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8-Oxoguanine incorporation into DNA repeats *in vitro* and mismatch recognition by MutSα

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ABSTRACT

DNA 8-oxoguanine (8-oxoG) causes transversions and is also implicated in frameshifts. We previously identified the dNTP pool as a likely source of mutagenic DNA 8-oxoG and demonstrated that DNA mismatch repair prevented oxidation-related frameshifts in mononucleotide repeats. Here, we show that both Klenow fragment and DNA polymerase α can utilize 8-oxodGTP and incorporate the oxidized purine into model frameshift targets. Both polymerases incorporated 8-oxodGMP opposite C and A in repetitive DNA sequences and efficiently extended a terminal 8-oxoG. The human MutSα mismatch repair factor recognized DNA 8-oxoG efficiently in some contexts that resembled frameshift intermediates in the same C or A repeats. DNA 8-oxoG in other slipped/mispaired structures in the same repeats adopted configurations that prevented recognition by MutSα and by the OGG1 DNA glycosylase thereby rendering it invisible to DNA repair. These findings are consistent with a contribution of oxidative DNA damage to frameshifts. They also suggest how mismatch repair might reduce the burden of DNA 8-oxoG and prevent frameshift formation.

INTRODUCTION

Oxidative DNA damage is a major threat to the genomic integrity of most living organisms. Mammalian cells are adapted to deal with this, and DNA repair plays a significant role in preventing oxygen-related genetic instability. 8-Oxoguanine (8-oxoG) is prominent among the potentially hazardous DNA oxidation products [reviewed in (1)]. The miscoding properties of 8-oxoG are well established and the oxidized base can direct incorporation of either C or A depending on the polymerase and the sequence context (2–5). This property is fully consistent with its involvement in AT→CG and GC→TA transversions (6–8). At least two complementary branches of the base excision repair (BER) pathway combine to minimize the toxic and mutagenic effects of this oxidized base [reviewed in (9)]. The OGG1 DNA glycosylase initiates BER of 8-oxoG by excising the oxidized purine from 8-oxoG:C base pairs. In a second pathway, the MYH DNA glycosylase removes adenine bases inappropriately inserted opposite 8-oxoG during replication. In the short term, this reduces 8-oxoG-mediated mutagenesis and it ultimately contributes to reducing the burden of DNA 8-oxoG. The particular importance of MYH-mediated BER is illustrated by its inactivation in some hereditary forms of human colorectal cancer (10).

Measurements of DNA 8-oxoG in mouse tissues generally support a central role for OGG1 and MYH in preventing a build-up of DNA 8-oxoG (11,12) and tumour formation (13). These studies also reveal alternative pathways that act independently to exclude, or remove 8-oxoG from DNA (14). Thus, genetic inactivation of OGG1 or MYH in knockout mice is associated with an increased steady-state level of DNA 8-oxoG in cultured embryonic fibroblasts (15,16) and in liver (12), a tissue with a relatively high oxidative metabolism and low cell turnover. DNA mismatch repair (MMR) is one of the alternative pathways for controlling DNA 8-oxoG levels, and the oxidized base accumulates extensively in the DNA of MMR-deficient human and mouse cells treated with oxidizing agents or low dose rate ionizing radiation (17–19).

MMR requires several dedicated proteins [reviewed in (20,21)]. Among these, the major mismatch recognition complex, MutSα, is a dimer of MSH2 and MSH6. It binds to mismatches and initiates their correction. The MLH1 protein together with PMS2 forms the MutLα heterodimer. This may

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stabilize the initial MutSα:mismatched DNA interaction and appears to act as an interface between mismatch recognition and subsequent excision. In addition to incorrectly paired bases, the unpaired mono or dinucleotides that arise by DNA strand slippage during replication of repetitive sequences are among the principal substrates for MutSα recognition and for MMR. Failure to repair these structural anomalies is reflected in the microsatellite instability and increased frequency of frameshifts that is characteristic of MMR-defective cells.

