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Characterization of Interstrand DNA–DNA Cross-Links Derived from Abasic Sites Using Bacteriophage ϕ29 DNA Polymerase

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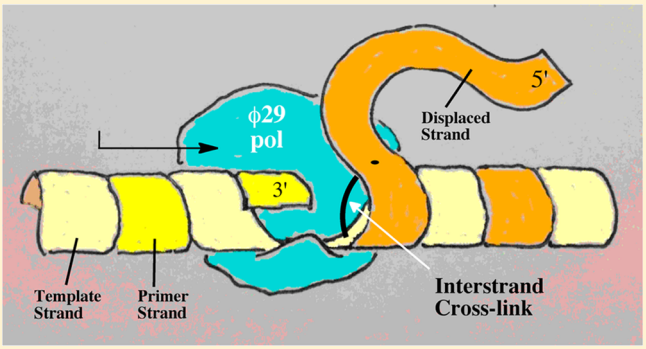
Supporting Information

ABSTRACT: Interstrand cross-links in cellular DNA are highly deleterious lesions that block transcription and replication. We recently characterized two new structural types of interstrand cross-links derived from the reaction of abasic (Ap) sites with either guanine or adenine residues in duplex DNA. Interestingly, these Ap-derived cross-links are forged by chemically reversible processes, in which the two strands of the duplex are joined by hemiaminal, imine, or aminoglycoside linkages. Therefore, understanding the stability of Ap-derived cross-links may be critical in defining the potential biological consequences of these lesions. Here we employed bacteriophage φ29 DNA polymerase, which can couple DNA synthesis and strand displacement, as a model system to examine whether dA-Ap cross-links can withstand DNA-processing enzymes. We first demonstrated that a chemically stable interstrand cross-link generated by hydride reduction of the dG-Ap cross-link completely blocked primer extension by φ29 DNA polymerase at the last unmodified nucleobase preceding cross-link. We then showed that the nominally reversible dA-Ap cross-link behaved, for all practical purposes, like an irreversible, covalent DNA–DNA cross-link. The dA-Ap cross-link completely blocked progress of the φ29 DNA polymerase at the last unmodified base before the cross-link. This suggests that Ap-derived cross-links have the power to block various DNA-processing enzymes in the cell. In addition, our results reveal φ29 DNA polymerase as a tool for detecting the presence and mapping the location of interstrand cross-links (and possibly other lesions) embedded within regions of duplex DNA.



Interstrand cross-links in cellular DNA are highly deleterious because they prevent the strand separation that is necessary for cellular machinery to extract genetic information from the double helix during transcription and replication.[1−3] We recently characterized two new structural types of interstrand cross-links derived from the reaction of abasic (Ap) sites with either guanine or adenine residues in duplex DNA (Scheme 1).[4−8] Ap sites are abundant in cellular DNA,[9−12] and cross-links derived from these lesions could play an important role in spontaneous mutagenesis, neurodegeneration, and aging.[13−19] Interestingly, these Ap-derived cross-links are forged by chemically reversible processes, in which the two strands of the duplex are joined by hemiaminal, imine, or aminoglycoside linkages (Scheme 1). Therefore, understanding the stability of Ap-derived cross-links may be critical in defining the potential biological consequences of these lesions. Early work showed that these cross-links are stable to gel electrophoretic analysis.[4,6−8] Furthermore, recent work has shown that, once formed in duplex DNA, the cross-links are stable for days at pH 7 and 37 °C.[4,7] Nonetheless, with regard to the potential biological activity of these lesions, it may be most important to consider whether the Ap-derived cross-links can block DNA-processing enzymes that induce strand separation.

In the studies described here, we employed bacteriophage φ29 DNA polymerase as a model system to examine whether dA-Ap cross-links can withstand DNA-processing enzymes. φ29 DNA polymerase displays remarkably high processivity and strand-displacement activity.[20] In fact, this polymerase is able to separate the strands of a DNA duplex and carry out DNA synthesis without assistance from helicases, clamp proteins, or other accessory factors.[21] Accordingly, φ29 DNA polymerase has been described as a hybrid polymerase-helicase.[22] We anticipated that these properties might make φ29 DNA polymerase a useful tool for examining whether Ap-derived cross-links can withstand enzyme-induced melting of the DNA duplex. As a test of this methodology, we first examined the action of φ29 DNA polymerase on substrates containing the chemically stable interstrand cross-link **5** (Scheme 1A) generated by hydride reduction of the dG-Ap cross-link.[4−6] This chemically stable cross-link blocked primer extension by φ29 DNA polymerase at the last unmodified nucleobase preceding the cross-link. We then characterized φ29

