Replication of an Oxidized Abasic Site in *Escherichia coli* by a dNTP-Stabilized Misalignment Mechanism that Reads Upstream and Downstream Nucleotides†

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ABSTRACT: Abasic sites (AP) and oxidized abasic lesions are often referred to as noninstructive lesions because they cannot participate in Watson−Crick base pairing. The aptness of the term noninstructive for describing AP site replication has been called into question by recent investigations in *E. coli* using single-stranded shuttle vectors. These studies revealed that the replication of templates containing AP sites or the oxidized abasic lesions resulting from C1′- (L) and C4′-oxidation (C4-AP) are distinct from one another, suggesting that structural features other than Watson−Crick hydrogen bonds contribute to controlling replication. The first description of the replication of the abasic site resulting from formal C2′-oxidation (C2-AP) is presented here. Full-length and single-nucleotide deletion products are observed when templates containing C2-AP are replicated in *E. coli*. Single nucleotide deletion formation is largely dependent upon the concerted effort of pol II and pol IV, whereas pol V suppresses frameshift product formation. Pol V utilizes the A-rule when bypassing C2-AP. In contrast, pol II and pol IV utilize a dNTP-stabilized misalignment mechanism to read the upstream and downstream nucleotides when bypassing C2-AP. This is the first example in which the identity of the 3′-adjacent nucleotide is read during the replication of a DNA lesion. The results raise further questions as to whether abasic lesions are noninstructive lesions. We suggest that abasic site bypass is affected by the local biopolymer structure in addition to the structure of the lesion.

Abasic sites (AP[1]), the most common DNA lesions formed, are produced in a variety of ways in DNA, including as intermediates during base excision repair (*1*−*3*). In addition, a variety of oxidized abasic lesions are produced when DNA is exposed to oxidative stress. DNA damaging agents selectively produce oxidized abasic lesions, including 2-deoxyribonolactone (L), and the C4′-oxidized abasic site (C4-AP) (Figure 1) (*4*−*7*). These agents take advantage of the accessibility and/or relatively low bond strength of the respective carbon−hydrogen bonds (*8*−*10*). The C2′-carbon−hydrogen bond is the strongest of the deoxyribose bonds in DNA. Consequently, the C2-AP lesion is only produced by highly reactive species, presumably via hydrogen atom abstraction of one of the C2′-carbon−hydrogen bonds (Scheme 1). These species include the hydroxyl radical, the product formed from water by γ-radiolysis, and the sigma radicals formed upon irradiation of 5-halopyrimidines in DNA (*11*−*15*). Until recently, when a method for synthesizing oligonucleotides containing C2-AP was reported, little was known about the lesion's biochemical effects (*16*). Subsequent in vitro experiments revealed that C2-AP in DNA is incised by the Type II *E. coli* repair enzymes, exonuclease III (Exo III) and endonuclease IV (Endo IV), albeit less

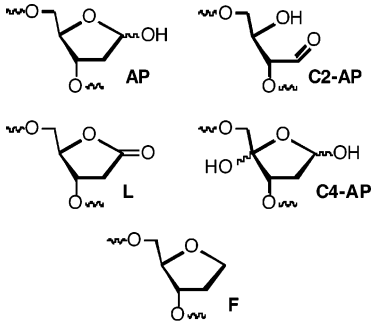
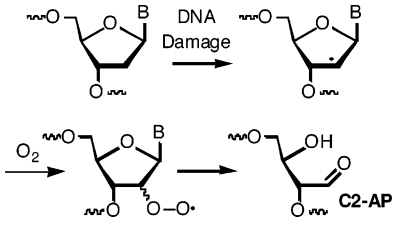


FIGURE 1: Structures of abasic lesions and an abasic site analogue (F).

Scheme 1: C2-AP Formation via Initial C2′-Hydrogen Atom Abstraction



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[1] Abbreviations: C2′-oxidized abasic site, C2-AP; C4′-oxidized abasic site, C4-AP; abasic site, AP; 2-deoxyribonolactone, L; tetrahydrofuran abasic site model, F; polyacrylamide gel electrophoresis, PAGE; DNA polymerase II, pol II; DNA polymerase IV, pol IV; DNA polymerase V, pol V; DNA polymerase beta, pol β.

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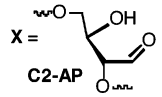
efficiently than other abasic lesions (*17*). Herein we describe the replication of templates containing the C2-AP and the role of SOS polymerases in bypassing the lesion in *E. coli* using single-stranded shuttle vectors. Our observations reinforce recent findings indicating that DNA polymerases discriminate between templates containing various abasic lesions, despite the inability to form Watson−Crick hydrogen bonds (*18*).

Abasic sites (AP) are incapable of forming Watson−Crick hydrogen bonds, which has led to their categorization as noninstructive lesions (*19*, *20*). Despite this, dA is preferentially incorporated opposite AP lesions in *E. coli* (*21*−*23*). However, the A-rule is not adhered to when AP lesions are bypassed in yeast (*24*−*26*). In addition, the replication of AP in templates by bypass polymerases (e.g., pol IV, Dpo4) reveals that the nucleotide 5′ to the AP site can interact with the incoming nucleotide triphosphate via a structure in which the abasic site is looped out (*27*−*29*). Substitution or −1-frameshifts occur depending upon whether the template undergoes realignment. Recent studies in *E. coli* on oxidized abasic sites (L, C4-AP) revealed that despite these molecules' inability to participate in Watson−Crick hydrogen bonds they distinctively affect replication. For instance, the replication of 2-deoxyribonolactone (L) does not follow the A-rule (*30*, *31*). The nucleotide 5′ to L does not influence the identity of the preferentially incorporated translesional nucleotide, which is frequently dG. Furthermore, unlike the replication of templates containing AP lesions, −1-frameshifts are only observed when the 5′ adjacent nucleotide to L is dC. The differences between the replication of templates containing C4′-oxidized abasic lesions (C4-AP) in *E. coli* and other abasic lesions is even more pronounced (*32*). Three nucleotide deletions are the exclusive products when templates containing C4-AP are replicated under SOS-induced conditions. These relatively large deletions were detected in two different nonrepeating local sequences. The distinctive behavior exhibited in *E. coli* by the AP, L, and C4-AP lesions piqued our curiosity with respect to the effects of the C2′-oxidized abasic site (C2-AP). We took advantage of the site specific generation of C2-AP in oligonucleotides to unambiguously examine the lesion's mutagenicity in *E. coli* using Delaney and Essigmann's M13 shuttle-vector method (*16*, *33*−*35*). These experiments further illustrate that despite the inability of abasic sites to form Watson−Crick hydrogen bonds, the replication of these lesions is diverse because of the transfer of other structural information.