The involvement of MMR in modulating DNA 8-oxoG is rather enigmatic. Most MMR events occur in close proximity to replication forks, whereas the majority of DNA 8-oxoG is in non-replicating DNA. *Saccharomyces cerevisiae* MMR appears to compensate for the apparent absence of an MYH function and removes misincorporated A from 8-oxoG:A base pairs (22). Both the base pairs formed by the oxidized purine, 8-oxoG:C and 8-oxoG:A, are, however, poorly recognized by mammalian MutSα (23) and are inefficiently corrected by MMR in human cell extracts (24). Guanine has a low redox potential and the dGTP pool is a significant target for oxidation. We have previously adduced evidence that incorporation of 8-oxodGMP from the oxidized dNTP pool provides a source of DNA 8-oxoG that is subject to removal by MMR (18,19). Incorporation of 8-oxodGMP into DNA is normally minimized by a family of hydrolases that prevent the build-up of intracellular 8-oxodGTP (25–27). MTH1, the human homolog of the *Escherichia coli* MutT protein, is an important member of this family (25). Cells derived from *Mth* knockout mice selectively accumulate single base frameshifts in repetitive sequences, consistent with the possible involvement of 8-oxodGTP and incorporated 8-oxoG in frameshift formation (28). In agreement with this idea, overexpression of MTH1 in MMR-defective human and mouse cells significantly attenuated their mutator phenotype and microsatellite instability. Strikingly, among *HPRT* mutations, MTH1 overexpression had a dramatic effect on single base frameshifts in an acknowledged target sequence that comprises six consecutive G residues (19). The important contribution of Mth to genome stability is emphasized by the cancer proneness of $Mth^{-/-}$ mice (29).

Previous studies of the interactions between MMR and the oxidized purine utilized model substrates in which 8-oxoG base pairs were placed in non-repetitive DNA sequences. Base misincorporation, mismatch recognition and MMR are all influenced by DNA sequence context, and particularly by repeats (30–32). We, therefore, examined incorporation of 8-oxoG by DNA polymerases and its recognition by MutSα in a series of repetitive DNA sequences based on the target 6G repeat of *HPRT*. We report that two DNA polymerases incorporate 8-oxodGMP opposite C and A in 6C and 6A runs and extend the terminal 8-oxoG:C and 8-oxoG:A pairs efficiently. In contrast to the weak recognition of regular 8-oxoG:C and 8-oxoG:A base pairs, 8-oxoG in slipped/mispaired model frameshift intermediates can be recognized very effectively by MutSα. Recognition is, however, dependent on the nature of the slipped/mispaired intermediate. The findings suggest how DNA 8-oxoG might contribute to frameshift mutagenesis and microsatellite instability. They also provide a possible explanation for how MMR reduces the level of the oxidized base in DNA. However, the behaviour of 8-oxoG in repeat sequences is not entirely predictable and in some circumstances it can become invisible to DNA repair.

MATERIALS AND METHODS

Enzymes

Klenow fragment of *E.coli* DNA polymerase I (Kf^{exo-}) was from New England Biolabs, MA. Mammalian DNA polymerase α (pol α) was purified from HeLa cells as described for calf thymus (33). The preparation used in this study contained 32 U/ml. The human OGG1 DNA glycosylase was from Trevigen, MD.

Oligonucleotide synthesis and labelling

Oligonucleotides (Invitrogen Corporation, CA) were purified from denaturing polyacrylamide gels (PAGE) and 5′ phosphorylated using T4 polynucleotide kinase (Boehringer Mannheim, GmbH, W-Germany) and [γ-^{32}P]ATP (Perkin Elmer, Italy). 6-carboxyfluorescein (6-FAM) labelled oligonucleotides and 8-oxodG-terminated primers were synthesized by the Oligonucleotide Synthesis Laboratory, Cancer Research, UK and further purified by PAGE.