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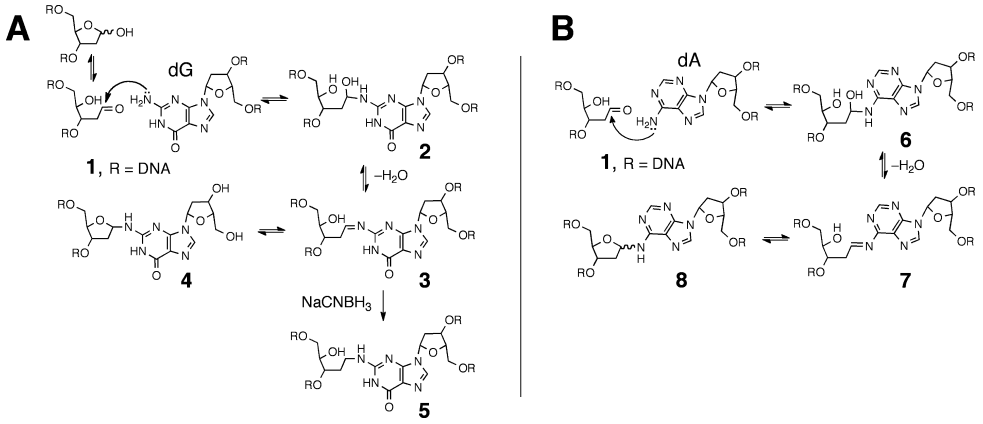
Scheme 1



DNA polymerase-mediated primer extension on substrates containing the chemically reversible dA-Ap cross-link (**8**, Scheme 1B) embedded in various locations and orientations within a region of duplex DNA. We found that the dA-Ap cross-link behaves like an irreversible, covalent DNA−DNA cross-link that completely blocks the progress of the φ29 DNA polymerase. The results suggest that, once formed in genomic DNA, Ap-derived cross-links may have the power to block various DNA-processing enzymes and exert significant biological effects. In addition, our results establish φ29 DNA polymerase as a tool for determining the presence and location of interstrand cross-links (and possibly other lesions) embedded within regions of duplex DNA.

EXPERIMENTAL SECTION

Reagents. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Uracil DNA glycosylase (UDG), φ29 DNA polymerase, and T7 DNA polymerase were from New England Biolabs (Ipswich, MA). A mixture of the four 2′-deoxynucleotide triphosphates (dNTP) was purchased from Promega (Madison, WI). [γ-^{32}P]-ATP (6000 Ci/mmol) was purchased from PerkinElmer. C-18 Sep-Pak cartridges were purchased from Waters (Milford, MA), and BS Poly prep columns were obtained from BioRad (Hercules, CA). Acrylamide/bis-acrylamide 19:1 (40% Solution/Electrophoresis) was purchased from Fisher Scientific (Waltham, MA), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Representative Procedure for Preparation of Cross-linked Templates Involving a Reduced dG-Ap Cross-link or dA-Ap Cross-link. A single-stranded, uracil-containing 2′-deoxy-oligonucleotide was annealed with its complementary strand and treated with the enzyme UDG (50 units/mL, final concentration) to generate duplexes containing an Ap site at a defined location.[23,24] The UDG enzyme was removed by phenol-chloroform extraction. The DNA was then ethanol precipitated,[25] and the duplexes were redissolved in either sodium acetate buffer (750 mM, pH 5.2) containing $NaCNBH_3$ (250 mM) and incubated at 37 °C for 24 h to form the reduced dG-Ap cross-link or redissolved in HEPES buffer (50 mM, pH 7.0) containing NaCl (100 mM) and incubated at 37 °C for 120 h to form the dA-Ap cross-link. The DNA was then ethanol precipitated, resuspended in formamide loading buffer,[25] and loaded onto a 2 mm thick 20% denaturing polyacrylamide gel, and the gel electrophoresed for 10 h at 200 V. The DNA bands in the gel were visualized by UV shadowing, and the slow-moving band corresponding to cross-linked duplex[6,8] was cut from the gel with a razor blade. The gel slice was crushed and then agitated in an elution buffer composed of aqueous NaCl (200 mM) and 1 mM EDTA (1 mM, pH 8) at 24 °C for 1 h. The gel fragments were removed by filtering through a Poly-Prep column, and the filtrate was desalted using a C18 Sep-Pak (100 mg size). The resulting solution was dried using a Speed-Vac concentrator, and the residue was redissolved in HEPES buffer (50 mM, pH 7.0) containing NaCl (100 mM) and stored frozen at −20 °C prior to use.

