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Effects of the C4′-Oxidized Abasic Site on Replication in *Escherichia coli*. An Unusually Large Deletion Is Induced by a Small Lesion†

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ABSTRACT: The C4′-oxidized abasic site (C4-AP) is produced in DNA as a result of oxidative stress by a variety of agents. For instance, the lesion accounts for ∼40% of the DNA damage produced by the antitumor antibiotic bleomycin. The effect of C4-AP on DNA replication in *Escherichia coli* was determined using the restriction endonuclease and postlabeling (REAP) method. Three-nucleotide deletion products are the sole products observed following replication of plasmids containing C4-AP under SOS conditions in wild-type cells. Full-length products are formed in varying amounts depending upon the local sequence in wild-type cells under non-SOS-induced conditions. The "A-rule" is followed for the formation of substitution products. C4-AP is the first example of a DNA lesion that produces significant levels of three-nucleotide deletions in a variety of sequence contexts. Experiments carried out in cells lacking specific polymerases reveal that formation of three-nucleotide deletion products results from a coordinated effort involving pol II and pol IV. This is the first example in which these SOS inducible polymerases are shown to work in concert during lesion bypass. Three-nucleotide deletions are not observed during the replication of other abasic lesions, and are rarely produced by bulky adducts. The effect of C4-AP on DNA replication suggests a significant role for this lesion in the cytotoxicity of bleomycin. Formation of the C4-AP lesion may also be responsible for the formation of mutant proteins containing single-amino acid deletions that exhibit altered phenotypes.

Maintaining the structure of DNA is essential for the accurate storage and conveyance of genetic information. Consequently, DNA lesions pose significant challenges to cells. Abasic sites (AP)[1] are ubiquitous lesions which, if not repaired, can be lethal and/or mutagenic (*1*−*3*). Oxidized abasic lesions, such as 2-deoxyribonolactone (L), are also unable to form Watson−Crick hydrogen bonds, but their in vitro and in vivo effects are significantly different from those of an AP lesion (*4*−*6*). The C4′-oxidized abasic lesion (C4′-keto abasic site, C4-AP) is produced by a variety of DNA damaging agents, including γ-radiation and antitumor agents

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[1] Abbreviations: AP, abasic lesion; C4-AP, C4′-oxidized abasic lesion; pol II, DNA polymerase II; pol IV, DNA polymerase IV; pol V, DNA polymerase V; L, 2-deoxyribonolactone; REAP, restriction endonuclease and postlabeling; ESI-MS, electrospray ionization mass spectrometry.

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such as the enediynes and bleomycin (*7*−*9*). C4-AP is a component of mono- and bistranded lesions produced by bleomycin selectively at the deoxycytidine of pyr-G-C sequences. The oxidized abasic site accounts for ∼40% of the DNA damage products produced by this drug. The frequent formation of the C4-AP lesion can be attributed partly to the high accessibility of the C4′ hydrogen at the edge of the minor groove, and the relatively low bond dissociation energy of the corresponding carbon−hydrogen bond (*10*). Reaction conditions that provide a means of forming C4-AP from the initially formed C4′ radical under oxygen deficient conditions also likely contribute to its frequent formation (*11, 12*). Despite the frequent occurrence of C4-AP, its biological effects are not well-understood. Studies in which the lesion is generated along with strand breaks and other forms of DNA damage, by treating lambda phage with bleomycin, were unable to definitively attribute the observed mutations to the oxidized abasic lesion (*13*). Similarly, although γ-irradiation and bleomycin produce high frequencies of three- and six-nucleotide deletions in a *Salmonella* tester strain, it is not possible to attribute these biological effects to a specific DNA lesion (*14*). The use of shuttle vectors containing specific lesions at defined sites is a powerful approach for examining their effects on DNA replication (*15, 16*). We sought to utilize this combination of chemistry and biology to determine the effect of the C4′-oxidized abasic lesion on DNA replication in *Escherichia coli*.