Primer extension reactions

8-oxodGTP was obtained from TriLink BioTechnologies CA. Analysis by high-performance liquid chromatography (HPLC) indicated that it contained <0.01% dGTP. In standard primer extension experiments, 5′ end-labelled primers were annealed to the template strands in a 1:1 molar ratio. For 8-oxodGMP incorporation and extension by Kf^{exo-}, ^{32}P-end labelled DNA substrates (30 nM) were incubated with the enzyme (2.5 nM) and increasing concentrations of 8-oxodGTP at 37°C in a buffer containing 20 mM Tris–HCl, pH 7.7, 2 mM $MgCl_2$ and 2 mM DTT. After 10 min, an equimolar dNTP mixture was added and incubated for 1 min. A second stage reaction measuring the extension of the 8-oxodG-terminated primer/template was performed by incubation with increasing concentrations of dNTPs at 37°C for 1 min.

6-FAM labelled DNA duplexes (50 nM) were initially preincubated with 4.8 U of pol α and non-radioactive competitor DNA in 25 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$ buffer for 1 min. Increasing concentrations of 8-oxodGTP were added to start the reaction. After 5 min at 37°C, dGTP was added for 2 min. Stop Solution (USB Corporation, USA) (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) was added, the products were denatured at 95°C for 5 min and separated on denaturing 20% polyacrylamide gels. Gels of radioactively labelled DNA samples were fixed in 10% acetic acid, dried and analysed by autoradiography. Densitometric analysis of the autoradiographs was performed using an ULTROSCAN XL laser densitometer (Pharmacia LKB Biotechnology AB, Sweden), and quantitation was performed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). Fluorescent bands were visualized using Typhoon 9200 Gel Imager (Amersham Biosciences Europe GmbH) and quantitated using ImageQuant TL software.

Kinetic analysis

Experiments with Kf^{exo-} and pol α were performed under the conditions described above using 0.01–1 μM dNTP and 0.5–100 μM dNTP, respectively. Data points were derived from densitometric analysis of the intensities of the products bands. To calculate the kinetic parameters for nucleotide incorporation, K_m and V_{max}, the values of integrated gel band intensities in dependence of the nucleotide substrate concentrations ([dNTP]) were fitted to the equation:

$$I^*_T/I_{T-1} = V_{max}[\text{dNTP}]/(K_m + [\text{dNTP}])$$

where T is the target site, the template position of interest; I^*_T is the sum of the integrated intensities at positions T, $T + 1, \ldots, T + n$.

Before being inserted in the above equation, the intensities of the single bands of interest were first normalized by dividing for the total intensity of the lane. This reduced the variability due to manual gel loading. An empty portion of the gel was scanned and the resulting value was subtracted as background. The goodness of fit of the interpolated curve was assessed by computer-aided calculation of the sum of squares of errors SSE and the correlation coefficient R^2. Interpolation, SSE, R^2 and standard errors determination were performed using the computer program GraphPadPrism.

MutSα purification and bandshift

MutSα was prepared from ~2×10^{10} Raji cells as described previously (32). The final concentrated Q Sepharose pool contained ~5 pmol MutSα per ml. For bandshift assays, 1 U of MutSα was defined as 5 fmol.

Bandshift experiments were carried out with ^{32}P-end labelled oligonucleotide duplexes as described previously. Briefly, MutSα (2–10 U) was pre-incubated (5 min at 20°C) with 2 pmol of non-radioactive matched competitor duplex in 20 μl reaction buffer containing 25 mM HEPES–KOH, pH 8.0, 0.5 mM EDTA, 0.1 mM $ZnCl_2$, 10% glycerol and 50 μg poly(dI:dC). An aliquot of 20 fmol substrate duplex was added and incubation continued for a further 20 min. Products were analysed by PAGE on 6% non-denaturing gels.

OGG1 cleavage

^{32}P-end labelled duplexes (1 pmol) were incubated with OGG1 in 10 μl of 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 100 μg/ml BSA. After 60 min at 37°C, reactions were stopped by the addition of 5 μl 95% formamide, 20 mM EDTA, 0.05% xylene cyanol and heating at 95°C for 5 min. Ten microlitre aliquots were applied to denaturing gels (15% polyacrylamide/7 M urea). Reaction products were separated by electrophoresis, detected by autoradiography on Kodak X-Omat film and quantitated by the Bio-Rad gel doc system.