Representative Procedure for ϕ29 DNA Polymerase Primer Extension Reactions. The primer was α-^{32}P 5′-end-labeled using standard procedures.[25] The labeled primer (100 000 cpm) was annealed to the template DNA (200 pmoles) in MOPS buffer (50 mM, pH 7.0) containing NaCl (100 mM) by incubation at 24 °C for 12 h to generate substrates for the polymerase assay. The DNA duplex was then ethanol precipitated, redissolved in an appropriate amount of concentrated (1.1×) solution of the polymerase assay buffer (see below) to give approximately 2222 cpm/μL, and aliquotted into the assays. For each primer extension assay, the polymerization reaction was started by addition of ϕ29 DNA polymerase (1 μL, 10 units) to 9 μL of the concentrated (1.1×) stock solution mentioned above to give an assay with a final volume of 10 μL that contained Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), the four canonical dNTPs (1 mM in each), bovine serum albumin (0.1 mg/mL), and 20 000 cpm [α-^{32}P]-labeled DNA substrate. After incubation at 24 °C for 30 min (for the un-cross-linked templates or templates containing a reduced dG-Ap cross-link) or 60 min (for the templates containing the dA-Ap cross-link), the reaction was stopped by addition of EDTA (1 μL of a 110 mM, pH 8 solution) to give a final concentration of 10 mM EDTA. Protein was removed from the sample by phenol-chloroform extraction,[25] the primer extension products ethanol precipitated, resuspended in formamide loading buffer, loaded onto a 20% denaturing polyacrylamide gel, and the gel electrophoresed for 9 h at 1000 V. Marker lanes were generated by hydroxyl radical cleavage, Maxam−Gilbert G-reactions, and Maxam−Gilbert A+G-reactions on a 5′-^{32}P-labeled single-stranded authentic synthetic standard of the full-length extension product using literature protocols.[26,27] The radiolabeled DNA fragments in the gel were visualized, and the amount of radioactivity in each band was measured by phosphorimager analysis using a Personal Molecular Imager (BioRad) with Quantity One software (v.4.6.5).

Representative Procedure for T7 DNA Polymerase Primer Extension Reaction. Substrates consisting of a template hybridized to a α-^{32}P 5′-end-labeled primer were prepared as described above, and 20 000 cpm of labeled DNA substrate was aliquotted into each reaction mixture. Primer extension reactions were initiated by addition of T7 DNA polymerase (10 units) to 10 μL of a solution containing Tris-HCl (20 mM, pH 7.5), $MgCl_2$ (10 mM), DTT (1 mM), bovine serum albumin (0.1 mg/mL), the four dNTPs (0.5 mM in each), and 20 000 cpm of the labeled substrate. The mixture was incubated at 24 °C for 8 min, followed by quenching by addition of formamide loading buffer (80 μL, composed of 95% deionized formamide, 25 mM EDTA (pH 7.5), 0.1% bromophenol and xylene cyanol). The DNA was loaded onto a 20% denaturing polyacrylamide gel, and the gel electrophoresed for 9 h at 1000 V, and the radiolabeled DNA fragments in the gel visualized by phosphorimager analysis.

Melting Temperature Measurement. The melting temperature of duplex B (no primer annealed) was determined by monitoring the

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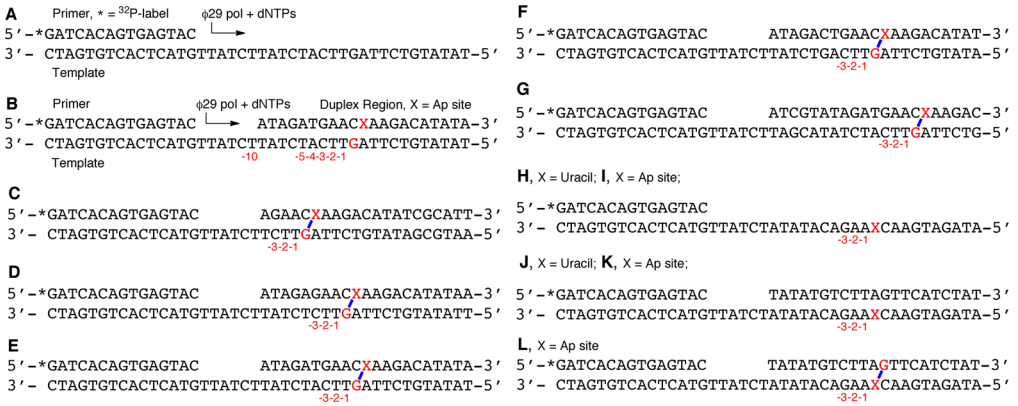

Figure 1. Polymerase substrates containing the chemically stable reduced dG-Ap cross-link (**5**).