Abasic sites (e.g. AP and L) are often termed noninstructional lesions. Shuttle vector studies on AP and 2-deoxyribonolactone (L) lesions using the restriction endonuclease and postlabeling (REAP) assay revealed that this is not the case (*4, 15*). Replication of these lesions in *E. coli* produces distinctive nucleotide substitution patterns. For instance, 2-deoxyribonolactone directs large amounts of deoxyguanosine incorporation opposite it, whereas the AP lesion adheres to the "A-rule" (*17*). The AP and L lesions also produce different distributions of frameshift and substitution products, and respond differently to sequence effects (*4*). The C4 hydroxyl group in C4-AP distinguishes it structurally from other AP lesions. The additional hydroxyl group provides another potential hydrogen bond donor and allows it to exist as a mixture of four stereoisomers, presumably in equilibrium with small amounts of an acyclic keto aldehyde (*18*). These studies were aimed at determining whether the structural uniqueness of the C4′-oxidized abasic site (C4-AP) carries over into its effects on DNA replication in *E. coli*. We discovered that the C4-AP lesion has a unique propensity to produce three-nucleotide deletions upon replication.

MATERIALS AND METHODS

Oligonucleotides containing the C4-AP lesion were prepared as described previously (*19*). The lesion was generated photochemically from **1** immediately prior to its incorporation into the plasmid. The extent of conversion to C4-AP was determined by base treating a 5′-^{32}P-radiolabeled aliquot of the oligonucleotide, followed by denaturing gel electrophoresis analysis. All other oligonucleotides, including those containing the tetrahydrofuran analogue (F) of an AP lesion, were prepared using commercially available reagents. The oligonucleotides were prepared with three different general sequences (differences are italicized and are used to name the respective sequences): 5′-GAA GAC C*TX G*GC GTC C (TXG), 5′-GAA GAC C*CX G*GC GTC C (CXG), and 5′-GAA GAC C*GX C*GC GTC C (GXC), where X is T, F, C4-AP, or **1**. Oligonucleotides containing the 4*S* diastereomer of an abasic site (4*S*-AP) were prepared in the TXG and CXG sequences.

In Vivo Replication and Analysis. M13 genomes were generated in triplicate for each 16-mer plasmid insert as described previously (*4, 15*). Briefly, lesion-containing oligonucleotide 16-mers were phosphorylated, and then ligated into EcoRI-digested M13mp7(L2). Ligation scaffolds were digested by reacting each mixture with T4 DNA polymerase. The DNA was purified by Centricon 100 filtration, following removal of the polymerase by phenol extraction. Ligation efficiency was determined by agarose gel electrophoresis. Cell growth was carried out in wild-type (K16), polymerase II deficient [STL1336 (SpcR)], polymerase IV deficient [Xs-1 (KanR)], polymerase V deficient [SR1157U (CamR)], and SOS polymerase triple knockout cells [SF2108 (SpcR, KanR, CamR)] under uninduced conditions or following SOS induction (*4*). Cells to be SOS-induced were grown in LB to an OD_{600} of 0.3, while cells to be electroporated without induction were grown to an OD_{600} of 0.5. To induce the cells, *E. coli* were pelleted, resuspended in 0.1 M $MgSO_4$ (50 mL), and irradiated at 45 J/m^2 with 254 nm light. SOS-induced cells were then added to 2×YT (50 mL) and grown for 40 min at 37 °C with orbital shaking (270 rpm). 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 the ligated M13 plasmid genome on ice, electroporated (∼2.5 kV, 4.74 ms), and then plated with X-Gal and IPTG. The percent bypass was determined by comparing the number of plaques formed from the plasmid with a C4-AP insert to that with the plasmid with an insert containing T instead of a lesion (*4*). Mutation analysis was carried out by the restriction endonuclease and postlabeling (REAP) assay (*15*). This method involves the PCR amplification of the progeny plasmid. The primers and methods used here were identical to those recently reported (*4*).

