown in Table 1. Commercially available oligonucleotide synthesis reagents were obtained from Glen Research or Dharmacon Research. DNA manipulation, including enzymatic labeling, was carried out using standard procedures (*23*). Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gel by electrophoresis (5% cross-link, 45% urea (by weight)). T4 polynucleotide kinase, uracil DNA glycosylase (UDG), Klenow, and Klenow exo$^-$ were obtained from New England Biolabs. Pol II, pol II exo$^-$, pol IV, and pol V/Rec A were obtained as previously described (*24*−*26*). [γ-^{32}P]-ATP was purchased from Amersham Pharmacia Biotech. Radioactive samples were quantitated by Cerenkov counting using a Beckman LS6500 liquid scintillation counter. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 Phosphorimager equipped with ImageQuant Version 5.1 software. Oligonucleotide photolyses were carried out in Pyrex tubes (0.25 in. i.d.) using a Rayonet Photoreactor (RPR-100) equipped with 16 lamps having a maximum output at 350 nm.

Table 1. Oligonucleotides Employed in Polymerase Experiments

5′-d(GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT)

a X = **1**
b X = **C4-AP**
c X = **2**
d X = **L**
e X = **dU**
f X = **AP**
g X = T

5′-d(AGG CTC AGC ACG TC)
h
5′-d(AGG CTC AGC ACG TCG T)
i
5′-d(AGG CTC AGC ACG TCG TA)
j
5′-d(AGG CTC AGC ACG TCG TG)
k

General Preparation of Primer−*Template Duplexes.* Primers were hybridized at 55 °C to template oligonucleotides (1.5 equiv) for 5 min and cooled to room temperature over 2 h. For experiments using Klenow exo$^-$ or Klenow exo$^+$, 25 pmol of 5′-^{32}P-labeled primer and 225 pmol of unlabeled primer were annealed to 375 pmol of template in 20 mM Tris (pH 7.5), 100 mM NaCl to produce a DNA working solution (3.215 μM). Duplexes used in these experiments are listed by number in Table 2.

Full-Length Primer Extension by Klenow exo$^-$/exo$^+$. Full-length primer extension in **3a**,**b** (75 nM) was carried out using Klenow exo$^-$ (1 nM) or Klenow exo$^+$ (10 nM). Reactions were carried out in 0.02% w/v BSA, 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 15 mM DTT. A 2× solution (25 μL) of **3a**,**b** (150 nM) and Klenow exo$^-$ (2 nM) or 20

Table 2. Oligonucleotide Duplexes Employed in Polymerase Experiments

5′-d-AGG CTC AGC ACG TC
3′-d-TCC GAG TCG TGC AGC A**X**A CGT CGT GCA CTG

3a X = C4-AP
3b X = T
3c X = AP

5′-d-AGG CTC AGC ACG TCG T
3′-d-TCC GAG TCG TGC AGC A**X**A CGT CGT GCA CTG

4a X = C4-AP
4b X = L
4c X = AP
4d X = **1**
4e X = **2**

5′-d-AGG CTC AGC ACG TCG T**Y**
3′-d-TCC GAG TCG TGC AGC A**X**A CGT CGT GCA CTG

5a X = C4-AP; Y = A
5b X = C4-AP; Y = G

nM Klenow exo$^+$ (20 nM) in buffer was added to a solution (25 μL) containing all four dNTPs (0.2 mM each). The reaction mixture was incubated at room temperature for 2 h. Aliquots (5 μL) were collected at 1, 5, 10, 20, 30, 45, 60, and 120 min and quenched immediately in formamide (10 μL) loading buffer. Samples were denatured and loaded on a 20% denaturing PAGE. Marker lanes were loaded with independently synthesized 5′-^{32}P-16mer and -17mer. Control experiments using **3b** were carried out as described above, except that aliquots were taken for 1 h.