MATERIALS AND METHODS

General Procedures. Oligonucleotide synthesis was carried out on an Applied Biosystems, Inc. 394 DNA synthesizer using standard protocols. Oligonucleotides containing the C2′-oxidized abasic site (C2-AP) were synthesized as described (*16*). Commercially available oligonucleotide synthesis reagents were obtained from Glen Research. Oligonucleotide separations were carried out on 20% polyacrylamide denaturing gel electrophoresis (5% cross-link, 45% urea (by weight)). Purified oligonucleotides were characterized by ESI-MS using an LCQ-Deca after precipitating them from NH_4OAc (*36*). The oligonucleotides used in these experiments are shown in Table 1. T4 polynucleotide kinase, EcoR I, T4 DNA ligase, Bbs I, and Hae III were obtained from New England Biolabs. T4 DNA polymerase

Table 1: Oligonucleotides Employed in Experiments

5'-d(GAA GAC CC**X** GGC GTC C)
5'-CXG
5'-d(GAA GAC CT**X** GGC GTC C)
5'-TXG
5'-d(GAA GAC CT**X** AGC GTC C)
5'-TXA
X =  **C2-AP**

was obtained from USB. Nuclease P1 and shrimp alkaline phosphatase were obtained from Roche. *E. coli* cells containing single SOS polymerase knockouts were obtained as previously described (*37*). [γ-^{32}P]-ATP was purchased from Amersham Pharmacia Biotech. Quantification of radiolabeled nucleotide monophosphates following cellulose TLC separation was carried out using a Molecular Dynamics Storm 840 Phosphorimager equipped with ImageQuant version 5.1 software.

Construction of M13 Genomes. M13 genomes were generated in triplicate for each oligonucleotide insert (5′-CXG, 5′-TXG, and 5′-TXA) using a modified version (*30*) of a previously reported procedure (*33*, *34*). The initial plasmid and scaffolds were identical to those used in the previous studies. To determine ligation efficiency, 0.5 pmol of the plasmid ligation reaction was added to 30% glycerol loading buffer and loaded onto a 1% TBE agarose gel. Uncut and linearized M13 vectors (0.5 pmol) were used as markers. After electrophoresing for 9 h at 100 V, the gel was stained with Sybr Green II for 30 min, followed by visualization using an 840 Storm Imager.

Preparation of E. coli for Electroporation. Wild type (K16), polymerase II- (STL1336 (SpcR)), polymerase IV- (Xs-1 (KanR)), polymerase V- (SR1157U (CamR)), and SOS-polymerase triple knockout cells (SF2108 (SpcR, KanR, and CamR)) were grown overnight from a genetic stock in 10 mL of LB at 37 °C with orbital shaking (270 rpm). SOS-induced and uninduced cells were prepared as described (*23*). The cells to be SOS-induced were grown in LB to an OD_{600} value of 0.3, whereas the cells to be electroporated uninduced were grown to an OD_{600} value of 0.5. To induce the cells, the *E. coli* were pelleted, resuspended in 0.1 M $MgSO_4$ (50 mL), and then irradiated at 45 J/m^2 with 254 nm light. The SOS-induced cells were then added to 50 mL of 2xYT and grown for 40 min at 37 °C with orbital shaking (270 rpm). Both SOS-induced and uninduced cells were then pelleted, resuspended in ice-cold H_2O, pelleted again, and then resuspended in ice-cold 10% glycerol (2 mL). The prepared cells (100 μL) were mixed with 1 pmol of ligated M13 plasmid genome on ice, electroporated (∼2.5 kV, 4.74 ms), and then plated with X-Gal and IPTG.

Electroporation of M13 Genomes into E. coli Cells. The prepared cells (100 μL) were mixed with 1 pmol of ligated M13 plasmid genome on ice and electroporated (∼2.5 kV, 4.74 ms). The electroporated cells were then transferred to a tube (15 mL) containing LB (10 mL). An aliquot (20 μL for the T-control plasmid and 100 μL for the lesion-containing plasmids) of the solution was added to plating

bacteria (375 μL, NR9050 cells, 2.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.07% thiamine, and 4.3 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)) and soft agar (2 mL). This was poured onto B broth plates and incubated overnight at 37 °C. Colonies containing a full-length replicated insert appear blue. The tubes of electroporated cells in LB were incubated at 37 °C with 270 rpm orbital shaking for 7 h and then centrifuged at 9500 rpm for 15 min at 4 °C. The supernatant, which contained the viral progeny, was decanted into fresh tubes and stored at 4 °C.

Bypass of C2-AP in E. coli. The percent bypass based on colony count was determined by dividing the number of blue colonies produced from electroporated M13-plasmid-containing C2-AP by the number of blue colonies derived from M13-containing dT instead of a lesion. The corrections for 18 nucleotide deletions were made as previously described (*30*).