Identification of Three-Nucleotide Deletions. The sequences of three-nucleotide deletions were identified via Southern blotting. Briefly, the M13 plasmid was isolated from electroporated *E. coli* and then PCR amplified. The PCR samples (33.3 pmol) and the complement to the probe to be used in the Southern blot (100 pmol) were then loaded in separate lanes on a 1% agarose gel. The denatured DNA was transferred to a nitrocellulose membrane, cross-linked, hybridized with the appropriate 5′-^{32}P probe, and subjected to phosphorimaging analysis. The probes used had the following sequences: 5′-GGA CGC CGT CTT C, 5′-GGA CGC GGT CTT C, 5′-GGA CGG GGT CTT C, and 5′-GGA CGA GGT CTT C.

UV melting experiments were carried out at a common duplex concentration (2.2 μM) in 10 mM PIPES (pH 7.0), 10 mM $MgCl_2$, and 100 mM NaCl in a total volume of 300 μL. Hybridization of oligonucleotides containing alkali-labile lesions were carried out as previously described (*19*).

RESULTS

Plasmid Preparation. Replication of plasmids containing the C4′-oxidized abasic site was examined in *E. coli* within three separate oligonucleotide sequences that differ in the identity of the 5′- and 3′-flanking nucleotides (designated TXG, CXG, and GXC where X is C4-AP). The corresponding plasmids containing the C4-AP photochemical precursor (**1**) or thymidine were prepared for use in control experiments. The sequence surrounding C4-AP of oligonucleotides TXG and CXG is identical to those prepared previously in studies on AP and L lesions (*4*). The nucleotide sequence flanking C4-AP in oligonucleotide GXC was chosen because it contains a sequence in which the lesion is efficiently formed by bleomycin (CGCC, the underlined C is where lesion is formed) (*13*). Oligonucleotides containing C4-AP were prepared photochemically immediately prior to ligation from **1** and were characterized by ESI-MS (*19*). The extent of photochemical conversion was greater than 92%, and the oligonucleotides were observed by ESI-MS as a mixture of the cyclic and acyclic (without water) isomers. The remainder is **1**, which independent experiments show affects replication in a manner very different from that of C4-AP (see below). The ratio of the isomers in GXC depends on the ionization conditions in the mass spectrometer. The relative amount of dehydration product increases as the inlet temperature is increased (see the Supporting Information), indicating that the presence of two ions is a consequence of the MS analysis, and not a chemical impurity. Shuttle vectors derived from the M13 plasmid containing the C4-AP lesion (or its precursor) suitable for analysis using the restriction endonuclease and postlabeling assay were prepared as previously described (*4*, *15*).

Bypass Efficiency of C4-AP. To determine how efficiently the C4-AP lesion is replicated in *E. coli*, the colonies produced in cells transfected with the plasmid containing the lesion were compared to those containing thymidine in place of C4-AP. Replication products were quantitated as previously described (*4*). The plasmid containing the photochemical precursor (**1**) was bypassed less than 1% compared to that containing thymidine. The combination of inefficient bypass and a high level of photochemical conversion indicates that the replication products cannot be attributed to the photochemical precursor. The C4-AP lesion was bypassed in uninduced wild-type cells considerably less efficiently than thymidine, independent of sequence (Figure 2A). Deletion of either pol II, pol IV, or pol V significantly reduced the bypass efficiency. Less than 1% of the plasmids containing C4-AP were bypassed in cells in which all three alternative polymerases were absent (data not shown). The effect of deleting polymerases is similar to that observed for AP sites in comparable sequences, but very different from what was observed during replication of L in *E. coli* where pol II and IV had little effect (*4*). Bypass efficiency of plasmids containing CXG and GXC oligonucleotides doubled when cells were subjected to SOS induction prior to transfection (Figure 2B). In addition, deleting any single polymerase had only a small effect on the overall bypass efficiency of C4-AP in SOS-induced cells.

