“Action-at-a-Distance” Mutagenesis

8-OXO-7,8-DIHYDRO-2’-DEOXYGUANOSINE CAUSES BASE SUBSTITUTION ERRORS AT NEIGHBORING TEMPLATE SITES WHEN COPIED BY DNA POLYMERASE β*

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8-Oxo-7,8-dihydro-2’-deoxyguanosine (8-oxo-dG), a common oxidative DNA lesion, favors a syn-conformation in DNA, enabling formation of stable 8-oxo-dGA base mispairs resulting in G-C → T-A transitions during replication. When human DNA polymerase (pol) β was used to copy a short single-stranded gap containing a site-directed 8-oxo-dG lesion, incorporation of dAMP opposite 8-oxo-dG was slightly favored over dCMP depending on “downstream” sequence context. Unexpectedly, however, a significant increase in dCMPA and dGMPA mispairs was also observed at the “upstream” 3’-template site adjacent to the error. Errors at these undamaged template sites occurred in four sequence contexts with both gapped and primed single-stranded DNA templates, but not when pol α replaced pol β. Error rates at sites adjacent to 8-oxo-dG were roughly 1% of the values opposite 8-oxo-dG, potentially generating tandem mutations during in vivo short-gap repair synthesis by pol β. When 8-oxo-dG was replaced with 8-bromo-2’-deoxyguanosine, incorporation of dCMP was strongly favored by both enzymes, with no detectable misincorporation occurring at neighboring template sites.

A significant increase in G-C → T-A mutation rates (1) takes place when oxidative damage to DNA occurs at C-8 of G to form 8-oxo-dG* (2, 3). In contrast to G, which exists predominantly in an anti-conformation in DNA and forms base pairs almost exclusively with C, base pairing properties of 8-oxo-dG tend to be ambiguous, having a syn-conformation when paired opposite A (4) and an anti-conformation when paired opposite C (5). Prokaryotic and eucaryotic cells contain highly conserved groups of enzymes to deal with the potentially lethal levels of 8-oxo-dG-stimulated mutations. Analogs of Escherichia coli MutM glycosylase excise 8-oxo-dG from DNA (paired opposite C) (6), whereas the MutY class of enzymes removes dAMP paired opposite 8-oxo-dG (1). Taken together, MutM and MutY, along with MutT, responsible for eliminating oxidized G from deoxy- and ribonucleoside triphosphate pools (7, 8), constitute the “8-oxo-dG repair system” (reviewed in Ref. 9). Although most 8-oxo-dG lesions are repaired by this system, those that escape repair are likely to encounter DNA polymerases during either replicative or repair DNA synthesis.

The nucleotide incorporation specificity opposite 8-oxo-dG may vary depending on which polymerase is used to copy the lesion in vitro (10). Furthermore, very little is known regarding the effects of sequence context on incorporation specificities for different polymerases (11). In this paper, we have used human DNA polymerase (pol) β and pol α to investigate specificity of incorporation opposite 8-oxo-dG, varying the template base immediately downstream from the lesion. During the course of this study, we observed unexpectedly that in addition to its own ambiguous base pairing properties, 8-oxo-dG also stimulates nucleotide misincorporations at adjacent upstream and downstream template sites. This paper is focused on the potential implications of 8-oxo-dG-stimulated “action-at-a-distance” mutagenesis in relation to pol β-catalyzed gap repair synthesis.

EXPERIMENTAL PROCEDURES

Materials

Enzymes—Human pol β was purified as described (12). pol α (200 units/mg), a kind gift from Dr. T. S.-F. Wang, refers to the large catalytic subunit of the holoenzyme and was purified as described (13). Both enzymes are highly purified and contain no detectable 3’exonuclease activity. One DNA polymerase unit is defined as the incorporation of 1 nmol of dNMP into DNA in 1 h at 37 °C. DNA polymerase activity for pol β was measured on gapped primer-template DNA and for pol α on ungapped DNA. T4 polynucleotide kinase was purchased from U. S. Biochemical Corp.

Nucleotides—Nonradioactive nucleotides were purchased from Pharmacia Biotech Inc. [γ-32P]ATP (2000 Ci/mmol) was purchased from NEN Radiochemicals.

Synthesis of DNA Primer-Templates—8-OxodG phosphoramidite was synthesized as described (14). Templates containing a single 8-oxo-dG site, control templates with no lesion, and various primers were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer. Oligodeoxyribonucleotides containing a 5’-phosphate group, needed to form 5-base gaps for pol β, were synthesized by Lynn Williams at the University of Southern California Comprehensive Cancer Center.