REAP Assay. The M13 progeny phage (100 μL) was used to infect SCS110 cells (10 mL, 1:10 dilution of an overnight culture) in LB. The samples were incubated at 37 °C with 270 rpm orbital shaking for 7 h and then centrifuged at 9500 rpm for 10 min at 4 °C. The supernatant (containing the progeny) was decanted into a clean tube and stored at 4 °C. To isolate the viral progeny from the regrowth, a QIAprep Spin M13 Kit was used following the manufacturer's protocol. The DNA was then amplified by PCR as previously described (*30*, *33*, *34*). The PCR product was then digested with Bbs I (5 U) and dephosphorylated as described in the aforementioned references. The resulting fragments were 5′-^{32}P-labeled and separated on a 20% denaturing gel (0.8 mm thick) for 2.5 h at 550 V. The substitution and deletion products (when appropriate) were excised, eluted from the gel, and desalted using G-25 Sephadex. The recovered DNA was then digested with nuclease P1 and the resulting nucleotide monophosphates eluted on cellulose TLC plates in 180 mL of saturated $(NH_4)_2HPO_4$ and 18 mL of H_3PO_4. The plates were exposed to a phosphorimaging screen for ~24 h, and the relative amounts of each nucleotide were calculated using ImageQuant 5.1. These procedures were carried out in a manner identical to that of previous experiments. Details of these procedures can be found in the appropriate references (*30*, *33*, *34*).

RESULTS

Summary of the Experimental Method.. Shuttle vectors were generated in M13mp7L2 plasmids using the design previously reported by Delaney and Essigmann for use with the restriction endonuclease and postlabeling (REAP) mutation detection system (*33*, *35*). We employed this system to characterize the replication of AP, F, L, and C4-AP in single-stranded shuttle vectors (*23*, *30*, *32*). The REAP method was attractive to us because it provided a statistically meaningful readout of nucleotide incorporation across from a lesion. In addition, the frameshift products were separated prior to mutation analysis and quantitated in the same experiment. The C2-AP lesion was introduced via synthetic 16-nucleotide-long synthetic oligonucleotides that are characterized by ESI-MS (*16*, *36*). The lesion was positioned within the shuttle vector that was constructed from M13 plasmid so that restriction digest of the duplex PCR product with Bbs1 yielded linearized material containing the nucleotide incorporated in place of the original lesion at the 5′-terminus of one of the strands. Radiolabeling was achieved at the 5′-termini of both strands using kinase, following dephosphorylation of the Bbs1 cleaved PCR product. The radiolabeled nucleotide of interest was liberated following a second restriction digest (Hae III), the isolation of the shorter (18mer) single-stranded oligonucleotide produced, and phosphodiesterase digestion. The identity of this nucleotide in a collection of progeny plasmids was ultimately determined by thin-layer chromatography analysis of ^{32}P-labeled nucleotide monophosphates.

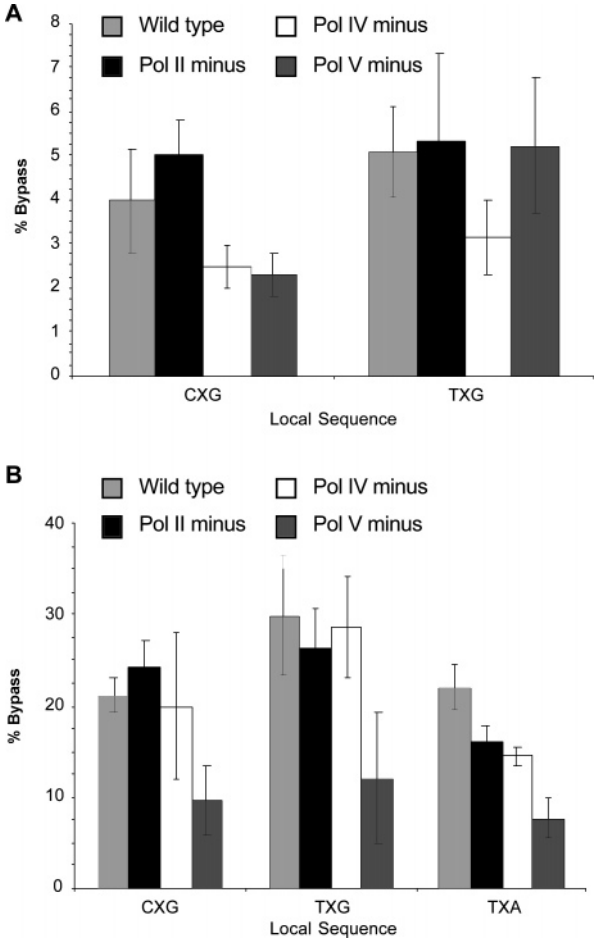


FIGURE 2: Percent bypass of the C2′-oxidized abasic site (C2-AP) in wild-type and bypass-polymerase-deficient *E. coli* transfected with plasmids containing the C2′-oxidized abasic site (C2-AP). (A) Uninduced cells. (B) SOS-induced cells.

Bypass of the C2-AP Lesion. The bypass efficiency following transfection was on the basis of a comparison with a control containing thymidine instead of a lesion in an otherwise identical plasmid as previously described (*30*). The bypass efficiency was examined in wild-type *E. coli* and a variety of bypass-polymerase-deficient cells under uninduced (5′-CXG, 5′-TXG, Figure 2A) and SOS-induced conditions (5′-CXG, 5′-TXG, 5′-TXA, Figure 2B). The values reported are the average of three replicates carried out simultaneously. In sequences where a direct comparison was possible, bypass efficiency increased 5- to 6-fold in wild-type and pol II-deficient cells that were transfected following SOS-induction (Figure 2B). Bypass efficiency increased as much as 10-fold following SOS-induction in pol IV-deficient cells (Figure 2B). In contrast, the bypass efficiency was only 2 to 3 times greater following inducement in pol V-deficient cells.