OGG1 bandshift

^{32}P-end labelled duplexes (0.3 pmol) were incubated with OGG1 for 30 min at 20°C. Reaction mixes (20 μl) contained 20 mM HEPES–KOH, pH 7.9, 100 mM KCl and 0.2 mM EDTA. Following addition of 2 μl loading buffer (0.05% bromophenol blue and 50% glycerol), samples (10 μl) were electrophoresed on non-denaturing 8% polyacrylamide gels in TBE buffer for 3.5 h at 4°C. Reaction products were detected by autoradiography on Kodak X-Omat film and quantitated by the Bio-Rad gel doc system.

RESULTS

Incorporation and extension of 8-oxodGMP—formation of 8-oxoG:C

The ability of the Kf^{exo-} to use 8-oxodGTP and incorporate 8-oxodGMP into templates based on the *HPRT* frameshift target sequence was investigated using primers that terminated at different positions within the C6 run. The 36mer template comprised the C6 sequence and 15 nt of flanking sequence from both 5′ and 3′ sides. This was annealed to different primers and used to direct incorporation of 8-oxodGMP at the beginning, middle or end of the C6 repeat (Figure 1A–C). Two-stage reactions were performed. In the first step, primer/templates were incubated with Kf^{exo-} and increasing concentrations of 8-oxodGTP. After 10 min, normal dNTPs (1.2 μM) were added and incubation continued for 1 min. During the first stage incubation, there was a concentration-dependent extension of all three primers by a single 8-oxodGMP residue (Figure 1A–C) with no detectable incorporation of a second. The electrophoretic mobility of primers extended by 8-oxodGMP was detectably lower than those with an incorporated dGMP (compare lanes 4, 6 and 8 with lane 10 in Figure 1A and B). This provided an indirect confirmation that the oxidized purine had been incorporated. The efficiency of 8-oxodGMP insertion was not significantly different among the three positions (Figure 1A–C). At 0.1 μM 8-oxodGTP, incorporation was just detectable (≤5% elongation) and at 3.8 μM, more than 70% of the primers had been elongated. As expected, addition of 8-oxodGMP was considerably (at least 100-fold) less efficient than dGMP. Even sub-optimal dGTP concentrations as low as 0.04 μM supported significant (>80%) extension of all three primers (Figure 1A–C).

The second stage reaction monitored the efficiency of elongation from the 3′ terminal 8-oxoG:C base pair. There was no indication that 8-oxodG-terminated primers were difficult to extend. In all cases, following addition of dNTPs, all substrates, including those with a terminal 8-oxoG:C, were converted to full-length 36mers in ≤1 min. Efficient elongation was independent of the position of the 8-oxoG:C pair within the run (Figure 1A–C). Extension from the terminal 8-oxoG involved insertion of a correct G and only dGTP supported further elongation when added as a single dNTP (data not shown). The ability of Kf^{exo-} to extend a terminal 8-oxoG:C pair was confirmed using synthetic primers with a 3′ terminal 8-oxoG. Elongation of these terminal 8-oxoG:C pairs–terminated primers was not significantly less efficient than extension from a terminal G:C pair. The percentage of elongated primer was similar in each case (Figure 1D) and was again unaffected by the position within the 6G run (data not shown).