DNA Substrates—To investigate the insertion specificity of pol β opposite 8-oxo-dG, 5-base gaps with the lesion in their center were formed by annealing 15-mer primers and 22-mer downstream oligodeoxyribonucleotides to 42-mer templates with a 8-oxo-dG lesion at position 18. The downstream oligodeoxyribonucleotides were phosphorylated at their 5’-end. This and a gap size of fewer than 6 nucleotides are required by pol β for processivity (15). For studies of insertion opposite normal G or 8-Br-dG, similar primer templates were used with either G or 8-Br-dG substituting for 8-oxo-dG at position 18. The same experiments were also carried out with pol α, but no downstream oligodeoxyribonucleotides were used since these inhibited pol α activity ~2-fold (data not shown). All primers and templates were purified by polyacrylamide gel electrophoresis.
Annealing was carried out by incubating 7.5 nM primer with 7.5 nM bovine serum albumin, and 1 mM 100, 200, 600, and 1200 μM dATP or dCTP; d, 10, 25, 100, 200, 600, and 1200 μM dGTP or dTTP; c, 25, 100, 200, 600 and 1200 μM dATP and 10, 25, 100, 200, 600, and 1200 μM dCTP. dATP (5 μM) was included in running-start reactions to extend the primer 3'-end to reach 8-oxo-dG. Template sequences are shown at the left. Arrows shown at the right indicate the locations of doublet bands. Reactions were carried out as described under “Experimental Procedures.”

**Methods**

**Conditions for Determining Kinetic Constants**—Primers were 5'-end-labeled with 32P using T4 polynucleotide kinase as described (16). Annealing was carried out by incubating 7.5 nm primer with 7.5 nm template and 38 nM downstream oligodeoxyribonucleotide in pol β reaction buffer (35 mM Tris-Cl, pH 8.0, 6.7 mM MgCl2, 100 mM NaCl, 0.2 mg/ml bovine serum albumin, 1.5 mM dithiothreitol, and 2% glycerol) at 37 °C for 1 h, followed by gradual cooling to room temperature. For pol α, 7.5 nm primer was annealed to 7.5 nm template in pol α reaction buffer (20 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 0.2 mg/ml bovine serum albumin, and 1 mM β-mercaptoethanol) at 37 °C for 1 h, followed by gradual cooling to room temperature.

**Kinetics of Insertion Opposite 8-Oxo-dG**—Time courses were run to establish the conditions for measuring incorporation efficiencies opposite 8-oxo-dG. Reactions were carried out by mixing equal volumes of annealed primer-template with reaction buffer containing deoxyribonucleotide (for buffer components, see annealing conditions described above). The final concentration of primer-template in the reactions was 3.8 nM. pol β (0.2 units) or pol α (0.02 units) was added, and the reactions were incubated at 37 °C for 10 min. Under these conditions, the data for both enzymes were in the linear range.

Four primer-template constructs were used to investigate the effect of the downstream nearest neighbor on incorporation efficiencies at the 8-oxo-dG site. For each reaction, the dNTP substrate for incorporation opposite the lesion was varied in concentration over a range of 10 μM to 1.2 mM. In the time course experiments, the dNTP substrate was held constant at 800 μM. The reactions were performed under running-start conditions with 5 μM dTTP for insertion opposite the two As upstream from 8-oxo-dG. Reactions were terminated by adding 2 volumes of 20 mM EDTA in 95% formamide and heating to 100 °C for 5 min. Samples were then cooled on ice, and the single-stranded primers were separated according to length by electrophoresis on 18% polyacrylamide gels.

The kinetics of incorporation of each of the four deoxynucleotides opposite the lesion were determined as a function of deoxynucleotide concentration (17, 18). Band intensities were measured by a PhosphoImager using Imagequant software (Molecular Dynamics, Inc., Sunnyvale, CA). Incorporation efficiencies were established as described (16).

**Dideoxynucleotide Incorporation**—To determine the nature of the dNMPs incorporated at or around the lesion site by pol β, primer extension reactions were carried out in the presence of 5 μM dTTP (running-start base) and a 50 μM concentration of either dATP or dCTP. At various time points (a range of 1–30 min), ddATP or ddCTP was added to a final concentration of 0.5 μM, and the reactions were allowed to continue for an additional 5 min at 37 °C. Reactions were terminated as described above. Controls were run under the same conditions, but in the absence of dideoxynucleotides. Reaction products were separated on 18% polyacrylamide gels.