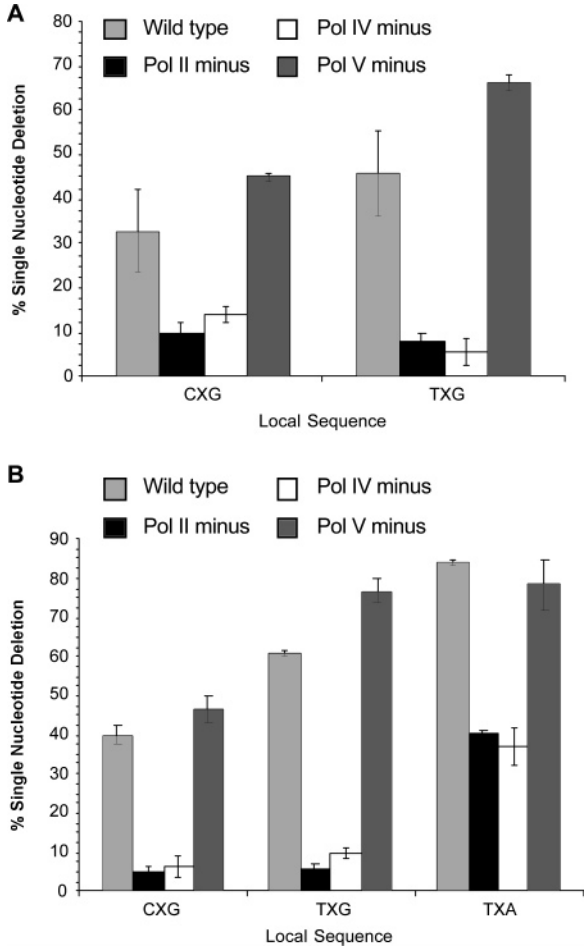


FIGURE 3: Percent single-nucleotide deletions from the replication of the C2′-oxidized abasic site (C2-AP) in wild-type and bypass-polymerase-deficient *E. coli* transfected with plasmids containing the C2′-oxidized abasic site (C2-AP). (A) Uninduced cells. (B) SOS-induced cells.

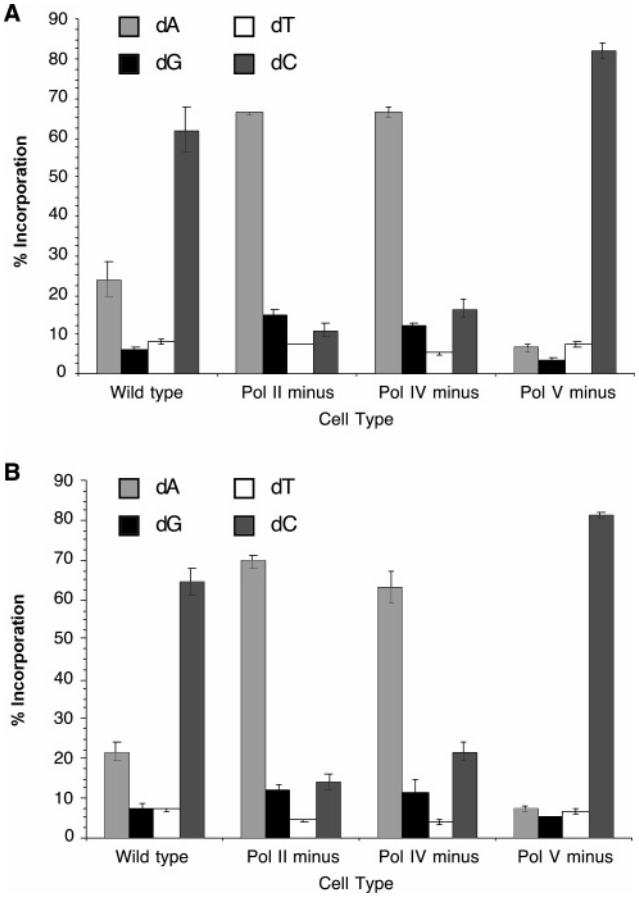


FIGURE 4: Nucleotide incorporation opposite the C2′-oxidized abasic site (C2-AP) in the full-length replication product formed from the replication of 5′-CXG in wild-type and various bypass-polymerase-deficient forms of *E. coli*. (A) Uninduced cells. (B) SOS-induced cells.

As was seen when other abasic lesions were bypassed, deleting all three alternative polymerases from the *E. coli* host resulted in an almost complete shut down of replication (<1%, data not shown) (*30*).

Sequence and Cellular Effects on the Relative Yields of Substitution and −1 Frameshift Products. Significant levels of single-nucleotide deletions were detected in wild-type cells upon replication of plasmids containing any of the 3 C2-AP inserts (Figure 3). SOS-induction increased the percentage of single-nucleotide deletions formed in the 5′-CXG and 5′-TXG sequences by 25−33% (Figure 3b). Minus one frameshifts accounted for ∼40−85% of the products detected in the three sequences examined under SOS-induced conditions (Figure 3b).

A fraction of the full-length product was affected by the deletion of individual bypass polymerases (Figure 3). Deleting pol II or pol IV significantly reduced the levels of single-nucleotide deletions. This effect was observed whether (Figure 3B) or not (Figure 3A) the cells were SOS-induced prior to transfection. For the 5′-CXG and 5′-TXG sequences the percentage of frameshift products were reduced 8−10-fold in SOS-induced cells deficient in either pol II or pol IV. The effect was considerably smaller (2−4-fold) in uninduced cells or when SOS-induced cells were transfected with plasmids containing the 5′-TXA insert (∼2-fold). Deleting pol V from *E. coli* had a qualitatively distinct effect compared to the loss of pol II or pol IV on the fraction of single-nucleotide deletion products. With one exception, deleting pol V resulted in an increase in the fraction of bypass products that consist of a single-nucleotide deletion compared to those observed in wild-type cells. The increases were observed regardless of whether the cells were induced. Only the 5′-TXA sequence (Figure 3B) showed a slight decrease (<10%) on average in the percentage of frameshift products compared to that in wild-type cells when pol V was deleted. However, the difference between pol V-deficient and wild-type cells was statistically indistinguishable from zero. Furthermore, 5′-TXA was the sequence in which the largest percentage of products consisted of deletions (>84%) in wild-type *E. coli*. Hence, it was not possible to significantly increase the percentage of this type of product.