Insertion Kinetics Opposite C4-AP, AP, L, or Photochemical Precursors 1 and 2 (27). A 2× DNA−enzyme solution (120 μL) was prepared containing **4a** (150 nM) and 2 nM Klenow exo$^-$ (2 nM)/exo$^+$ (20 nM) in 0.02% w/v BSA, 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 15 mM DTT. The DNA−enzyme solution (5 μL) was added to the appropriate 2× dNTP solution (5 μL). The reaction was allowed to run for a fixed period of time at room temperature and then quenched with 95% formamide loading buffer containing EDTA (20 μL). The samples were denatured by heating at 90 °C for 3 min and then immediately placed on ice before being loaded (4 μL) on a 20% PAGE gel. The concentration ranges and reaction times for Klenow exo$^-$ reactions were as follow: dATP, 10−250 μM, 3 min; dGTP, 10−250 μM, 15 min; dCTP, 0.1−1.5 mM, 15 min; dTTP, 0.5−10 mM, 9 min. The concentration ranges and reaction times for Klenow exo$^+$ reactions were as follow: dATP, 10−1000 μM, 15 min; dGTP, 10−1000 μM, 20 min.

Insertion kinetics by Klenow exo$^-$ opposite 2-deoxyribonolactone (L) or an abasic site (AP) were carried out using duplexes **4b** and **4c**, respectively. The concentration ranges and reaction times for templates containing an AP site (**4c**) were as follow: dATP, 10−1000 μM, 1 min; dGTP, 10−1000 μM, 15 min. The concentration range and reaction time for template containing a 2-deoxyribonolactone site (**4b**) were as follows: dATP, 10−500 μM, 3 min. The concentration range and reaction time for template containing the photochemical precursors to 2-deoxyribonolactone (**4d**) or C4-AP (**4e**) were as follow: dATP, 10−500 μM, 15 min.

Klenow exo$^-$/exo$^+$ Primer Extension Past C4-AP Site. Attempts to obtain kinetic parameters for the incorporation of dTTP opposite dA on the 5′-side of C4-AP were carried out under the same conditions as described above for insertion reactions. When **5a** (dA opposite C4-AP) or **5b** (dG opposite C4-AP) was used as substrate, reactions were carried out for 30 min in the presence of 10−1000 μM dTTP. Reaction times and dTTP concentration ranges were independent of the form of the Klenow fragment.

Full-Length Primer Extension by Pol II, Pol II exo$^-$, or Pol IV. Reactions were carried out in a manner similar to those reported previously (*26*, *28*). Full-length primer extension of **3a**−**c** (2 nM) was carried out in 20 mM Tris (pH 7.5), 8 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 25 mM sodium glutamate, 4% glycerol, and 4 μg/mL BSA. The four native dNTPs were present at 0.5 mM each. Polymerase was added to a final concentration of 500 nM using stock solutions of pol II, pol II exo$^-$, and pol IV at 2, 15, and 40 μM, respectively, yielding a total reaction volume of 100 μL. The reactions were incubated at 37 °C for 60 min. Aliquots (10 μL) were quenched with formamide loading buffer (10 μL) and analyzed by 20% denaturing PAGE.

Full-Length Primer Extension by Pol V. Primer extension of **3a**−**c** (10 nM) was carried out in solution (20 μL) containing 0.25 μM Rec A and 0.5 mM ATPγS in addition to the buffer components employed in the bypass polymerase experiments described above. The reaction was incubated at 25 °C for 3 min, and then all four dNTPs were added to a final concentration of 0.5 mM each. Pol V was added (0.4 μL from a 12.5 μM stock solution) to a final concentration of 250 nM. Reactions were incubated at 37 °C, and aliquots (10 μL) were removed after 10 and 30 min, quenched with formamide loading buffer (10 μL), and analyzed by 20% denaturing PAGE.

Translesional Synthesis by Klenow and Pol II exo$^-$. Reactions (100 μL) were carried out on **4a**,**d** using 2.5 μL (for Klenow reactions) or 5 μL (for pol II exo$^-$ reactions) of a 0.2 μM DNA stock solution to produce final DNA concentrations of 5 and 10 nM, respectively. Reactions were carried out in 20 mM Tris·HCl (pH 7.5), 8 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 25 mM sodium glutamate, 4% glycerol, and 4 μg/mL BSA. Each dNTP (final concentration of 0.5 mM) was added individually to the mixture, and then Klenow (2.72 μL of a 0.368 μM stock solution, final concentration of 10 nM) or pol II exo$^-$ (0.67 μL of a 15 μM stock, final concentration of 100 nM) was added to the reactions, resulting in a final volume of 100 μL. Reactions were incubated at 37 °C for 2 min, and then an aliquot (10 μL) was quenched with formamide loading buffer (10 μL) and analyzed by 20% denaturing PAGE.