**Sequence Markers**—We identified each product sequence based on the dideoxynucleotide sequencing data. An independent sequence confirmation was obtained first by synthesizing each “full size” oligonucleotide sequence on a DNA synthesizer (Applied Biosystems 392 DNA/ RNA synthesizer). The oligonucleotides were gel-purified and 5'-end-labeled with 32P. A sequencing ladder containing a digest of the labeled oligonucleotide was obtained by 3'-exo-exonucleolytic digestion using E. coli DNA polymerase III core (containing the e-proofreading exonuclease) under the following conditions. Labeled oligonucleotide (13 nt) was incubated with 0.5 μM pol III core for 2–5 min at 37 °C in pol III reaction buffer (20 mM Tris-Cl, pH 7.5, 40 μg/ml bovine serum albumin, 50 mM dithiothreitol, 50 mM NaCl, and 8 mM MgCl2). Reactions were quenched as described above. Samples were run alongside pol β reaction products on 18% polyacrylamide gels.

**RESULTS**

We have measured the effects of sequence context on dAMP and dCMP insertion efficiencies opposite 8-oxo-dG using human polymerases β and α. Synthesis by pol β involves filling in a 5-nucleotide template gap, 3'-AA 8-oxo-dG NC-5', where the nucleotide N, immediately downstream from the lesion, is varied (Fig. 1 and Table I). Two Ts are incorporated opposite the two running-start As, followed by incorporation of each of the four dNMPs opposite 8-oxo-dG. We have also used pol α to copy a subset of these sequences (Table I).
8-oxo-dG-induced Tandem Misincorporation

### Table I
Nucleotide incorporation specificities for pol b and a opposite 8-oxo-dG, 8-Br-dG, and G

| Primer-template DNA structure and sequence | Enzyme | dATP | dCTP | dGTP | dTTP |
|-------------------------------------------|--------|------|------|------|------|
| 5‘-AA-GO-CC- | pol b   | 637a | 322a | ND   | ND   |
| 5‘-AA-GO-AC- | pol b   | 273a | 149a | ND   | ND   |
| 5‘-AA-GO-GC- | pol b   | 157a | 146a | ND   | ND   |
| 5‘-AA-GO-TC- | pol b   | 240a | 152a | ND   | ND   |
| 5‘-AA-GC-   | pol b   | 1375 |      | ND   | ND   |
| 5‘-AA-GO-CC- | pol b   | 0.9  | 0.7  | ND   | ND   |
| 5‘-AA-GO-GC- | pol b   | 0.9  | 0.7  | ND   | ND   |
| 5‘-AA-GO-GC- | pol a   | 3.4  | 0.7  | ND   | ND   |
| 5‘-AA-GO-CC- | pol a   | 4.9  | 0.5  | ND   | ND   |
| 5‘-AA-BG-CC- | pol β   | 0.2  | 5.2  | 7.9  | 0.01 |
| 5‘-AA-BG-CC- | pol β   | 0.1  | 1.9  | 3.7  | 0.01 |

a Letters in boldface indicate where template bases have been changed, e.g. G or 8-Br-dG (BG) replacing 8-oxo-dG (GO). The double lines on the right indicate primer-templates containing a 5-nucleotide gap.

b $V_{max}/K_m$ values represent an average from at least three separate measurement with an S.E. of ±30%.

c ND, not determined. Typically, bands corresponding to incorporation of dGMP or dTMP opposite 8-oxo-dG were small, but detectable. We did not carry out further analysis.

Nucleotide Incorporation Specificities Opposite 8-Oxo-dG—An analysis of the data reveals that pol β inserts dAMP slightly more efficiently than dCMP opposite 8-oxo-dG with three of the four 5’-nearest-neighbor bases and inserts dAMP and dCMP with about equal efficiency with the other nearest-neighbor base (G) (Table I). Both dAMP and dCMP are incorporated slightly more efficiently when C is the 5’-nearest neighbor. The efficiency of dCMP incorporation opposite 8-oxo-dG is 4-fold less than that of dCMP incorporation opposite G in this sequence context. This same template sequence, having an abasic site in place of 8-oxo-dG, also showed the highest misincorporation efficiency (16). Low intensity gel bands corresponding to the incorporation of either dGMP or dTMP opposite 8-oxo-dG are clearly visible (Fig. 1, a and b). However, their corresponding $V_{max}/K_m$ values are <1% of the values for incorporation of either A or C and are excluded from Table I. pol α also preferentially incorporates A opposite 8-oxo-dG. However, in contrast to pol β, which incorporates A with almost a 2-fold higher efficiency compared with C, pol α favors incorporation of A with much higher selectivity, ~5–10-fold (Table I).