Translesion Synthesis in Full-Length Products. 2′-Deoxycytidine is the major nucleotide incorporated opposite C2-AP in SOS-induced wild-type *E. coli* when the lesion is flanked by a 3′-dG (Figures 4 and 5). In the 5′-TXG, sequence incorporation of dA is slightly favored over dC in uninduced wild-type cells. This slight difference in the replication of plasmids containing 5′-CXG and 5′-TXG sequences disappears when experiments are carried out in *E. coli* that are deficient in one of the three bypass polymerases. The changes in nucleotide incorporation frequencies are very similar in pol II- and pol IV-deficient

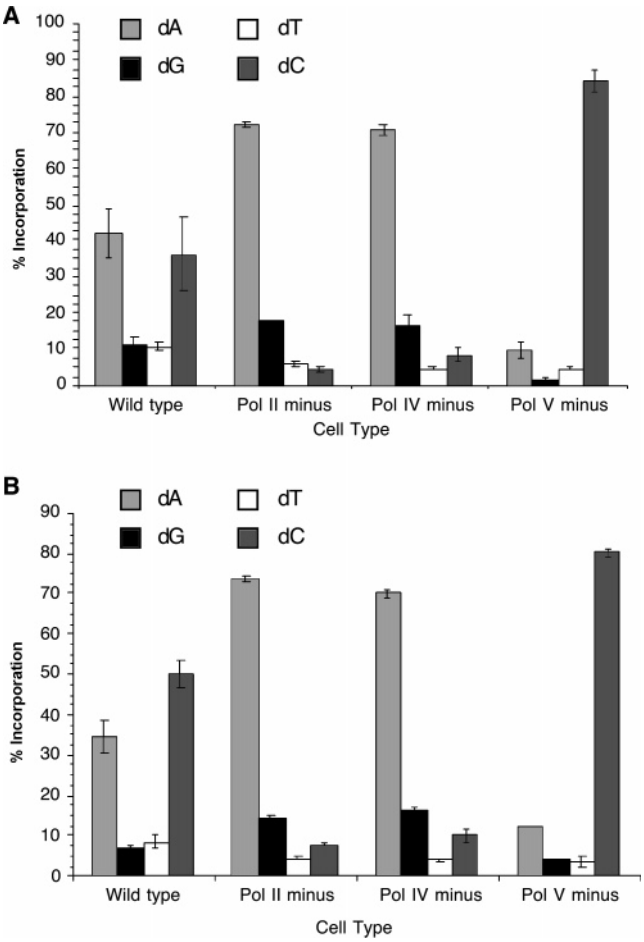


FIGURE 5: Nucleotide incorporation opposite the C2′-oxidized abasic site (C2-AP) in the full-length replication product formed from the replication of 5′-TXG in wild-type and various bypass-polymerase-deficient forms of *E. coli*. (A) Uninduced cells. (B) SOS-induced cells.

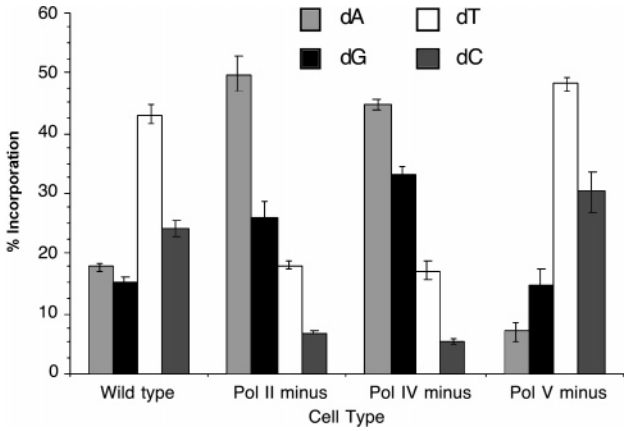


FIGURE 6: Nucleotide incorporation opposite the C2′-oxidized abasic site (C2-AP) in the full-length replication product formed from the replication of 5′-TXA in wild-type and various bypass-polymerase-deficient forms of *E. coli*. (A) Uninduced cells. (B) SOS-induced cells.

E. coli. Substitution (full-length) products produced from the replication of plasmids containing C2-AP in the 5′-TXG or 5′-CXG sequences in either of these cell lines contained dA opposite the lesion more than 60% of the time. The high frequency of dA incorporation is observed in these cells whether or not they are SOS-induced. Moreover, the large increase in dA incorporation opposite C2-AP in the 5′-CXG and 5′-TXG sequences when pol II (Figure 7A) or pol IV (Figure 7B) is deleted is accompanied by a commensurate decrease in translesional dC incorporation. Deleting pol V has a very different effect when C2-AP is bypassed in the 5′-TXG or 5′-CXG sequences. 2′-Deoxycytidine is incorporated opposite the lesion >80% of the time regardless of whether the cells are in an induced state. Furthermore, the increase in translesional dC incorporation is compensated by a decrease in the level of dA found opposite C2-AP (Figure 7C).