RESULTS

Oligonucleotide Synthesis. A 30-nucleotide-long oligonucleotide template (Table 1) containing the photochemical precursor (**1**) of C4-AP was prepared and purified by gel electrophoresis, as previously described (*22*). The precursor containing oligonucleotide was hybridized to the appropriate primer and photolyzed immediately prior to each experiment. Hybridization was carried out at 55 °C. Photochemical conversion was determined by treating a duplex in which the template strand was radiolabeled with NaOH (0.1 M, 37 °C, 20 min). These conditions cleave the C4-AP site but not its photochemical precursor (**1**) (*22*). Greater than 90%

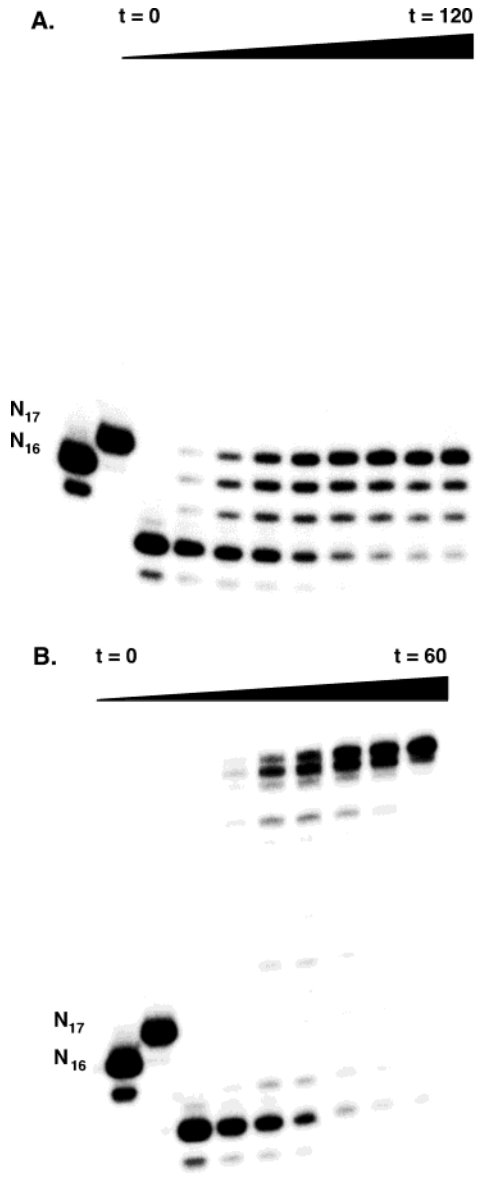


FIGURE 3: Klenow exo⁻ (1 nM)-mediated extension of **3a**,**b** (75 nM) in the presence of native dNTPs (0.1 mM each): (A) **3a** and (B) **3b**.

conversion to the C4-AP lesion was achieved. Template−primer duplexes containing "regular" abasic (AP) or 2-deoxyribonolactone (L) sites in otherwise identical sequences were prepared immediately prior to enzyme experiments using published procedures (*12*, *16*).

Primer Extension by Klenow exo⁺/exo⁻. Qualitative analysis of Klenow exo⁻'s ability to read through a C4-AP site was carried out using duplex **3a** in which the enzyme was given a running start. Although translesion synthesis was achieved, Klenow exo⁻ was unable to bypass the lesion after 2 h (Figure 3A). Complete extension of a comparable duplex (**3b**) containing thymidine in place of the lesion was observed within 60 min under identical conditions (Figure 3B). Bypass of the C4-AP site was not achieved, even when the concentration of Klenow exo⁻ was increased to 10 nM (see Supporting Information). Klenow exo⁺ was also unable to bypass the C4-AP when reactions were carried out at 10 nM enzyme, whereas the control was fully extended (see Supporting Information).