8-Oxo-dG-stimulated Nucleotide Misincorporation at Neighboring Template Sites Catalyzed by pol b—The gel band patterns in Fig. 1 demonstrate that dAMP and dCMP are both readily incorporated opposite 8-oxo-dG by pol β and are also extended 1 nucleotide beyond the lesion by incorporation of a mismatched base pair. Remarkably, however, in addition to its ambiguous base pairing and promiscuous extension properties, 8-oxo-dG also stimulates nucleotide misincorporations at adjacent upstream template sites. Correct and incorrect incorporations opposite a single template site appear as doublet gel bands (Fig. 1). When a combination of dTTP (at 5 μM, for incorporation opposite the running-start As) and either dCTP or dGTP at increasing concentrations is included in pol β reactions, doublet bands occur upstream, downstream, or directly opposite 8-oxo-dG in a sequence-dependent manner. In contrast, there are no doublet bands detected when dATP is used as a substrate for pol β, even when high-resolution (20% polyacrylamide) gels are used (data not shown). Data illustrating the presence of doublets are presented in which C (Fig. 1a), A (Fig. 1b), G (Fig. 1c), or T (Fig. 1d) is immediately downstream from 8-oxo-dG on the template strand.

We can identify individual doublet band sequences unambiguously, in almost all instances, using the following criteria. Dideoxynucleotides (ddCTP or ddATP) are added to the reaction at different time points, resulting in termination of primer extension following incorporation of each ddNMP by pol β. Each specific oligonucleotide sequence corresponding to the partial sequences deduced from the termination data, along with permutations of other possible sequences, are then synthesized; the synthetic oligonucleotides are end-labeled with 33P and used as molecular mass markers for comparison with polymerase reaction products (Fig. 2). For example, the doublet appearing opposite template A in the dGTP lane (Fig. 2a) contains G and T as upper and lower bands, respectively, based on the marker digest shown to the right. In a similar way, the doublet in the dCTP lane (Fig. 2b) is resolved into a lower C and an upper T.

With C as the adjacent downstream neighbor (Fig. 1a), doublets occur only when dTTP + dCTP are present in the reaction. The lesion is flanked by both upstream and downstream doublets. The upstream doublet opposite A is easily observed, even at the lowest concentration of dCTP (10 μM), and reflects the correct incorporation of dTMP (upper band in doublet) and the misincorporation of dCMP opposite A (lower band in doublet). The downstream doublet is much less intense, appearing at dCTP concentrations above 200 μM. The downstream doublet results from incorporation of two dCMPs (opposite 8-oxo-dG and then opposite C) from each upstream doublet. No other dNTP gives rise to doublets in this sequence context.

Note that the 5-nucleotide gap is filled to the end when dGTP is present in the reaction. However, in contrast to incorporation of either dCMP or dAMP, incorporation of dGMP is unlikely to be occurring directly opposite 8-oxo-dG. Instead, G incorporation is likely to be taking place opposite the template C downstream from 8-oxo-dG (3′-AA 8-oxo-dG CC) (Fig. 1a) by “skipping over” 8-oxo-dG using a transiently misaligned primer 3′-end (Fig. 3; see “Discussion”) (16).

In the template sequence 3′-AA 8-oxo-dG GC (Fig. 1c), both dAMP and dCMP are incorporated almost equally, primarily because of a reduction in $V_{max}/K_m$ for incorporation of dAMP directly opposite 8-oxo-dG as opposed to an increase in the dCMP incorporation efficiency, e.g. compare $V_{max}/K_m$ values in the dATP and dCTP columns for the four gapped primer-template DNAs containing 8-oxo-dG (Table I, first four sequences). Note that $V_{max}/K_m$ for incorporation of dCMP is no higher than for the other three sequences (Table I), suggesting that misalignment misincorporation may not be occurring.