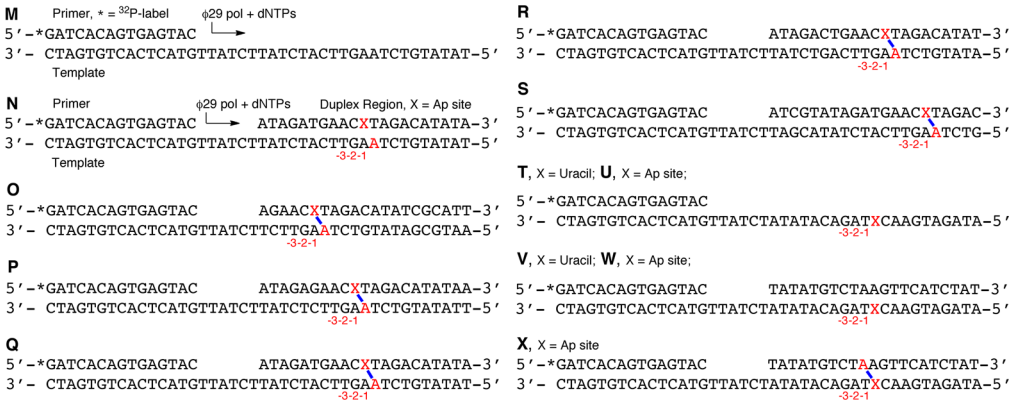

Figure 2. Polymerase substrates containing dA-Ap cross-link (**8**).

increase in absorbance at 260 nm as a function of temperature. The temperature was increased from 20 to 70 °C at a rate of 0.5 °C/min. The melting temperature was determined using Cary WinUV-Thermal software.

RESULTS AND DISCUSSION

Preparation and Characterization of Polymerase Substrates Containing Ap-Derived Cross-links. The polymerase templates used here were 42 nucleotide (nt), mixed-sequence 2′-deoxyoligomers (Figures 1 and 2). At the 5′-end of most templates was located a 21 base pair (bp) duplex region. The duplex region of some substrates contained Ap-derived cross-links, indicated by the blue lines joining the complementary strands in Figures 1 and 2. These cross-linked substrates were prepared using previously described methods.[6,8] Briefly, Ap sites were generated at defined locations by treatment of the corresponding 2′-deoxyuridine-containing duplexes with the enzyme uracil DNA glycosylase (UDG).[23,24] Substrates **C**−**G** and **L**, containing the chemically stable, reduced dG-Ap cross-link (**5**, Scheme 1A) in the duplex region were generated by incubation of the Ap-containing duplex in sodium acetate buffer (750 mM, pH 5.2) containing $NaCNBH_3$ (250 mM) at 37 °C for 24 h. Templates **O**−**S** and **X**, containing a dA-Ap cross-link at various locations in the duplex region, were prepared by incubation of the requisite Ap-containing duplex in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) at 37 °C for 120 h. The cross-linked DNA duplexes were separated from un-cross-linked material by electrophoresis on a 20% denaturing polyacrylamide gel. The cross-linked DNA was excised from the gel and desalted prior to use in the polymerase experiments. The location of interstrand cross-linking (shown in Figures 1 and 2) in the sequences used here has been established previously.[4−8]

We first set out to demonstrate that the 21 bp duplex region of the un-cross-linked polymerase substrates used here remains annealed to the template strand under the conditions used for the primer extension experiments. A UV−vis thermal melting experiment on the un-cross-linked duplex **B** (freshly prepared Ap site, without the primer bound) showed a T_m of 51.3 ± 0.9 °C in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)SO_4$ (10 mM), DTT (4 mM), containing 0.1 mg/mL bovine serum albumin at a duplex concentration of approximately 1 μM (Figure S1). In addition, we examined duplex **B** as a substrate for T7 DNA polymerase. T7 DNA polymerase is not capable of efficient strand displacement synthesis and was expected to stall at, or near, the single strand-duplex junction of this substrate.[28] A 15-nt ^{32}P-labeled primer was hybridized to the 3′-end of the template strand, and T7 DNA polymerase was allowed to extend the primer in the presence of all four dNTPs. As expected, we observed that the primer extension by T7 DNA polymerase stalled near the beginning of the double helical region of substrate **B** (Figure S2). A control experiment showed that T7 DNA polymerase was able to fully extend a primer annealed to the single-stranded template **A** (Figure S2). Together, these results provided evidence that the duplex region of the polymerase substrates used in these studies remains hybridized under conditions identical to (T_m experiments) or similar to (T7 experiments) those used for the φ29 DNA polymerase experiments described below.