We also investigated the ability of a mammalian DNA polymerase, pol α, to incorporate 8-oxodGMP in the same sequence context (Figure 2). The template with the 15mer primer terminating immediately 5′ of the C6 repeat was incubated with pol α and 8-oxodGTP (3–100 μM). In a second phase reaction, normal dGTP (10 μM) was added after 5 min,

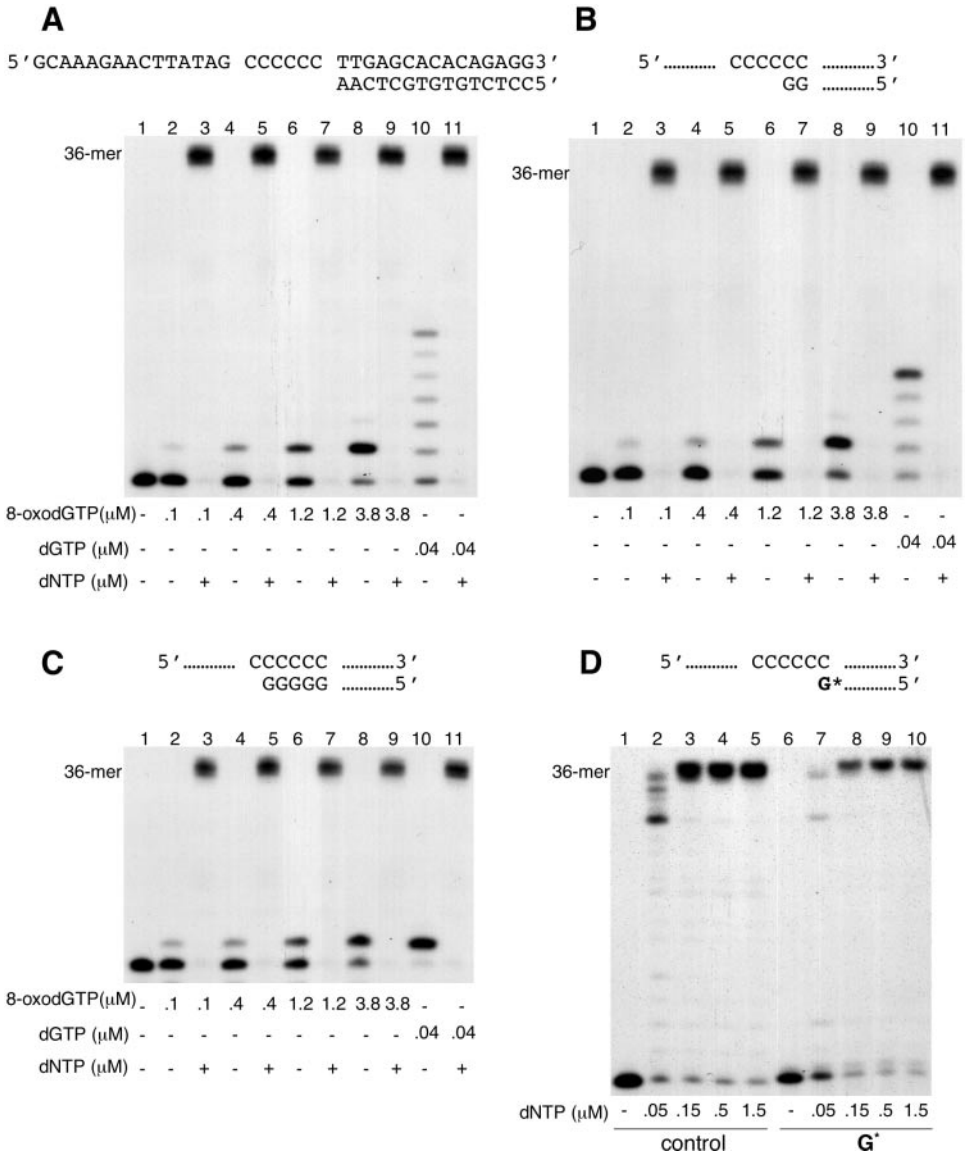

Figure 1. Primer extension: incorporation and extension of 8-oxodGMP by Kf^{exo-} DNA polymerase. Incorporation of 8-oxoG at the beginning (**A**), middle (**B**) and the end (**C**) of the C6 run of the 36mer primer/templates of the indicated structures is shown. First stage incorporation reactions contained 30 nM primer/template and 2.5 nM Kf^{exo-} supplemented with increasing concentrations of 8-oxodGTP (0.1–3.8 μM) (lanes 2, 4, 6 and 8) or dGTP (0.04 μM) (lane 10). Controls (lane 1) were incubated without enzyme or dNTP. Subsequent second stage elongation reactions (lanes 3, 5, 7, 9 and 11) were initiated by the addition of an equimolar mixture of normal dNTPs (1.2 μM). (**D**) Elongation of a terminal 8-oxoG:C (lanes 6–10) or G:C (lanes 1–5) base pair by Kf^{exo-} (0.5 nM) in the presence of increasing concentrations of dNTPs (0.05–1.5 μM). In this substrate, the primer contained a synthetic terminal 8-oxoG.

and incubation continued for further 2 min. Figure 2 shows the concentration-dependent 8-oxodGMP incorporation by pol α. As we observed with Kf^{exo-}, the terminal 8-oxoG:C base pair was no impediment to elongation and 8-oxoG-terminated primers were elongated as efficiently by pol α as those with a terminal G, albeit with somewhat lower processivity. Interestingly, at high (30–100 μM) 8-oxodGTP concentrations, pol α was even capable of partially extending the terminal 8-oxoG:C base pair by addition of further 8-oxodGMP residues.