Table 3. Steady-State Analysis of Translesion Synthesis on **4a**−**c** by Klenow exo⁻

lesion	dNTP	V_{max} (%·min^{-1})	K_m (μM)	V_{max}/K_m (%·min^{-1}·M)
C4-AP (**4a**)	A	6.5 ± 1.8	64.4 ± 10.4	(1.1 ± 0.4) × 10^5
C4-AP (**4a**)	G	0.6 ± 0.1	46.0 ± 11.6	(1.4 ± 0.4) × 10^4
AP (**4c**)	A	11.9 ± 2.3	89.5 ± 3.4	(1.3 ± 0.3) × 10^5
AP (**4c**)	G	1.1 ± 0.2	90.8 ± 9.1	(1.2 ± 0.2) × 10^4
L (**4b**)	A	1.0 ± 0.4	193.8 ± 32.3	(5.2 ± 0.9) × 10^3

Table 4. Steady-State Analysis of C4-AP Translesion Synthesis on **4a** by Klenow exo⁺

dNTP	V_{max} (%·min^{-1})	K_m (μM)	V_{max}/K_m (%·min^{-1}·M)
A	0.8 ± 0.3	33.7 ± 1.5	(2.3 ± 0.9) × 10^4
G	0.1 ± 0.01	25.2 ± 5.9	(3.6 ± 0.9) × 10^3

Steady-state experiments were carried out using **4a** in order to measure nucleotide incorporation opposite (translesion synthesis) the C4-AP lesion (Table 3) by Klenow exo⁻. Klenow exo⁻ showed an ∼10-fold preference for incorporating dA over dG opposite the lesion. Although the apparent K_m of dG translesion synthesis was approximately one-third lower than that of dA, the bulk of the selectivity was attributable to a larger observed apparent V_{max} for dA incorporation, which could reflect a difference in the rate of product release. Incorporation of dT or dC opposite the C4-AP site was extremely inefficient. Despite increasing the reaction time from 3 to 9 min, no incorporation of dT was observed in the presence of as much as 10 mM dTTP, and less than 1% of the primer was extended after 15 min in the presence of 1.5 mM dCTP. Hence, kinetic analysis of translesion incorporation of dCTP or dTTP by Klenow exo⁻ was unfeasible. Translesion incorporation was also checked on a template containing the C4-AP precursor (**4e**), but no incorporation was detected in 3 min in the presence of as much as 1 mM dATP.

For comparison purposes, translesion synthesis by Klenow exo⁻ on templates containing 2-deoxyribonolactone (L, **4b**) or an AP site (**4c**) was examined. The preference for translesion deoxyribonucleotide incorporation of dA versus dG in the presence of a typical AP site was comparable to that observed for the C4-AP template (Table 3). In contrast, the efficiency for incorporation of dA opposite the C1′-oxidized abasic site (L) was ∼20-fold lower (Table 3) compared to that opposite the C4-AP and AP sites. No extension of a primer opposite a template containing the photochemical precursor to L (**4d**) was detected in the presence of dATP (0.5 mM) after 15 min.

The efficiency for deoxyribonucleotide incorporation opposite the C4-AP site by Klenow exo⁺ was less efficient than that observed using Klenow exo⁻ (Table 4). The apparent K_m's measured for dA and dG incorporation opposite C4-AP were ∼2-fold lower than those when Klenow exo⁻ was used. However, V_{max} was 6−8 times slower when Klenow exo⁺ was used. Although translesion synthesis by Klenow exo⁺ was less efficient on the C4-AP template, the overall selectivity for dA incorporation relative to dG was comparable to that observed using Klenow exo⁻.

The qualitative results indicating that Klenow exo⁻/exo⁺ cannot extend the primer past the C4-AP lesion (Figure 3, Supporting Information) were examined more thoroughly using a standing start. No extension of the primer by either

Table 5. Percent Extension by Bypass Polymerases

enzyme[a]	C4-AP (**3a**)	AP (**3c**)	control (**3b**)
pol II	3.5	0.6	89[b]
pol II exo$^-$	14.5	24.8	88[b]
pol IV	1.8	2.1	78
pol V[c]	0.6	0.8	38

[a] [Enzyme] = 500 nM, except where noted otherwise. [b] [Enzyme] = 100 nM. [c] [Enzyme] = 250 nM, Rec A = 250 nM.

form of Klenow was observed over the course of 30 min when dA (**5a**) or dG (**5b**) was opposite the lesion in the presence of 1 mM dTTP (data not shown).