Significantly different observations were made when the C2-AP lesion is flanked by a 5′-dT and a 3′-dA (Figure 6). Of the three sequences examined, full-length products are produced in the smallest amount (~16%) in the 5′-TXA sequence. Moreover, the distribution of nucleotides incorporated opposite C2-AP when the 5′-TXA template is replicated is very distinctive. In wild type *E. coli*, thymidine is preferentially incorporated (43.0 ± 1.3%). In contrast, thymidine is incorporated less than 10% of the time when the 5′-TXG or 5′-CXG sequences are replicated in wild-type cells (Figures 4 and 5). The effects of deleting pol II or pol IV from *E. coli* upon nucleotide incorporation opposite C2-AP in the 5′-TXA sequence are qualitatively similar to those observed when the lesion is flanked by a 3′-dG. Translesion incorporation of dA is preferred when either pol II or pol IV is absent (Figure 6). However, the preference is smaller than that observed when the lesion is incorporated in either the 5′-CXG (Figure 4) or 5′-TXG sequences (Figure 5) because of an increase in the frequency of dG incorporation in the 5′-TXA sequence. Translesion incorporation of dG reached 33% when the 5′-TXA sequence was replicated in pol IV-deficient cells, whereas dG incorporation opposite C2-AP never reached 18% when bypassed in the other sequences. The relationship between translesional dA and dT incorporation upon the bypass of 5′-TXA was analogous to that observed between dA and dC in the sequences in which C2-AP was flanked by a 3′-dG. The increased level of dA incorporation upon C2-AP bypass in pol II- or pol IV-deficient cells correlated with a decrease in dT incorporation (Figure 7A and B). Deleting pol V from *E. coli* restores the preference for incorporation of thymidine opposite the C2-AP lesion that is flanked by a 3′-dA. Again, the preference is not as great as that observed in the 5′-TXG and 5′-CXG sequences where dC is preferably incorporated. However, the increase in dT incorporation in the pol V-deficient cells is commensurate with a decrease in dA (Figure 7C).

DISCUSSION

Abasic lesions (e.g., AP) are referred to as noninstructive lesions because they lack the heterocyclic base that is used to form Watson−Crick hydrogen bonds (*19*, *20*). However, recent studies using nonnative nucleotides designed to be nonhydrogen bonding isosteres of native nucleotides suggest that Watson−Crick hydrogen bonding provides an incomplete analysis of replication fidelity (*18*). 2′-Deoxyadenosine is preferentially incorporated opposite an AP site during replication in *E. coli*. This observation contributes to the formulation of the A-rule that predicts preferential incorporation of dA opposite noninstructive lesions in *E. coli* (*19*, *20*). Thermodynamic studies on the stability of duplexes contain-

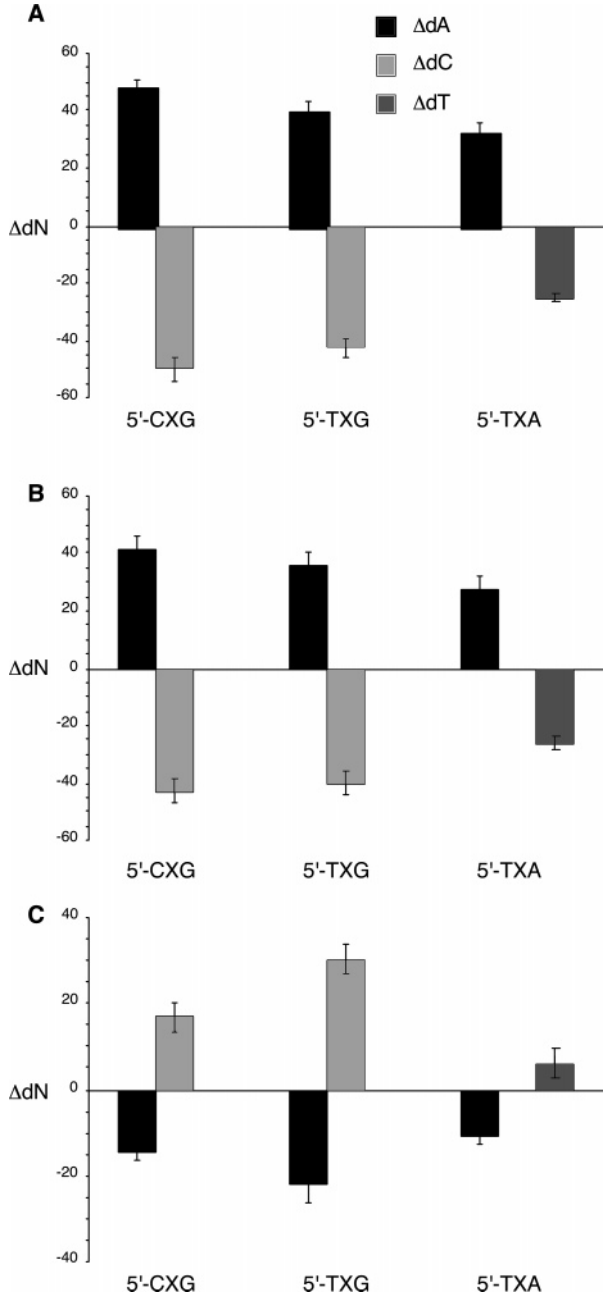


FIGURE 7: Change in nucleotide (ΔdN) incorporation opposite C2-AP in full-length products as a function of cell type. (A) Pol II-deficient cells ($\Delta dN = \%\ dN_{pol\ II\ deficient} - \%\ dN_{wild\ type}$) (B) Pol IV-deficient cells ($\Delta dN = \%\ dN_{pol\ IV\ deficient} - \%\ dN_{wild\ type}$) (C) Pol V-deficient cells ($\Delta dN = \%\ dN_{pol\ V\ deficient} - \%\ dN_{wild\ type}$).

ing a 3′-dangling base indicate that its preferential incorporation may be a consequence of the more favorable base stacking of this nucleotide at the 3′-terminus compared to that in other native nucleotides (*38*). However, the empirical A-rule has been modified to accommodate information acquired from studies on translesion synthesis involving a growing list of polymerases. For example, dNTP-stabilized misalignment (*39*), which involves the interactions of the downstream nucleotide, polymerase, and incoming dNTP, is an amendment that explains −1-frameshift formation upon replication by pol IV (*27*). Pre-steady-state kinetic experiments on pol IV and structural studies on AP bypass by Dpo4 support this mechanism (*28*, *40*).