Three-Nucleotide Deletions Result from Replication of C4-AP. An advantage of the REAP method is that substitution, insertion, and deletion products can be not only detected but also separated in a single experiment (*4*, *15*). The replication product distribution resulting from C4-AP was extremely unusual. Under uninduced conditions, −3 deletions were formed in significant amounts in wild-type cells from plasmids containing TXG (42.5 ± 6.1%) or CXG (66.9 ± 6.6%) oligonucleotides (Figure 3A; see the Supporting Information). The "bleomycin sequence" (GXC) gave rise to more than 96% −3 frameshift product. Minor amounts of the −1 frameshift product (∼1%) and the full-length product (∼3%) were also observed. The product distribution

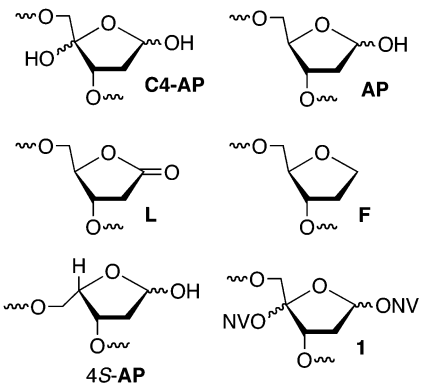

FIGURE 1: Structures of abasic lesions: C4′-oxidized abasic site (C4-AP), "regular" abasic lesion (AP), 2-deoxyribonolactone (L), tetrahydrofuran analogue of AP (F), C4 epimer of an AP site (4*S*-AP), and the photochemical precursor of C4-AP (**1**).

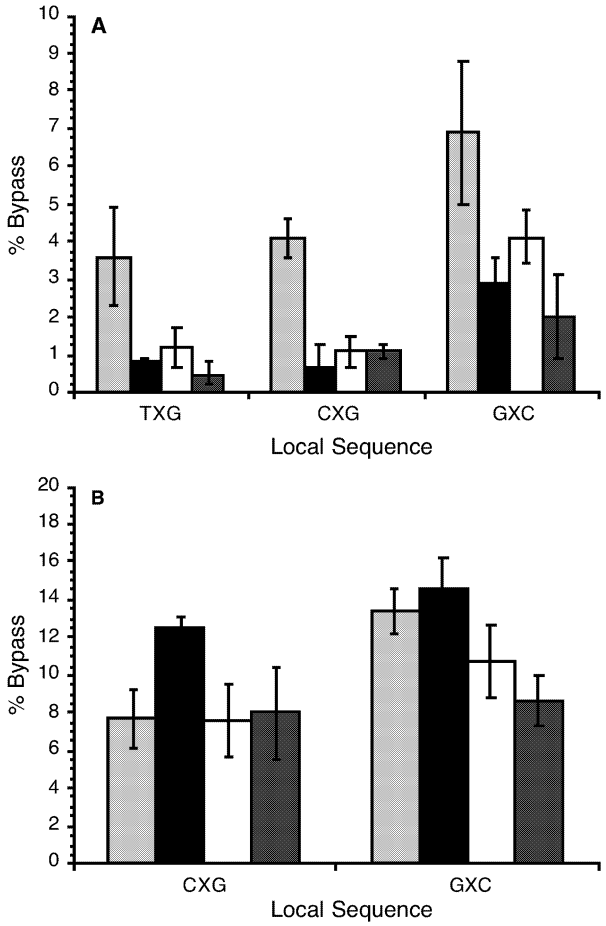

FIGURE 2: Percent bypass of the C4′-oxidized abasic site (C4-AP) relative to an identical plasmid containing thymidine in *E. coli*, (A) without SOS induction and (B) after SOS induction: wild type (light gray), pol II minus (black), pol IV minus (white), and pol V minus (dark gray).