Primer Extension by Escherichia coli SOS-Induced Bypass Polymerases. The ability of pol II, pol II exo$^-$, pol IV, and pol V in the presence of Rec A to read through the C4-AP lesion (**3a**) was examined in a qualitative manner using a running start (Table 5). The percent extension reported is defined by the sum total of products equal to or longer than that resulting from translesion synthesis. Reactions using a control template (T substituted at the position of C4-AP, **3b**) and an non-oxidized AP site (**3c**) were run side by side. With the exception of Pol V, large amounts of full-length product were obtained from the control template (**3b**) when large excesses of bypass polymerases were used (see Supporting Information). Only modest extension was achieved using pol V (250 nM) in the presence of Rec A (250 nM), and no improvement was observed upon increasing enzyme concentration (*29*). Primers hybridized to templates containing an AP (**3c**) or C4-AP lesion (**3a**) were extended far less efficiently. This was particularly true for pol IV and pol V. Extension by pol II was compromised by the enzyme's exonuclease activity. The enzyme's exonuclease activity made it difficult to gauge the extent that the AP sites were bypassed, but this was not an issue when pol II exo$^-$ was used. Extension past the C4-AP (**3a**) by pol II exo$^-$ was the most proficient of the four bypass polymerases, but extension was approximately half as efficient compared to that of the respective AP-site-containing template (**3c**).

The high concentrations of pol II exo$^-$ required for extension of the primer opposite C4-AP precluded carrying out kinetic analysis of translesion synthesis. However, a qualitative picture of the enzyme's preference was obtained by measuring the percent extension in **4a** in the presence of each dNTP individually (Figure 4A). Comparable measurements were carried out on templates containing an AP site (**4c**, Figure 4B). Pol II exo$^-$ incorporated dT followed by dG most efficiently under these conditions. In contrast, pol II exo$^-$ preferred to incorporate dA and dG opposite an AP site. The pol II exo$^-$ observations were calibrated by carrying out comparable extension experiments on **4a** and **4c** using Klenow exo$^+$ (Figure 4C,D). As expected, incorporation levels for all of the dNTPs were higher. Moreover, the polymerase preferred to incorporate dA > dG > T > dC opposite the C4-AP and AP lesions.

DISCUSSION

The C4′-hydrogen atom is a likely target for reactive species that react with DNA. The respective carbon−hydrogen bond is a relatively weak one, and the C4′-hydrogen atom lies on the outer edge of the minor groove (*7*, *9*). These physical features of DNA provide an explanation for why the C4-AP site is a commonly formed lesion. We investigated the mutagenic potential of the C4-AP lesion. As an abasic site, the C4-AP lesion can be described as a noninformative one. Consequently, we compared the C4-AP lesion to the regular abasic site (AP) and 2-deoxyribonolactone (L), whose effects on polymerases have been previously characterized (*12*, *30*). We carried out more limited studies on the latter two lesions in order to provide a benchmark for comparison to literature values, which were obtained from templates containing different sequences.

Qualitative primer extension experiments (Figure 3, Supporting Information) using either form of Klenow suggest that C4-AP is a blocking lesion. Both forms of Klenow pause prior to incorporating a nucleotide opposite the C4-AP lesion. Moreover, neither polymerase extends the primer past the lesion. The C4-AP lesion's effect on Klenow is similar to that exhibited by a "regular" AP site and 2-deoxyribonolactone (L). Klenow enzymes were able to extend past the lactone lesion only under forcing conditions, and bypass was achieved most efficiently on templates whose sequences were prone to introducing deletions (*30*). Kinetic experiments reveal that the regular abasic site (AP) is also a strong blocking lesion. Nucleotide incorporation opposite an AP site by Klenow exo$^+$ was approximately 1000-fold slower than that when a native nucleotide was present. Extension (bypass) of a primer containing a 3′-terminal dA opposite the AP site was retarded by yet another 1000-fold (*12*).