We conclude that Kf^{exo-} inserts 8-oxodGMP at a similar frequency at three different positions of the G repeat. Although the use of 8-oxodGTP is inefficient compared with normal dGTP, once inserted a terminal 8-oxoG:C base pair in each position is extended by Kf^{exo-} accurately and without significant impediment. DNA pol α can also incorporate 8-oxodGMP in this sequence and efficiently extend terminal 8-oxoG:C base pairs. Unlike Kf^{exo-}, DNA pol α has the surprising ability to catalyse the formation of successive 8-oxoG:C base pairs.

8-OxoG:C base pairs and recognition by MutSα

To investigate whether a G repeat might influence 8-oxoG:C recognition by MMR, we analysed MutSα binding to duplexes containing an 8-oxoG:C pair in the 6G sequence or with a single additional base in either the 8-oxoG strand or the complementary strand. These substrates were generated by

annealing oligos with a single 8-oxoG at position 1, 3 or 6 of the central 6G tract to complementary strands containing 5, 6 or 7 Cs (Figure 3A). It is well established that 8-oxoG:C base pairs in non-repeat sequences are recognized inefficiently by MutSα (23,24). This also proved to be the case for this repeated sequence, and the 6C:6G*[1], 6C:6G*[3] and 6C:6G*[6] duplexes (G* indicates 8-oxoG, the suffix denotes its position relative to the 5′ end of the 6G repeat) with an 8-oxoG:C pair at the beginning, middle or end of the 6G run, were all poor substrates (Figure 3B). In addition, none of the 5C:6G* duplexes in which the extra base was in the G repeat was detectably bound by MutSα. As expected, the analogous 5C:6G duplex without 8-oxoG was recognized efficiently (Figure 3B and C). MutSα did, however, recognize 8-oxoG in the context of a single base loop when the C-containing strand contained an additional repeat member. Binding to each of the 7C:6G* duplexes was efficient, comparable with a 7C:6G control, and independent of the position of the 8-oxoG at the 3′, 5′ end, or in the centre of the repeat (Figure 3C).