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Chemically Stable, Reduced dG-Ap Cross-Links (5) Block Primer Extension by φ29 DNA Polymerase. To establish φ29 DNA polymerase as a useful tool for probing the properties of Ap-derived cross-links, we first examined the ability of this enzyme to carry out primer extension on five different substrates (duplexes **C**−**G**) containing the chemically-*stable*, reduced dG-Ap cross-link (**5**, Scheme 1A) in various locations. A ^{32}P-labeled primer was hybridized to the template and incubated with φ29 DNA polymerase in the presence of all four dNTPs (1 mM in each) for 30 min at 24 °C. The resulting primer extension products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. In each case, the longest extension product observed corresponded to stalling at the −1 position immediately preceding the cross-linked guanine residue on the template strand (Figures 1 and 3). The −1 product was generated alongside a collection of shorter products corresponding to extension of the primer to locations within the duplex region of the substrate (the position of the arrow in the Figures corresponds to extension of the primer to the last base in the single-stranded region of the template). In the case of duplex **G**, bands in the −2 to −8 region were relatively weak. Control experiments showed that φ29 DNA polymerase fully extended a labeled primer that was annealed to the single-stranded substrate **A** and the non-cross-linked, duplex-containing substrate **B** (Figure S3).

We investigated the effect of dNTP concentration on these primer extension reactions. At higher dNTP concentrations larger fractions of the −1 extension product were observed (Figure 4). Conversely, at the lower dNTP concentrations, larger fractions of the −2 and −3 products as well as products resulting from stalling approximately 2−3 bases past the single strand-duplex junction were observed. Control experiments showed that all of the dNTP concentrations used here supported extension of the primer to full length on the un-cross-linked substrate **B** (Figure S4). These experiments showed that the chemically stable reduced dG-Ap cross-link completely blocks primer extension by φ29 DNA polymerase. For the purposes of detecting the presence and mapping the location of a cross-link within a region of duplex DNA, higher dNTP concentrations were most effective.

dA-Ap Cross-Links In Duplex DNA Block Primer Extension by φ29 DNA Polymerase. We next examined the ability of φ29 DNA polymerase to carry out primer extension on five different substrates **O**−**S** containing the chemically reversible dA-Ap cross-link **8** at different locations within the duplex region. The 5′-^{32}P-labeled primer was hybridized to the templates and incubated with φ29 DNA polymerase in the presence of all four dNTPs (1 mM in each) for 60 min at 24 °C. In each case, we observed that primer extension was blocked primarily at the −1 position, the last unmodified base preceding the cross-link (Figure 5). The intensity of other bands corresponding to stalling at other sites preceding the cross-link was relatively weak. Importantly, no significant extension of the primer past the cross-link was observed. Analogous results were observed when primer extension reactions were carried out at 37 °C (Figures S5 and S6).

Time Courses For Primer Extension By φ29 DNA Polymerase On Various Substrates. We examined time courses for primer extension on six different substrates containing the reduced dG-Ap cross-link and the dA-Ap cross-link at different locations within the duplex region (Figures 6 and 7). Early in the reactions, the major products

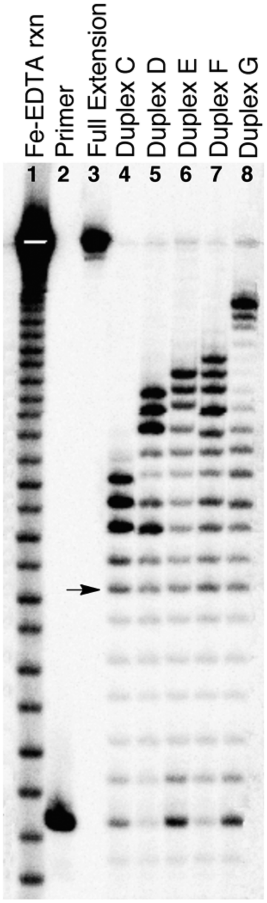

Figure 3. Chemically stable, reduced dG-Ap cross-link 5 blocks primer extension by φ29 DNA polymerase. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 30 min at 24 °C. After reaction workup, the primer extension products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. Lane 1 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA), lane 2 is the 15 nt, 5′-^{32}P-labeled primer, lane 3 is the 5′-^{32}P-labeled full-length extension product, and lanes 4−8 depict the results of primer extension on templates **C**−**G**. The arrow corresponds to extension of the primer to the last base in the single-stranded region of the template. The slight difference in gel mobility observed for the iron-EDTA cleavage products and the primer extension products reflects the fact that the cleavage products generated by iron-EDTA possess 3′-phosphate termini,[26] while the primer extension products possess 3′-hydroxyl termini.