The similarities and differences between the effects of the C4-AP, AP, and L lesions are evident under more quantitative analysis as well. Translesional synthesis (nucleotide incorporation opposite a lesion) is considerably less efficient than replication opposite a native nucleotide. Both forms of Klenow preferentially incorporate dA opposite any of the three abasic sites, followed by dG (Table 3). Pyrimidine incorporation opposite the C4-AP site was too slow to measure accurately. This, too, is consistent with previous reports on the AP and L lesions in which pyrimidines are incorporated less efficiently than purines by 10−100 times or more (*12*, *30*, *31*). The absolute specificities for dA or dG incorporation by Klenow exo$^-$ opposite the C4-AP and AP lesions are within experimental error of one another for each respective nucleotide (Table 3). Furthermore, the kinetic parameters measured for translesional synthesis by Klenow exo$^-$ on the latter (**4b**) are comparable to those previously reported in a different sequence (*12*). Klenow exo$^-$ exhibits an approximately 8- and 11-fold preference for dA versus dG incorporation opposite the C4-AP and AP lesions, respectively. A slightly more modest preference for dA incorporation (V_{max}/K_m, dA:dG $\approx$ 6.4) is observed using Klenow exo$^+$, a trend which is also consistent with a reported study on a template containing a regular AP site (*12*). In contrast, the 2-deoxyribonolactone lesion was recognized more weakly by Klenow exo$^-$ than either of the other lesions. The kinetic parameters for dA incorporation opposite L measured using **4c** were very similar to those previously reported (*30*). The apparent K_m is significantly higher, and the V_{max} is ~10-fold lower than that measured for incorporation of dA opposite AP or C4-AP (Table 3).

It is tempting to postulate a structural reason for the quantitative differences observed between replication of templates containing 2-deoxyribonolactone in comparison to the AP and C4-AP lesions. Limited UV-melting studies