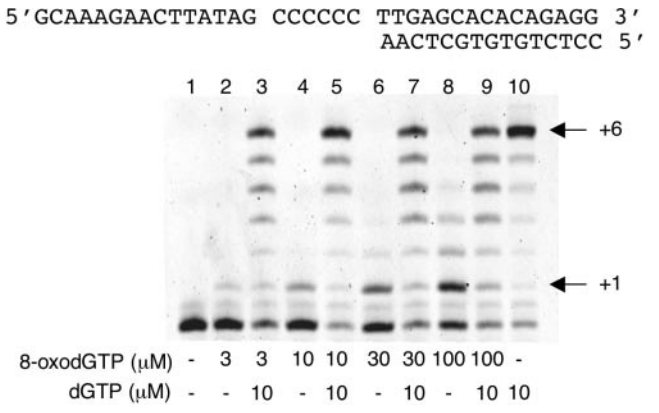

Figure 2. DNA polymerase α: incorporation of 8-oxodGMP opposite C at the beginning of the C6 run and extension. Primer/templates (50 nM) were pre-incubated with pol α. After 1min, reactions were initiated by the addition of 8-oxodGTP (3–100 μM) (lanes 2, 4, 6 and 8) or 10 μM dGTP (lane 10). Second stage elongation reactions (lanes 3, 5, 7 and 9) were initiated by the subsequent addition of dGTP (10 μM).

These binding studies with MutSα indicate that the likelihood that an 8-oxoG:C base pair will trigger MMR is not improved when it lies within a G repeat. More importantly, the efficient binding to 7C:6G* substrates containing 8-oxoG opposite a strand with a supernumerary base provides the first indication that an incorporated or template 8-oxoG might, under certain circumstances, provoke MMR in human cells. The intriguing observation that MutSα binding is inefficient when the 8-oxoG-containing G repeat contains an extra base