correspond to unextended primer and primer extended to the −1, −2, and −3 positions preceding the cross-links. This is consistent with processive extension of the primer to stall sites near the cross-link. No significant amounts of full-length extension products resulting from bypass of the cross-link were generated at any time point. For both the reduced dG-Ap cross-link (**5**) and the dA-Ap cross-link (**8**), longer incubation times led to increases in the intensity of several shorter extension products. For the most part, these shorter products corresponded to primers extended to locations near the single strand-duplex junction of the template (Figures 6 and 7). The size distribution of these shorter products, presumably

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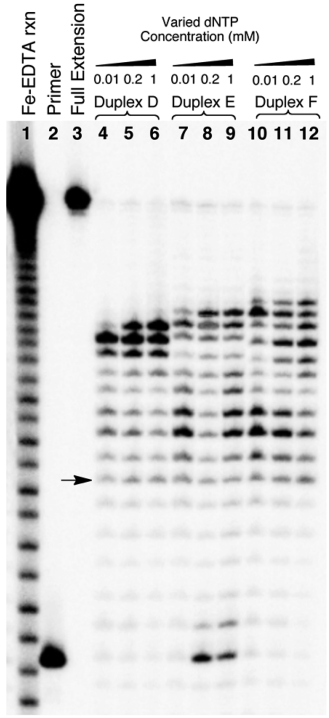

Figure 4. Higher dNTP concentrations favor extension of primers to the −1 position immediately preceding the reduced dG-Ap cross-link 5. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (0.01−1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 30 min at 24 °C. After reaction workup, the primer extension products were subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel. Lane 1 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA), lane 2 is the 15 nt, 5′-^{32}P-labeled primer, lane 3 is the 5′-^{32}P-labeled full-length extension product, and lanes 4−12 depict the results of primer extension on templates **D**−**F** in the presence of the indicated dNTP concentrations. The arrow corresponds to extension of the primer to the last base in the single-stranded region of the template.

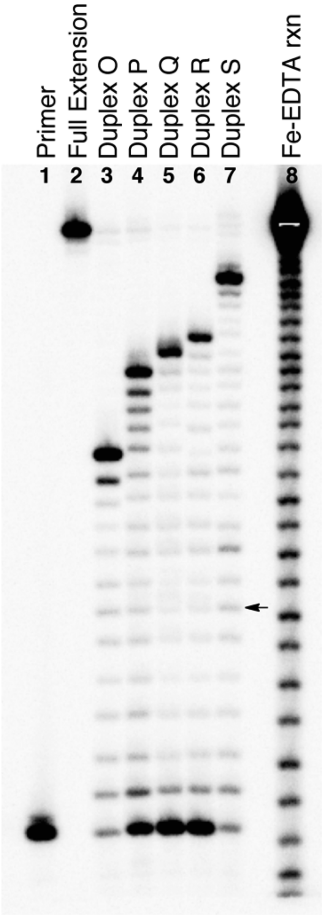

Figure 5. The dA-Ap (**8**) cross-link blocks primer extension by φ29 DNA polymerase. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 60 min at 24 °C. After reaction workup, the primer extension products were subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel. Lane 1 is the 15 nt, 5′-^{32}P-labeled primer, lane 2 is the 5′-^{32}P-labeled full-length extension product, lanes 3−7 depict the results of primer extension reactions on substrates **O**−**S**, and lane 8 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA). The arrow corresponds to extension of the primer to the last base in the single-stranded region of the template.

generated by the exonuclease function of φ29 DNA polymerase,[29−33] differ somewhat for the two types of cross-link examined here. The intensity of these truncated products was greater in the case of the substrates containing the reduced dG-Ap cross-link. With respect to the use of φ29 DNA polymerase as a tool for detecting the presence and location of cross-links in duplex DNA, it is clear that, under the conditions of our experiments, shorter incubation times yield a sharper picture of cross-link location.

Cross-Linked Substrates Containing the Abasic Site in the Template Strand. We examined primer extension on substrates in which the Ap site of the cross-link was located in the template strand rather than the displaced strand. We first examined primer extension on the 2′-deoxyuridine-containing duplexes (**H** and **J**) that served as precursors to the abasic duplexes (**I** and **K**). As expected, these substrates were fully extended by φ29 DNA polymerase (Figure 8, lanes 6 and 8). On the other hand, the Ap site in the template strand of substrates **I** and **K** acted as a partial block to primer extension under the conditions used here (Figure 8, lanes 7 and 9). Substrates **L** and **X**, containing the reduced dG-Ap (**5**) and dA-Ap cross-link (**8**), respectively, blocked primer extension by φ29 DNA polymerase. This shows that the dA-Ap cross-link blocks primer extension by φ29 DNA polymerase regardless of whether the Ap component of the cross-link is located in the template or displaced strand.