Single-stranded shuttle-vector studies using the REAP analysis (*33*, *35*) method on a variety of abasic sites in *E. coli* suggest that the A-rule and the term noninstructional may need to be amended further. An analysis of the replication of a genuine AP lesion and its tetrahydrofuran analogue (F) in this system is consistent with the A-rule (*23*). However, the replication of the oxidized abasic lesions L and C4-AP produced unique results. The REAP analysis of 2-deoxyribonolactone (L) replication reveals that the A-rule is not adhered to (*30*). Large amounts of dG are incorporated translesionally in full-length products. Furthermore, −1-frameshifts are observed only when the nucleotide immediately downstream from L is dC. In contrast to the AP (or F) bypass, a misinsertion−misalignment mechanism is believed to control the replication of L (*27*, *30*). Substitution products formed by replicating plasmids containing C4-AP abide by the A-rule (*32*). However, the yield of substitution products is highly dependent upon the flanking sequence, and they are only observed in non-SOS-induced cells. The unique aspect of C4-AP bypass is that 3-nucleotide deletions are produced exclusively in wild-type *E. coli*.

The data presented above using plasmids containing the C2-AP lesion increases the diversity of effects exhibited by abasic lesions on DNA replication in *E. coli*. Unlike AP, C4-AP, and F, the translesion incorporation of dA is not preferentially observed in substitution products formed from the C2-AP lesion. In addition, unlike substitution products produced from the replication of templates containing L, the translesion dG incorporation is observed infrequently. The C2-AP bypass is also distinguished from that of the L in that single-nucleotide deletions are formed in a wider variety of flanking sequences. A more striking difference is that unlike C4-AP, 3-nucleotide deletions are not produced by the replication of C2-AP. This is not to say that there are no similarities between the replication of single-stranded plasmids containing C2-AP and other abasic lesions. For instance, the level of single-nucleotide frameshift products resulting from the bypass of C2-AP or other abasic lesions is strongly dependent upon the presence of pol II and pol IV in *E. coli* (Figure 3) (*23*, *30*, *32*, *41*). The effect of pol V on deletion product formation is also common among C2-AP and the other abasic lesions. Deleting pol V from *E. coli* that are transfected with plasmids containing any of the abasic lesions typically results in deletion product levels similar to, or greater than those observed in wild-type cells. Indeed, with the exception of when the 5′-TXA sequence is replicated in SOS-induced cells, the fraction of products obtained from replicating C2-AP-containing plasmids that are single-nucleotide deletions increases in pol V-deficient cells compared to those in wild-type ones (Figure 3).

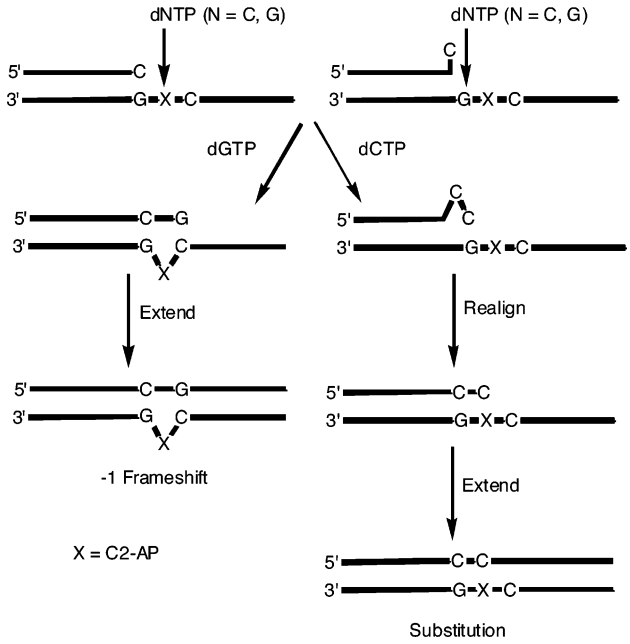
The most distinctive aspect of C2-AP replication is the distribution of nucleotides incorporated opposite it in substitution products. Two of the three sequences were examined in uninduced and SOS-induced cells. The distributions of nucleotides incorporated opposite C2-AP in the 5′-CXG (Figure 4) and 5′-TXG (Figure 5) sequences depended weakly on whether the cells were in a SOS-induced state, as was the fraction of substitution products formed (Figure 3). The identity of the preferred nucleotide incorporated opposite C2-AP in the 5′-TXG sequence was different in uninduced and SOS-induced cells (Figure 5). However, in this instance, the preference for one nucleotide over another

was small, and therefore, it was easier to alter the identity of the favored one between dA and dC. Remarkably, with but one exception, dC was the most frequently incorporated nucleotide in 5′-TXG and 5′-CXG templates. The exception was the replication of the 5′-TXG sequence in uninduced cells where dA incorporation opposite C2-AP was slightly favored (Figure 5A). The bypass of AP, F, L, and C4-AP was examined previously in identical sequence contexts using the same cell lines. In only one instance was dC the most frequently incorporated nucleotide opposite an abasic site. 2′-Deoxycytidine incorporation opposite F was favored over dA in SOS-induced wild-type *E. coli* (*23*). In all other instances, dA was preferentially incorporated opposite AP, F, and C4-AP lesions in full-length products. The replication of templates containing 2-deoxyribonolactone (L) predominantly resulted in the translesional incorporation of purine (often with dG favored over dA), in violation of the A-rule. However, one can rationalize that dA and dG could be preferably incorporated opposite a noninstructive lesion because they stabilize the growing strand more than the native pyrimidine nucleotides (*38*). One cannot utilize thermodynamics to explain preferential dC incorporation opposite C2-AP. Consequently, we considered the possibility of a unique, specific structural interaction to explain this selectivity.