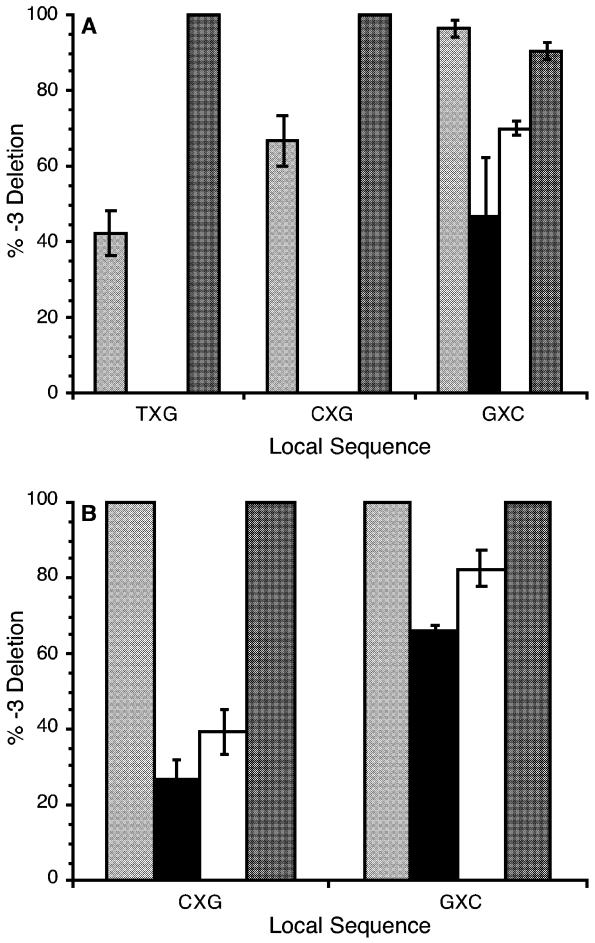

FIGURE 3: Percent deletion products formed from replication past the C4′-oxidized abasic site (C4-AP) in *E. coli*, (A) without SOS induction and (B) after SOS induction: wild type (light gray), pol II minus (black), pol IV minus (white), and pol V minus (dark gray).

resulting from C4-AP replication in various polymerase deficient cells was determined to elucidate the role of individual enzymes in the formation of the different families of products. The plasmid containing GXC responded more modestly than TXG and CXG to deletions of pol II or IV (Figure 3A). Replication of the latter two plasmids produced 100% full-length product when either polymerase was removed from the cell, whereas the bleomycin sequence (GXC) showed a much smaller reduction in the amount of three-nucleotide deletion product. It is noteworthy that regardless of sequence or induction status, removing pol II or pol IV had the same effect on product distribution. Deleting pol V from cells had the opposite effect of pol II or IV. Replication of plasmids containing TXG or CXG yielded 100% three-nucleotide deletion product. Full-length product was not observed from GXC either, but replication in pol V minus cells also produced ~10% −1 frameshift product. These results suggest that pol II and pol IV work in concert with each other, but separate from pol V, during the replication of the C4′-oxidized abasic lesion.

Specific sequences of three-nucleotide deletions were detected via Southern blots. Assuming that the C4-AP lesion is contained within the loop of deleted nucleotides, three different events are possible for TXG (Figure 4) and CXG inserts. The sequence within GXC prevents one from distinguishing these. Hybridization with the three appropriate

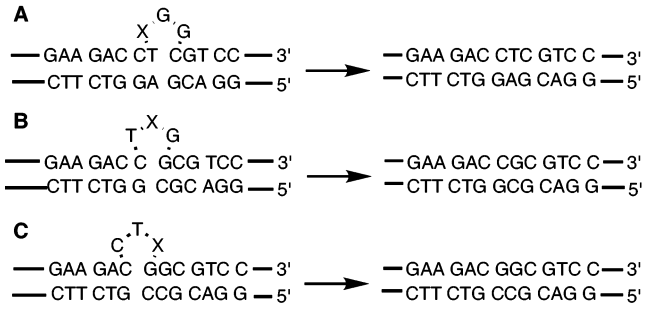

FIGURE 4: Possible three-nucleotide deletions involving the C4-AP lesion. X is C4-AP.

radiolabeled probes for CXG and TXG revealed that deletions in which the lesion is the furthest upstream nucleotide (i.e., Figure 4C) or second nucleotide encountered (i.e., Figure 4B) during replication are formed. The latter is observed in an amount approximately 4 times greater.