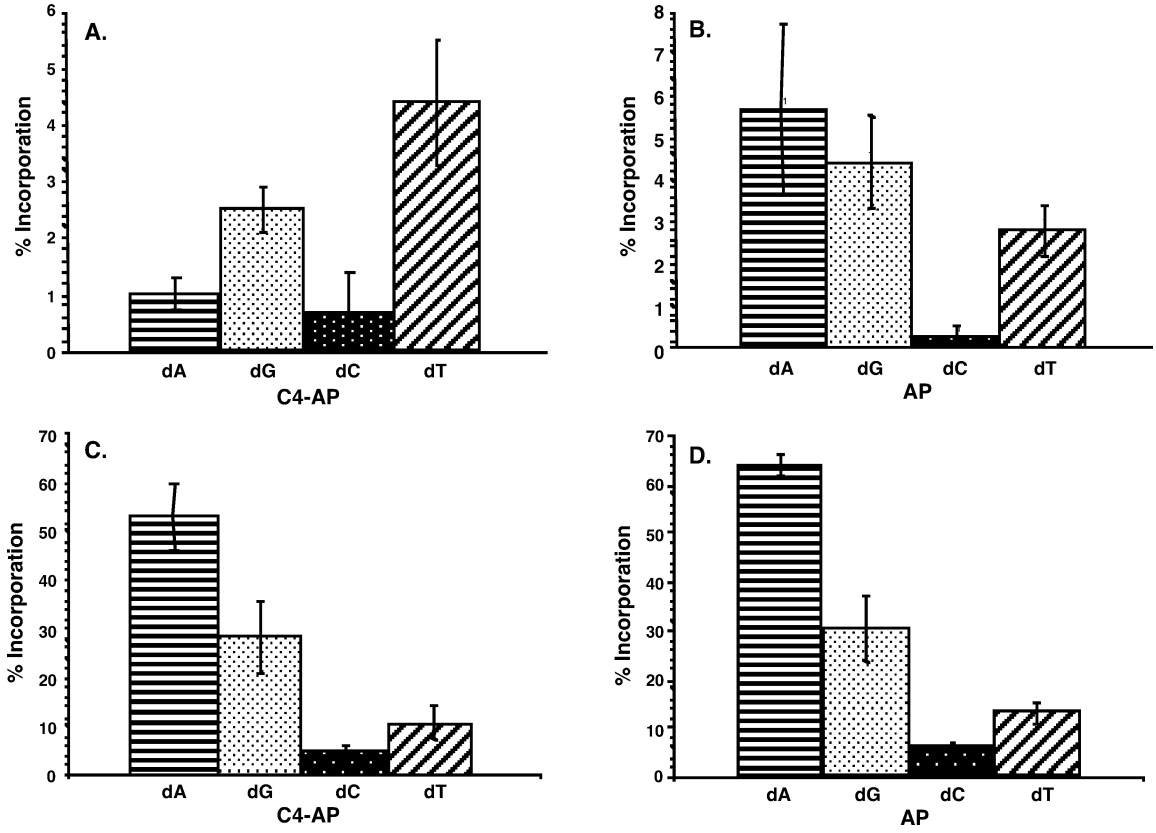


FIGURE 4: Single time point measurement of translesional nucleotide incorporation by Pol II exo$^-$ opposite (A) C4-AP (**4a**) or (B) AP (**4c**) and by Klenow exo$^+$ opposite (C) C4-AP (**4a**) or (D) AP (**4c**).

indicate that the abasic lesions destabilize duplex DNA to comparable extents (*22*, *32*, *33*). Structural information on DNA containing the C4-AP site is unavailable at this time, and variable effects have been observed in NMR studies of an AP site (or its stable tetrahedral analogue) (*34*−*36*). However, examination of a duplex that is otherwise identical to that containing L indicated that, overall, the structures of duplexes containing this lesion or an AP site are comparable (*37*, *38*). We suggest that the structural differences between 2-deoxyribonolactone (L) and the other two abasic sites are subtle and are concentrated at carbon one. All three abasic sites should exist predominantly in their cyclic isomers, particularly L, for which ring opening is not readily reversible. The C1-carbonyl of 2-deoxyribonolactone (L) represents a hydrogen bond acceptor, whereas the hemiacetal forms of the AP and C4-AP lesions present a potential hydrogen bond donor in two possible configurations. Furthermore, sp^2 hybridization at C1 in 2-deoxyribonlactone results in a different shape than those exhibited by the sp^3-hybridized C4-AP and AP lesions (*39*).

The kinetic experiments also suggest that the polymerases do not detect the structural differences present at C4 between the C4-AP and AP lesions. The 4*S*-C4-hydroxyl group in C4-AP (Figure 1) should alter the phosphate backbone significantly more than the 4*R*-isomer. The 4*S*-diastereomers of C4-AP presumably decrease the thermal stability of duplex DNA more than the 4*R*-diastereomers. Consequently, it is possible that these comprise minor constituents of the equilibrium mixture. Alternatively, it is possible that the altered backbone introduced by the 4*S*-C4-AP isomers may affect bypass more than translesional synthesis. The former was too slow for us to measure and is very inefficient for AP lesions as well (*12*).

The reduced rate of nucleotide incorporation opposite the lesions compared to undamaged templates, and the inability to detect C4-AP bypass by Klenow exo$^+$/exo$^-$, led us to investigate primer extension by *E. coli* alternative polymerases. The observed effects were calibrated by comparison to templates containing thymidine or AP sites. The total amount of extension was summed over all nucleotides. Only pol II exo$^-$ was able to extend a significant fraction of primers hybridized to AP (**3a**) or C4-AP (**3c**) templates, despite being present in large excess. Full-length product was less than 1% of the total DNA. The absence of full-length product is not surprising because the bypass polymerases are known to exhibit low processivity, and short DNA templates such as those employed here are typically poor substrates. These observations are consistent with previous studies in which AP sites are a substrate for pol II exo$^-$ (*28*). Previous studies suggested that including accessory proteins would not enhance extension of **3** or **4** due to their short length (*40*). Realizing that this is only a model of what occurs within a cell, we focused on the relative preference for nucleotide incorporation by pol II exo$^-$ opposite C4-AP, and AP for comparison, because this enzyme exhibited the greatest activity on **3a** (Figure 4). Nucleotide incorporation by Klenow exo$^+$ was carried out under the same conditions in order to calibrate the results with pol II exo$^-$. The results of these single-point measurements with Klenow exo$^+$ (Figure 4C,D) were consistent with the steady-state kinetic measurements (Table 3), suggesting that the method was reliable. Nucleotide incorporation by pol II exo$^-$ opposite

the AP lesion followed the "A-rule" (Figure 4B) (*41*). However, the bypass polymerase preferentially incorporated thymidine opposite the C4-AP site (Figure 4A).