Three doublets are observed when G is located downstream from 8-oxo-dG when the reaction is carried out in the presence of dCTP + dTTP. The doublets occur opposite both upstream running-start As and directly opposite 8-oxo-dG (Fig. 1c, dCTP lanes). Weak intensity bands running just below the bands...
of dNMPs opposite 8-oxo-dG (GO) by DNA polymerase β was measured using template sequences AA 8-oxo-dG AC (a) and AA 8-oxo-dG CC (b) at 1200 μM dNTP concentrations in the presence of dTTP (5 μM) to extend the primer 3'-end to reach 8-oxo-dG. A sequencing ladder containing a digest of 32P-end-labeled oligonucleotides is shown at the right of each gel (see “Sequence Markers” under “Experimental Procedures”).

FIG. 2 Identification of doublet band nucleotides. Incorporation of dNMPs opposite 8-oxo-dG (GO) by DNA polymerase β was measured using template sequences AA 8-oxo-dG AC (a) and AA 8-oxo-dG CC (b) at 1200 μM dNTP concentrations in the presence of dTTP (5 μM) to extend the primer 3'-end to reach 8-oxo-dG. A sequencing ladder containing a digest of 32P-end-labeled oligonucleotides is shown at the right of each gel (see “Sequence Markers” under “Experimental Procedures”).

FIG. 3 Misinsertion mechanisms at 8-oxo-dG sites. In direct insertion (upper panel), dGTP is inserted directly opposite 8-oxo-dG (GO). In dNTP-stabilized misalignment (lower panel), 8-oxo-dG rotates out of the helical plane, and an incoming dNTP substrate (dGTP), bound at the pol β active site, is inserted opposite template C situated immediately downstream from 8-oxo-dG.

corresponding to incorporation of each running-start T (Fig. 1c, lower two arrows) are generated by misincorporation of dCMP opposite both As upstream from 8-oxo-dG. Further extension of each of the lower doublets gives rise to weak doublet bands opposite 8-oxo-dG (Fig. 1c, upper arrow). The weak bands corresponding to incorporation of C opposite 8-oxo-dG are efficiently extended by incorporation of another dCMP opposite the downstream G, followed, most likely, by misincorporation of dCMP opposite C.

We also find that dAMP 8-oxo-dG base pairs are easily extended by two consecutive misincorporations of dAMP opposite the template bases G and C downstream from the lesion (Fig. 1c, dATP lanes). The relative ease with which nucleotides can be misincorporated by pol β downstream from 8-oxo-dG (Fig. 1c, dATP and dCTP lanes) may result from significant template distortions by 8-oxo-dG and by primer termini distortions caused by the non-Watson-Crick 8-oxo-dG-A base pairing structure. For the template sequence having T proximal to 8-oxo-dG, there is a single upstream doublet when dCTP is included in the reaction, originating from correct incorporation of dTMP and misincorporation of dCMP opposite the second running-start A (Fig. 1d).

Since the doublets are sequence-dependent, we decided to mask the template sequence downstream from 8-oxo-dG, by reducing the gap length from 5 to 3 bases, to see if the upstream doublet bands were altered by eliminating the downstream region of ssDNA. Indeed, the doublet bands were not altered. An example is shown where the template region to the 5'-side of 8-oxo-dG is annealed to a downstream oligonucleotide (Fig. 4). The doublet upstream from 8-oxo-dG is present at the same dCTP concentration (10 μM) either with or without the covered downstream Cs (compare Figs. 4 and 1a).

FIG. 4 Doublet bands persist when pol β copies 8-oxo-dG-containing DNA in the presence of a masked downstream template sequence. The entire downstream sequence from 8-oxo-dG (GO), with C as the downstream nearest neighbor of 8-oxo-dG, was covered with a 5'-phosphorylated oligonucleotide. Incorporation of dAMP and dCMP was measured at 0.1, 0.5, 2, 10, 50, 200, and 800 μM dTTP (5 μM) was used in a running-start reaction to extend the primer to 8-oxo-dG. The template sequence is shown at the left, and the arrow shown at the right indicates the location of doublet bands. Reactions were carried out as described under “Experimental Procedures.”

Misincorporations Proximal to 8-Oxo-dG Are Catalyzed by pol β, but Not by pol α—The 8-kDa amino-terminal domain of pol β has been shown to interact with the 5'-phosphoryl group on the downstream gap-forming DNA when functioning as a deoxyribose phosphate lyase during base excision repair (19, 20). Therefore, it is necessary to determine if the doublets occur only in the presence of gapped DNA. Since pol β is much more active and processive with primer-template DNA containing a 1–6-nucleotide gap compared with primed ssDNA (15), we carried out reactions with both gapped and primed ssDNAs over a 10-fold range of enzyme concentration (Fig. 5a). The substrates for the reaction, dTTP for the running start and dCTP for incorporation opposite 8-oxo-dG, are those that give rise to doublet bands opposite the upstream A proximal to 8-oxo-dG (Fig. 1a, dCTP lanes).