Our attention was drawn to the identity of the nucleotide on the 3′-side (upstream) of the C2-AP lesion. There is precedence for the upstream nucleotide affecting the efficiency of F bypass by pol β in vitro (*39*). However, the identity of the 3′-flanking nucleotide did not alter the nucleotide incorporation preference. We explored the possibility that the 3′-adjacent dG influenced the incorporation of the dC opposite C2-AP using the 5′-TXA sequence with the anticipation that elevated levels of dT incorporation opposite C2-AP would be observed if the upstream dA was involved. 2′-Deoxyadenosine was chosen as the upstream nucleotide because thymidine is infrequently incorporated opposite C2-AP in the other sequences examined (Figures 4 and 5), and this nucleotide is least likely to benefit from thermodynamic stabilization of the growing chain (*38*). The replication past C2-AP in the 5′-TXA sequence was examined in SOS-induced cells only because of the aforementioned lack of effect of SOS-induction on nucleotide incorporation. Preferential incorporation of thymidine (43 ± 1.5%) opposite C2-AP in wild-type cells (Figure 6) was fully consistent with the postulated role of the upstream nucleotide in the translesion synthesis of templates containing C2-AP.

Experiments carried out in *E. coli* lacking one of the three bypass polymerases using the C2-AP templates indicate that pol II and pol IV are largely responsible for the incorporation of the nucleotide that is complementary to the upstream nucleotide (dC or dT) opposite the lesion (Figures 4−6). In each instance, deleting either pol II or pol IV resulted in preferential translesion incorporation of dA and a corresponding decrease in the incorporation of the nucleotide complementary to the nucleotide that is 3′-adjacent to C2-AP (Figure 7A and B). The levels of translesional dA incorporation in pol II- and pol IV-deficient cells were comparable to those observed in similar studies on AP and F replication in *E. coli* (*22*, *23*). The differences in translesion nucleotide incorporation observed in wild-type and pol V-deficient cells were more subtle. The amount of nucleotide

Scheme 2: Representative dNTP-Stabilized Misalignment Process for Pol II/Pol IV Bypass of C2-AP in a 5′-CXG Sequence



that is complementary to that which is 3′-adjacent to C2-AP increased in pol V-deficient cells relative to that in wild-type ones. Furthermore, the level of translesion dA incorporation decreased in pol V-deficient cells compared to the level observed in wild-type cells. These data indicate that pol V follows the A-rule upon bypassing the C2-AP lesion, as it does when copying templates containing F (*42*).

The data also indicates that pol II and pol IV dominate C2-AP bypass in wild-type cells, where they work in concert to produce substitution products (Figures 4−6) and single-nucleotide deletions (Figure 3). The dominance of pol II and pol IV is evidenced by the large reduction in levels of single-nucleotide deletions when either pol II or pol IV is absent (Figure 3). In contrast, deleting pol V gives rise to levels of single-nucleotide deletions that are often only modestly higher than those observed in wild-type cells.

The relative contributions of pol IV/pol II and pol V in wild-type cells were also estimated by comparing the changes in translesion nucleotide incorporation in wild-type and pol V-deficient cell types (Figure 7C). These calculations assume that pol V utilizes the A-rule when copying C2-AP but that pol II and pol IV do not. They also assume that there is no synergistic effect between pol V and the other bypass polymerases. Hence, the difference in translesion dA incorporation between wild-type cells and cells lacking pol V provides a lower limit on the contribution of pol V toward C2-AP bypass in wild-type cells. If one assumes that pol V incorporates dA opposite an abasic lesion ~60% of the time, then its participation toward C2-AP replication is estimated to range from ~18−37% (*23*, *42*).

Competition between pol II and pol IV with pol V has been observed in studies on the replication of templates containing other abasic lesions in *E. coli* (*23*, *30*, *32*, *43*). However, this is the first instance in which the identity of the upstream nucleotide in the DNA template appears to play a direct role on translesional nucleotide incorporation. We are also unaware of any in vitro studies in which a

polymerase reads the 3′-adjacent nucleotide when bypassing a lesion (*27*, *39*, *42*, *44*−*46*). We propose that pol II/pol IV bypass C2-AP via dNTP-stabilized misalignments (Scheme 2) (*27*). This process is preferred over misinsertion misalignment or Streisinger misalignment because it enables us to account for the observed substitution products as well as single-nucleotide deletions using a single mechanism. The deletion product results from initial template slippage followed by nucleotide addition to form a looped-out structure (Scheme 2, left). In contrast, full-length products result from initial primer slippage followed by dNTP pairing with the upstream nucleotide (Scheme 2, right). A recent structural study on Dpo4 supports the recognition of the incoming dNTP by the upstream nucleotide (*28*). However, this structure did not support subsequent bond formation, as is observed in our experiments. An extension of the looped-out primer intermediate would result in insertion product (+1 frameshift) formation, which was not observed. Nucleotide incorporation to the distorted primer strand opposite C2-AP is presumably too slow to compete with realignment, after which extension proceeds to produce the observed substitution products.

Conclusions. Despite lacking a nucleobase that is capable of forming Watson−Crick hydrogen bonds, C2-AP affects DNA replication of a template containing it in a unique manner. C2-AP is the first DNA lesion to induce bypass polymerases to read the upstream (3′-adjacent) nucleotide. The distinctive product profiles produced by the replication of C2-AP by different bypass polymerases suggest that the lesion could be an effective tool for determining the role of other polymerases in vivo.

The observations reported here reinforce the growing body of data, which demonstrate that molecules that are incapable of forming Watson−Crick hydrogen bonds interact with polymerases in distinct ways. We suggest that the effects of a nucleotide, nucleotide analogue, or lesion on the structure of a DNA·polymerase complex that determines dNTP incorporation should be expanded beyond the consideration of the atoms contained within the molecule being bypassed. In particular, when considering the bypass of a hydrogen bond compromised molecule such as C2-AP, one should also consider how it affects the transient biopolymer structure at neighboring nucleotides.

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SUPPORTING INFORMATION AVAILABLE

ESI-MS of the oligonucleotides (3) containing C2-AP used to prepare the single-stranded plasmids and a figure describing the REAP experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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