The formation of three-nucleotide deletion products was accentuated in wild-type cells following SOS induction, where replication of both plasmids that were examined, the CXG and bleomycin (GXC) sequence, yielded 100% of this product (Figure 3B). Removing either pol II or pol IV led to a smaller reduction in the amount of deletion product under these conditions than in uninduced cells. This was particularly noticeable in the CXG sequence, where without induction the three-nucleotide deletion product was not observed when either enzyme was absent. By comparison, deletion of pol V led to complete formation of the three-nucleotide deletion product, an observation identical to that without SOS induction.

Nucleotide Incorporation in Replication Products. Full-length replication products obtained from TXG, CXG, and GXC in wild-type *E. coli* abided by the A-rule (see the Supporting Information). The frequency of dA incorporation opposite C4-AP (58−61%) was comparable to that observed for plasmids containing an AP lesion in comparable sequences using the REAP method (~55%) (*4*). Deleting either pol II or pol IV resulted in a modest increase in the level of translesion dA incorporation to 82 or 78%, respectively. This trend paralleled the effects observed in the replication of AP-containing plasmids. The level of dA incorporation opposite C4-AP in full-length products formed in pol II and pol IV deficient cells was unaffected by SOS induction, a trend which was also echoed by the behavior of the AP lesion. The differences in translesional incorporation levels of the other three native nucleotides between one another were insignificant and were not affected by polymerase deletions or SOS induction (data not shown).

Replication of the C4 Epimer of an Abasic Site (4S-AP). To determine if the ability of C4-AP to isomerize at the C4 position via the keto aldehyde intermediate was the structural source for the lesion's unique ability to induce three-nucleotide deletions, plasmids containing 4*S*-AP were prepared and transfected into *E. coli*. The 4*S*-AP lesion was incorporated into chemically synthesized oligonucleotides in place of C4-AP in sequences that were otherwise identical to CXG and TXG via a procedure analogous to that described for the naturally occurring (4*R*) isomer of an AP site (*20*). Plasmids containing 4*S*-AP in a sequence otherwise identical to TXG and CXG were bypassed with efficiency comparable to the efficiency of those containing AP and C4-AP.

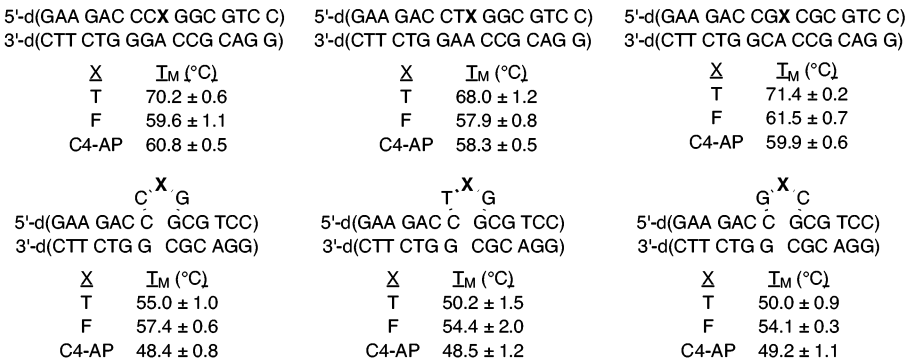

FIGURE 5: Comparison of UV melting temperatures of duplex DNA containing the C4′-oxidized abasic site (C4-AP), the tetrahydrofuran analogue of an abasic site (F), and thymidine (T).

However, three-nucleotide deletion products were not observed upon replication of these plasmids. The mixtures consisted mostly of −1 frameshifts (≥90%) and a small amount of full-length products.

UV Melting Temperatures of Duplexes Containing C4-AP Lesions. Abasic lesions and their analogues (e.g., F) thermally destabilize duplex DNA (*19*, *21*). To determine if three-nucleotide deletion formation was the result of selective stabilization of three-nucleotide loops by the C4-AP lesion, the T_m values of duplexes (2.2 μM) with and without three-nucleotide loops were measured (Figure 5). Substitution of either C4-AP or the tetrahydrofuran analogue (F) of an AP lesion with dT reduced the T_m of a 16-mer duplex by approximately 10 °C. However, the respective duplexes containing three-nucleotide loops in which C4-AP, F, or dT occupies the central position exhibited insignificantly different melting temperatures.