CONCLUSIONS

Our results show that the dA-Ap cross-link blocks primer extension by the bacteriophage φ29 helicase-polymerase. On all substrates examined here, primer extension was blocked at the last unmodified base prior to the cross-link. The dA-Ap cross-linkage is nominally reversible but functioned, for all practical purposes, as an irreversible covalent cross-link in these experiments. Previous measurements using optical tweezers showed that the fork junction destabilization energy of φ29 is

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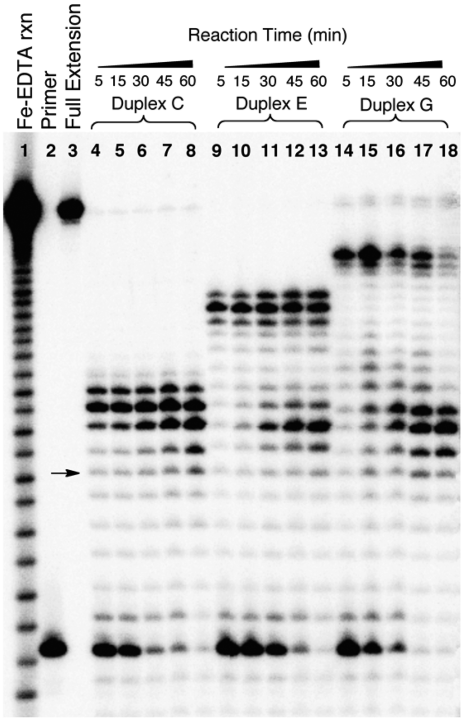

Figure 6. The effects of incubation time on the collection of products generated by φ29 DNA polymerase-mediated primer extension on substrates containing the reduced dG-Ap cross-link 5. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 5−60 min at 24 °C. After reaction workup, the primer extension products were subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel. Lane 1 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA), lane 2 is the 15 nt, 5′-^{32}P-labeled primer, lane 3 is the 5′-^{32}P-labeled full-length extension product, and lanes 4−18 depict primer extension reactions on templates **C**, **E**, and **G** for the indicated times. The arrow corresponds to extension of the primer to the last base in the single-stranded region of the template.

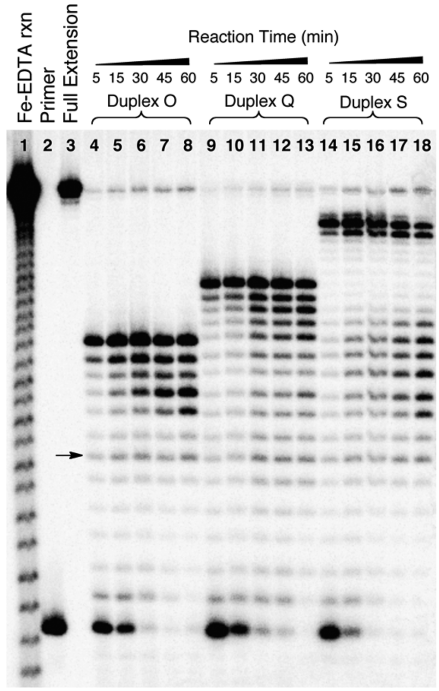

Figure 7. The effects of incubation time on the collection of products generated by φ29 DNA polymerase-mediated primer extension on substrates containing the dA-Ap cross-link 8. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 5−60 min at 24 °C. After reaction workup, the primer extension products were subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel. Lane 1 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA), lane 2 is the 15 nt, 5′-^{32}P-labeled primer, lane 3 is the 5′-^{32}P-labeled full-length extension product, and lanes 4−18 depict primer extension reactions on duplexes **O**, **Q**, and **S** for the indicated times. The arrow corresponds to extension of the primer to the last base in the single-stranded region of the template.

greater than that of other polymerases and helicases examined.[22] This suggests that φ29 polymerase serves as a stringent test of the ability of the dA-Ap cross-link to withstand enzyme-driven strand separation.

DNA in the cells of normal mammalian tissue carry a substantial burden of Ap sites.[9−12] Certainly, not all of the Ap sites generated in cellular DNA go forward to forge interstrand cross-links. Cross-link formation exists in competition with other processes including repair and strand cleavage.[9,34−42] However, even small numbers of cross-links in cellular DNA can lead to profound consequences including mutagenesis, cell death, or cell senescence.[43] Our work suggests that, to the extent that Ap-derived cross-links are generated in cells, these lesions have the potential to exert significant biological effects via blockage of helicases, DNA polymerases, and RNA polymerases.