Doublet bands opposite A are observed at each pol β level in the gap-filling reaction (Fig. 5a, right panel). However, despite significantly reduced synthesis using primed ssDNA, a doublet band is nevertheless detectable opposite the upstream A at the highest pol β concentration used (Fig. 5a, left panel). Therefore, we conclude that the interaction between the 8-kDa domain of pol β and the 5'-phosphoryl is not linked exclusively to the presence of the dCMP-A upstream misincorporation band. Although the downstream oligonucleotide is not necessary for doublet formation, its presence is required for continued synthesis beyond 8-oxo-dG. A similar experiment performed using pol α shows that doublets are not observed with either primer-template DNA (Fig. 5b), suggesting that these unusual misincorporation events are dependent not only on the presence of 8-oxo-dG, but also on properties specific to pol β. Again, the misincorporations upstream of 8-oxo-dG could be linked to 8-oxo-dG-induced distortions in the template (see “Discussion”).

pol β-catalyzed Doublet Misincorporation Bands Require the Presence of 8-Oxo-dG—Templates with normal G in place of 8-oxo-dG were incubated with pol β (Fig. 6) or pol α (data not shown). Both polymerases incorporate two dTPs opposite the two running-start As and dCMP opposite G. At high dCTP concentrations (≥100 μM), pol β catalyzes weak misincorporation of dCMP opposite the downstream C. However, no doublets are formed by pol β using templates in which either G (Fig. 6) or C (data not shown) is used in place of 8-oxo-dG.

NMR studies have shown that 8-oxo-dG favors a syn-conformation when paired opposite A (4, 21). Data showing favored hydrolysis of both 8-oxo-dGTP (22) and 8-Br-dGTP (23) over dGTP by the MutT hydrolase suggest that 8-Br-dG also favors
a syn-conformation (24), although direct NMR evidence is not yet available to substantiate this point. To investigate whether a syn-nucleotide is required to generate mispair-containing doublet bands proximal to the template lesion, 8-Br-dG was substituted for 8-oxo-dG and copied using reactions similar to those displayed in Fig. 1 (with C as the 5′-neighbor). Reactions were carried out using pol β (Fig. 7a) and pol α (Fig. 7b).

Both polymerases exhibit similar primer extension patterns in the presence of 8-Br-dG. Following incorporation of the running-start Ts, dCMP and dAMP are incorporated directly opposite the modified template base. However, in contrast to the templates containing 8-oxo-dG, for which incorporation of dAMP is favored over dCMP (Table I), the reverse is now true, with dCMP strongly favored for incorporation opposite 8-Br-dG by both polymerases (Fig. 7, a and b). Note that, as before, the highly efficient incorporation of dGMP is unlikely to be taking place directly opposite the lesion, but more likely results from pairing an incoming dGTP opposite a downstream template C using a dNTP-stabilized misalignment mechanism (Fig. 3).

The most important point is that the doublet patterns so obvious with 8-oxo-dG in this sequence context (Fig. 1) do not occur with 8-Br-dG. Thus, the emergence of upstream doublet bands when synthesis is carried out using pol β appears to require the presence of 8-oxo-dG on the template strand.

**Doublets Can Be Driven Out by Adding the dNTP Complementary to the Downstream Neighbor of 8-Oxo-dG**—In an attempt to drive out the upstream doublet on the template with C located 5′ to 8-oxo-dG (Fig. 1a), incubations were carried out using three dNTPs: dTTP for the running start, a high concentration of dCTP (1.2 mM) to form the doublet, along with increasing concentrations of dGTP, complementary to the downstream Cs (Fig. 8). The doublet is clearly visible in the absence of dGTP (Fig. 8, first lane). However, as the concentration of dGTP is increased, the lower band of the doublet, corresponding to misincorporation of dCMP opposite the second running-start A, diminishes in intensity concomitant with complete filling of the 5-nucleotide gap (Fig. 8, dGTP lanes). We suggest that the dCMP-A mispair is eliminated because the binding of dGTP on the polymerase-primer-template DNA complex is reduced by mass-action competition with dGTP. However, in contrast to 8-oxo-dG-stimulated dCMP-A mispair formation, 8-oxo-dG does not stimulate misincorporation of dGMP opposite the upstream A.
8-oxo-dG-induced Tandem Misincorporation

8-oxo-dG DNA template lesions are believed to contribute significantly to the large increase in mutation rates observed in oxidatively damaged cells. NMR studies have shown that 8-oxo-dG can assume two conformations, a favored syn-conformation, in which base mispairs are formed with A (24), or an anti-conformation, forming “correct” base pairs with C (21). Previous measurements using DNA polymerase in vitro (10), including this investigation of the effect of sequence context on the specificity of incorporation by pol β opposite 8-oxo-dG (Fig. 1), confirm that both dAMP and dCMP are incorporated, suggesting that 8-oxo-dG exhibits both anti- and syn-conformation base pairing properties during replication.