DISCUSSION

DNA Replication Substitution Products Reveal Similarities between C4-AP and AP Lesions. The SOS inducible polymerases pol II, pol IV, and pol V are involved in the bypass of the C4-AP lesion. Under non-SOS-induced conditions where constitutive levels are low, deleting individual enzymes significantly reduced bypass efficiency. Deleting any one polymerase under SOS-induced conditions has little effect on bypass efficiency because the other enzymes compensate, albeit with different outcomes. However, the extent of replication is reduced to less than 1% relative to a control containing thymidine when all three inducible polymerases are deleted, indicating that at least one SOS inducible polymerase is necessary for bypass. Although there were a number of similarities, replication of C4-AP was readily distinguishable from that of a non-oxidized abasic site (AP). While a number of investigators have reported on the replication of an AP site (or its tetrahydrofuran analogue, F) in *E. coli*, we utilized recent results from our own group for comparison because these were obtained using the same assay (REAP), cell lines, and sequence context (*4*). The formation of substitution (full-length) products was dependent upon pol V, which is similar to what has been observed for AP sites and some bulky adducts (*22*, *23*). Selective translesional dA incorporation is consistent with the similarity between the structures of the C4-AP and AP lesions (*4*, *24*). UV melting studies showed that the presence of the C4 hydroxyl group in C4-AP does not alter the thermal stability of duplex DNA compared to one containing the tetrahydrofuran analogue of an AP site (F). Hence, barring significant distortion of the phosphate backbone, such as one might expect due to the presence of 4*S*-C4-AP, a template containing an "intrahelical" C4-oxidized abasic site should appear the same to a polymerase as an AP lesion, resulting in the observed adherence to the A-rule. This would be true regardless of whether one considered the hydrogen bonding pattern presented by C4-AP or its shape (*25*).

Replication of C4-AP Produces Three-Nucleotide Deletions. Pol II and pol IV are responsible for the formation of deletion products, and experiments in polymerase deficient cells indicate that these enzymes work in concert during replication of C4-AP. To our knowledge, this is the first example in which these two enzymes work in a coordinated manner during lesion bypass. In wild-type cells, the activity of these enzymes overshadows that of pol V following SOS induction. This qualitative trend is reminiscent of studies on AP lesions where pol II and pol IV were separately shown to produce deletion products (*26*−*28*). However, it is the size of the deletion product (three nucleotides) formed upon replication of C4-AP that is unique. Single-nucleotide deletions produced from *in vitro* and *in vivo* replication of abasic lesions are common, as are −2 frameshift products when the lesion is surrounded by the proper flanking sequences (*6*, *28*, *29*). Bulky DNA lesions also produce frameshift products with as many as four nucleotides, but their size can be highly dependent upon sequence (*22*, *30*, *31*). Three-nucleotide deletions are rarely observed in non-triplet repeat sequences, yet the C4-AP lesion produces a mixture of two different three-nucleotide deletion products in two different sequence contexts (TXG and CXG) under non-SOS-induced conditions. A third oligonucleotide sequence (GXC) yields even larger amounts of three-nucleotide deletions, but blotting experiments cannot distinguish between the possible loops that are formed. The three-nucleotide deletion products account for ∼40−100% of the replication products. Under SOS-induced conditions, −3 deletions are the sole products observed in the two sequences (CXG and GXC) that were examined. We do not know the mechanism for the formation of the three-nucleotide deletions at this time. The variety of deletions formed in different sequence contexts argues against a process involving nucleotide incorporation followed by misalignment, as has been suggested for other lesions (*22*, *31*). However, the data are

consistent with either dNTP-stabilized misalignment or Streisinger misalignment (*26*, *32*).