Analysis of φ29-DNA cocrystal structures suggested that the template strand of the DNA duplex is fed into the polymerase active site via a tunnel that extends 5−6 nt downstream.[21,44] This tunnel, composed of the TPR2 and exo domains of the protein, plays an important role in the remarkable processivity and strand-displacement activity of φ29.[21] Our data support the idea[44] that the active site entry tunnel observed in the crystal structures of φ29 is created by a dynamic flap that can open to allow substrate access. If the tunnel were a rigid structure, the interstrand cross-links in our substrates likely would have stalled primer extension several nucleotides prior to the cross-link lesion, rather than at the last unmodified nucleotide as observed.

Finally, these studies introduce φ29 DNA polymerase as a tool for detecting the presence and mapping the location of interstrand DNA cross-links in duplex DNA. Our results showing that an uncross-linked Ap site serves as a partial block to primer extension further suggest that φ29 polymerase may also be useful for the detection of non-cross-linked lesions on the template strand. This method could provide a useful alternative to T7 RNA polymerase as a tool for mapping the location of DNA adducts generated by natural products such as anthramycin, ecteinascidin, saframycin, or tomaymycin that are stable in duplex DNA but potentially *unstable* in single-stranded DNA.[45−50]

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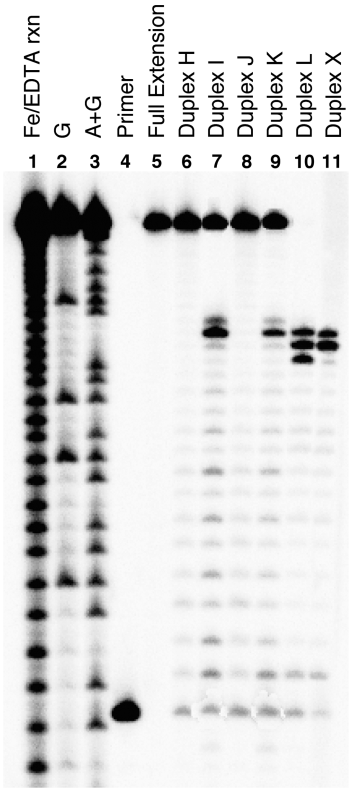

Figure 8. Cross-links containing the abasic site in the template strand block primer extension by φ29 DNA polymerase extension, while an uncross-linked abasic site in the template strand is a partial block. The ^{32}P-labeled primers were extended by incubation of the DNA substrates with φ29 DNA polymerase (10 units) and the four dNTPs (1 mM in each) in Tris-HCl (50 mM, pH 7.5), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (10 mM), DTT (4 mM), and bovine serum albumin (0.1 mg/mL) for 60 min at 24 °C. After reaction workup, the primer extension products were subjected to electrophoretic analysis on a 20% denaturing polyacrylamide gel. Lane 1 is an iron-EDTA cleavage reaction on a synthetic standard of the full-length extension product (5′-^{32}P-GAT CAC AGT GAG TAC AAT AGA ATA GAT GAA CTA AGA CAT ATA); lanes 2 and 3 are Maxam−Gilbert G- and A+G-reactions carried out on the 5′-^{32}P-labeled full-length extension product; lane 4 is the 15 nt, 5′-^{32}P-labeled primer; lane 5 is the 5′-^{32}P-labeled full-length extension product; lane 6, primer extension on the single-strand substrate **H** containing dU in the template; lane 7, single-strand substrate **I** containing Ap in the template; lane 8, duplex substrate **J** containing dU in the template; lane 9, duplex substrate K containing an uncross-linked Ap site in the template strand; lane 10, duplex substrate **L** containing reduced dG-Ap cross-link **5** with the Ap residue in the template strand; lane 11, duplex substrate **X** containing the dA-Ap cross-link in which the Ap residue is in the template strand.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

UV−vis thermal melting analysis of substrate B, T7 DNA polymerase-mediated primer extension on substrate B, control reactions involving φ29-mediated primer extension on substrate B, and primer extension reactions at 37 °C. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00482.

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Notes

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■ ABBREVIATIONS

Ap, DNA abasic site; dA, 2′-deoxyadenosine; dG, 2′-deoxyguanosine; UDG, uracil DNA glycosylase; dNTP, 2′-deoxynucleoside triphosphate; HEPES, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, 1,4-dithio-D-threitol; nt, nucleotide; bp, base pair

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