8-Oxo-dG Reduces pol β Fidelity at Neighboring Template Sites—When synthesis is carried out using pol β, the data reveal an interesting and unexpected gel band pattern, namely the appearance of doublet bands at template sites adjacent to 8-oxo-dG (Fig. 1). A doublet band upstream from 8-oxo-dG corresponds to incorporation of a different nucleotide prior to reaching the lesion site and thus reflects the ability of 8-oxo-dG to stimulate misincorporation errors at neighboring template sites.

The doublet bands upstream from 8-oxo-dG vary, depending on the sequence downstream from the lesion. In the sequence 3′-AA 8-oxo-dG CC, an upstream doublet opposite A occurs, generated by a correct incorporation of dTMP opposite A and a misincorporation of dCMP opposite A (Fig. 1a). Extension of each of the doublets by incorporation of dCMP opposite 8-oxo-dG followed by misincorporation of dCMP opposite C also gives rise to downstream doublet bands.

The nature of the doublets changes for the sequence 3′-AA 8-oxo-dG AC, where a doublet is formed by correct incorporation of dTMP and misincorporation of dGMP opposite the upstream running-start A (Fig. 1b). For the sequence 3′-AA 8-oxo-dG GC, doublets corresponding to dCMP misincorporation (and correct dTMP incorporation) are observed opposite both upstream running-start As, and a doublet band is also formed opposite 8-oxo-dG (Fig. 1c). For the sequence 3′-AA 8-oxo-dG TC, a doublet occurs opposite the upstream A, corresponding, as before, to incorporation of dTMP and dCMP (Fig. 1d). This action-at-a-distance effect appears to be a novel property of the interaction of pol β with 8-oxo-dG, reducing the fidelity of DNA synthesis at neighboring template positions while, at the same time, resulting in mutations at the site of the lesion.

Doublet Band Dependence on 8-Oxo-dG and pol β—Doublets are not observed in the following three series of experiments. (i) Synthesis carried out by pol α on either gapped or primed ssDNA fails to give rise to doublet bands adjacent to 8-oxo-dG, even when high levels of enzyme are used (Fig. 5b). Indeed, none of the 8-oxo-dG sequence contexts giving rise to doublets when copied by pol β results in detectable doublet bands using pol α (data not shown), suggesting that these bands arise from a specific interaction between pol β and 8-oxo-dG, as opposed to the presence of base composition heterogeneity in the synthetic templates. (ii) There are no doublet bands observed when 8-oxo-dG is replaced by G (Fig. 6) or T (data not shown) and copied with pol β. Thus, the presence of pol β alone is insufficient to generate doublets. (iii) No doublets are observed when 8-oxo-dG is replaced by 8-Br-dG and copied with pol β (Fig. 7a). 8-Br-dG, like 8-oxo-dG, is believed to favor a syn-conformation. Thus, it appears that substitutions at the C-8 atom on the purine ring causing a 180° rotation about the glycosidic bond might not be the sole basis for misincorporations occurring at neighboring template positions, but that some additional property of 8-oxo-dG is also required. For example, should the C-8 substituted purine ring adopt an anti-conformation, the significant steric hindrance with the template phosphodiester backbone could be a cause of the misincorporations at neighboring template bases.

The 8-kDa domain of pol β is in direct contact with the 5′-phosphoryl group on the downstream portion of the gap, giving rise to a relatively processive gap-filling reaction (15). pol β is much more active on DNA containing short, 1–6 nucleotide gaps (15, 25) than on primed ssDNA templates, as confirmed in Fig. 5a. However, an upstream doublet band is still observed when synthesis is carried out at a high concentration of pol β on primed ssDNA (Fig. 5a, left panel), despite its reduced activity on ungapped DNA. Therefore, the doublet bands are not solely attributable to protein-DNA interactions involving the 8-kDa domain taking place downstream from 8-oxo-dG. Doublets are not observed using levels of pol α comparable to pol β on either gapped or ungapped DNA (Fig. 5b).