Our inability to carry out comparable in vitro experiments using pol IV and pol V does not eliminate the possibility that these polymerases may incorporate thymidine or other nucleotides preferentially opposite the C4-AP lesion in vivo. However, preferential incorporation of thymidine opposite the C4-AP lesion by pol II exo$^-$ is unique with regard to all other in vitro experiments that we are aware of. All available information indicates that prokaryotic DNA polymerases preferentially incorporate dA followed by dG opposite AP and L lesions in vitro (*12*, *30*, *31*). The A-rule is followed by the pol V bypass polymerase when replicating AP sites (*42*). Deoxyadenosine is also preferentially incorporated opposite AP sites in *E. coli* (*43*).

The reason for enhanced thymidine incorporation opposite C4-AP by pol II exo$^-$ is unknown. The 5′-adjacent nucleotide relative to C4-AP in the template (**4a**) is dA. We cannot rule out a mechanism in which thymidine is incorporated via a misalignment mechanism in which the lesion is looped out, as is seen when pol IV acts on AP sites (*26*, *44*). However, previous studies involving pol II and AP sites discounted this explanation for translesion incorporation of dA (*45*). Regardless of the mechanism for increased thymidine incorporation by pol II opposite the C4-AP lesion, these experiments point out a difference between the handling of this abasic site compared to the AP lesion. Presumably, the difference is due to the presence of the C4-hydroxyl group. However, we cannot determine at this time if thymidine insertion is ascribable to a particular stereoisomer (e.g., 4*S*) at the position, which differentiates C4-AP and AP lesions. Furthermore, the in vitro reaction of pol II exo$^-$ may be significantly different than what happens in *E. coli* in the presence of other proteins.

Finally, it is interesting to note that previous studies on the mutations induced in *E. coli* by bleomycin revealed significant levels of G:C → T:A transversions (*18*). These transversions were concentrated at 5′-d(CG*C*C) sequences (site of mutation italicized), in which the chemical selectivity of bleomycin would lead one to predict high levels of C4-AP production from deoxycytidine oxidation (*5*). The in vitro experiments described above suggest that the bleomycin mutations involving G:C → T:A transversions may be attributable at least in part to C4-AP bypass by pol II. If pol II is involved in these transversions, a misalignment mechanism cannot explain the observed substitution. Misalignment in the 5′-d(CGCC) sequence would result in misincorporation of dC, not thymidine.

CONCLUSIONS

Despite a significant structural difference, the in vitro examination of the effects of the C4-AP site on Klenow exo$^+$/exo$^-$ and bypass polymerase activity indicate that in many respects the oxidized abasic lesions parallel those previously reported for regular abasic sites (AP). A notable exception is the preferential thymidine incorporation by pol II exo$^-$ opposite the C4-AP lesion. Thymidine incorporation opposite C4-AP may be attributable to a misalignment mechanism. However, this would distinguish the lesion from a regular abasic site, which does not interact with pol II in this manner (*45*). Pol II is not believed to play a significant role in bypassing "regular" AP sites (*46*). When the observations reported here are evaluated in the context of bleomycin mutagenesis studies, they suggest that pol II may play a role in the mutagenesis induced by the C4-AP lesion. These results provide motivation for examining the possible role of pol II in replicating DNA containing the C4-AP in vivo.

SUPPORTING INFORMATION AVAILABLE

Figures showing Klenow exo$^+$-mediated extension of **3a,b** and exo$^-$-mediated extension of **3a** in the presence of native dNTPs, bypass polymerase extension of **3a**−**c**, and pol V, Rec A extension of **3a**−**c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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