These observations suggest that the structure of the C4-AP lesion is capable of interacting with the polymerases and/or neighboring nucleic acid in a unique manner that gives rise to three-nucleotide deletions. Since three-nucleotide deletions are not formed from the replication of any other abasic lesion (AP and L) using the same sequences of oligonucleotides in an identical shuttle vector assay, we focused our attention on the C4 hydroxyl group as the structural driving force for these products (*4*). We explored two possible effects of the C4 hydroxyl group. The C4′-oxidized abasic site can exist as an interconvertible mixture of four cyclic stereoisomers. Although we are unaware of structural studies on duplex DNA containing a single 4*S* nucleotide component, the two 4*S* diastereoisomers of C4-AP could distort the phosphate backbone and induce the looping out of three nucleotides containing the lesion. Plasmids containing the configurationally stable analogue 4*S*-AP were synthesized and transfected into wild-type cells. The epimeric abasic lesion was bypassed as efficiently as C4-AP or AP. However, the major products observed from replication of both sequences corresponded to a −1 frameshift (≥90%), with the remaining material corresponding to the full-length product. Because the C4 hydroxyl group is missing, this model study cannot exclude the involvement of the 4*S* diastereomers during replication. However, the data indirectly support the hypothesis that the C4 hydroxyl group is necessary for formation of three-nucleotide deletions.

To probe the possibility that the C4 hydroxyl in C4-AP stabilized the three-nucleotide loop, we measured the UV melting temperatures of duplexes containing the lesion opposite dA and as the middle nucleotide (corresponding to the major deletion products formed) of such a loop (Figure 5). The T_m values of the analogous duplexes containing either dT or F were also measured for comparison. The reduction in melting temperature of duplexes containing C4-AP opposite dA relative to those containing dT was comparable to the case when F is present, and is consistent with previous measurements. Placing either abasic lesion in the central position of a three-nucleotide loop eliminated the destabilization compared to the duplexes containing the native nucleotide. Moreover, the looped duplexes containing C4-AP did not exhibit any additional stabilization. These initial experiments led us to propose that the interaction between the C4 hydroxyl of C4-AP and pol II and/or pol IV is responsible for the formation of the three-nucleotide deletions. However, structural data like those obtained for ternary complexes containing AP DNA, incoming nucleotide triphosphate, and Dpo4 that address this possibility are unavailable (*33*). Consequently, this hypothesis will need to be tested soon using additional DNA analogues.

Possible Physiological Significance of Three-Nucleotide Deletions from C4-AP Replication. DNA damage can cause cancer, and is a goal of some agents used to treat this disease. Consequently, identifying lesions that exert significant biological effects provides valuable information regarding the origin and treatment of this disease. The experiments described provide a definitive role for the C4-oxidized abasic site in the formation of three-nucleotide deletions when cells are exposed to bleomycin and γ-radiolysis (*14*). C4-AP is a common product of oxidative stress, and the formation of such large deletions as major products in nonrepeat sequences by an abasic lesion is to our knowledge a previously unreported process.

The combination of these facts suggests that the lesion may be involved in the development of diseases in which oxidative stress is a causative factor and proteins lacking a single amino acid are produced. We are aware of two recent examples where the deletion of a single amino acid exhibits a significant phenotype. Deletion of Leu62 from caspase-8 produced a protein without proapoptotic activity in mammalian cells (*34*). Such a mutation could provide a mechanism by which cancer cells resist undergoing apoptosis. In another example, a three-nucleotide deletion resulted in the loss of Glu2347 from a calcium-release-channel protein involved in malignant hyperthermia (*35*). Deletion of three nucleotides from the very large RYR1 gene resulted in a mutant protein that is believed to contribute to abnormal calcium regulation in skeletal muscle. Although a connection between these mutations and oxidative stress has not been made, the observations described here could provide the impetus for appropriate investigations.

SUPPORTING INFORMATION AVAILABLE

ESI-MS of oligonucleotides containing C4-AP, **1**, and 4*S*-AP, a phosphorimage showing the distribution of full-length products and deletions from C4-AP replication in wild-type cells, and a table describing incorporation of dA opposite C4-AP in full-length replication products. This material is available free of charge via the Internet at http://pubs.acs.org.

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