Sequence Context Dependence of Nucleotide Misincorporation Directly Opposite 8-Oxo-dG—The biological significance of 8-oxo-dG lies in its ability to cause a sizable increase in mutation rates caused by preferential base pairing with A rather than C (23, 29). In contrast to pol α, which strongly favors the incorporation of A opposite 8-oxo-dG (Table I) (10), pol β is much more “even-handed” in the four sequence contexts examined, favoring the incorporation of A compared with C opposite 8-oxo-dG by no more than 2.4-fold (Table I).

In the sequence 3′-AA 8-oxo-dG GC, we expected that dCMP incorporation would be strongly enhanced by skipping past 8-oxo-dG and pairing directly with the nearest-neighbor downstream G, owing to the propensity of pol β to copy past lesions via dNTP-stabilized misalignment (Fig. 3) (16, 26). However, this was not the case since there is an ∼2-fold reduction in the incorporation rate of dCMP (and also of dAMP) compared with the 3′-AA 8-oxo-dG CC sequence. Although transient primer-template DNA misalignment may be occurring, we cannot distinguish this mode unambiguously from direct incorporation of dCMP opposite 8-oxo-dG followed by extension opposite the downstream G site.

However, evidence that pol β can carry out dNTP-stabilized misalignment synthesis is found with the sequence 3′-AA 8-oxo-dG CC (Fig. 1a, dTTP lanes). Here, incorporation of dGMP is unlikely to occur directly opposite 8-oxo-dG, but is instead taking place 1 base downstream from 8-oxo-dG, opposite C. In support of this model, we find that extension beyond the lesion using dGTP as substrate is reduced significantly when A is the template base downstream from 8-oxo-dG (3′-AA 8-oxo-dG DC) (Fig. 1b, dGTP lanes), whereas extension past 8-oxo-dG does take place using dTTTP as substrate. At high dGTP concentrations (600 μM), however, trace bands can be detected correspond-
ing to misincorporation of dGMP directly opposite 8-oxo-dG followed by correct additions of T and G opposite the next 2 downstream template bases. A single upstream doublet, occurring only at high dGTP concentrations (200 and 600 μM) when A is located 5’ to 8-oxo-dG (Fig. 1b), is attributable to formation of a dGMP-A mispair.

Comparison with Previous In Vitro Data Using Polymerases to Copy 8-oxo-dG and Possible Biological Consequences—In an earlier in vitro study, Shibutani et al. (10) reported that pol α strongly favors formation of dAMP-8-oxo-dG over dCMP-8-oxo-dG mispairs by a ratio of 200:1. Our data using pol α also demonstrate preferential incorporation of dAMP opposite 8-oxo-dG, but with a lower specificity of −10:1. For pol β, we find that incorporation of A is only slightly favored over C in the four sequence contexts examined.

In contrast to our data, Shibutani et al. (10) found a reversal in the specificity of pol β favoring dCMP-8-oxo-dG over dAMP-8-oxo-dG by 4:1, although kinetic data for pol β were not reported. As mentioned above, the activity of pol β is much greater when filling a short gap than when extending primed ssDNA (see, for example, Fig. 5 and Table I). The previous measurements were made using primed ssDNA (10), whereas we focused our attention on short gap-filling reactions (Fig. 1), which are believed to be most relevant for base excision and short nucleotide excision repair in vivo (27, 28). However, similar measurements using pol β to copy primed ssDNA gave a dAMP/dCMP incorporation efficiency ratio of ~1.8 (Table I), which is similar to the incorporation efficiencies found for gapped DNA. As expected, the activity of pol β was much greater (~200-fold) using gapped primer-template DNA. Because the 18-mer template used by Shibutani et al. (10) contained no Gs downstream of 8-oxo-dG, the preference for C cannot be attributed to enhanced dCMP incorporation by transient primer-template DNA misalignment. A difference in the primer-template sequence context remains the most likely explanation for the differences between our data and those of Shibutani et al. (10).

Sequence context-dependent errors occurring at residues adjacent to 8-oxo-dG were reported by Kuchino et al. (10) challenged both observations, noting that Klenow fragment was not suitable for the dideoxynucleoside dNTPs. Shibutani et al. (10) reported that 8-oxo-dG-directed misincorporation at sites adjacent to 8-oxo-dG were reported by Kuchino et al. (10) con-

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