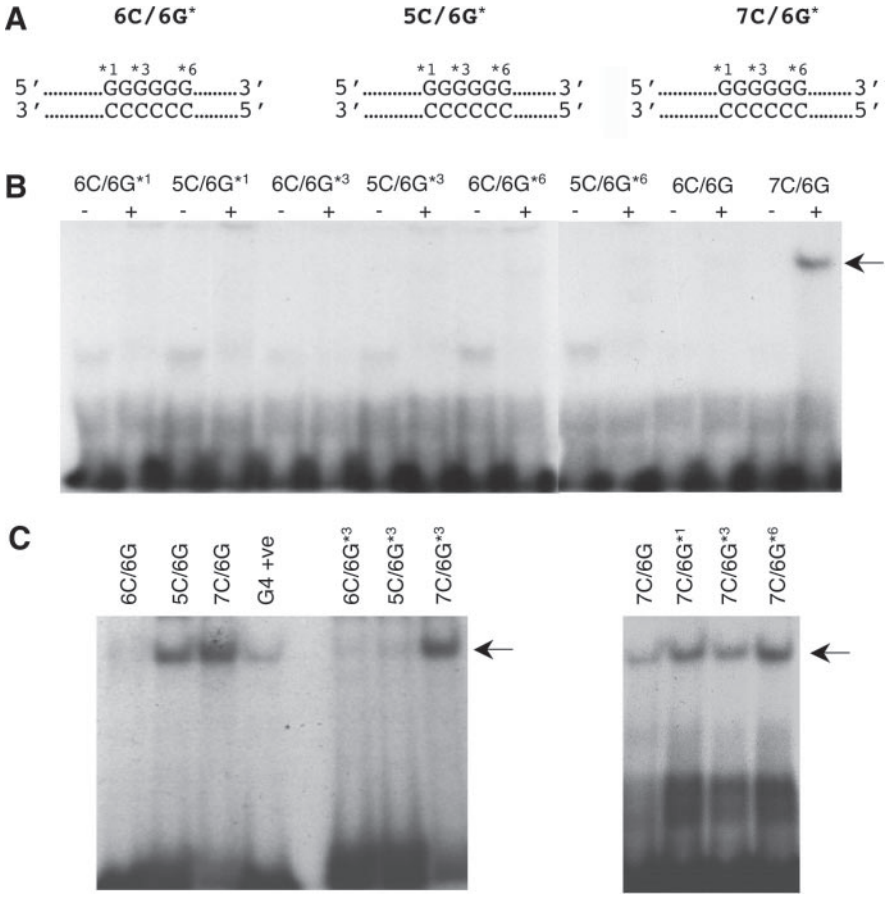

Figure 3. MutSα binding to 8-oxoG/C duplexes. (**A**) Sequences of repeat regions in substrate duplexes. (**B**) Bandshift of C5 and C6 duplexes. The 8-oxoG duplexes shown and a 7C:6G control duplex were incubated with (+) or without (−) 1 U (1 μl) purified MutSα. After 20 min at 20°C, products were analysed by non-denaturing PAGE. The position of the MutSα:DNA complex is arrowed. (**C**) Bandshift of C7 duplexes. 8-OxoG:5C, 6C or 7C duplexes and control 6G:5C, 6C or 7C duplexes as indicated were incubated with 1 U purified MutSα and products analysed as above. G4+ve is a 4G:3C substrate of unrelated structure (32).

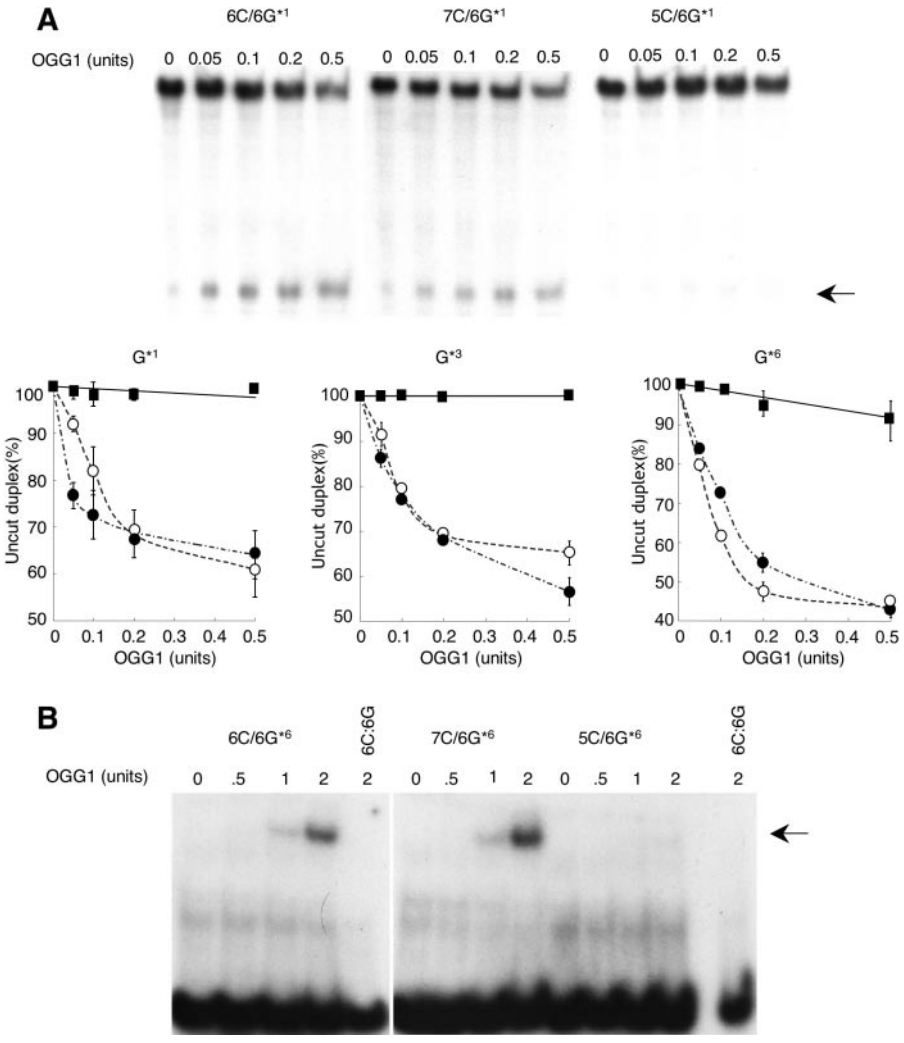

Figure 4. OGG1 as a probe for 8-oxoG conformation. (**A**) Cleavage. A 5′ ^{32}P-end labelled 8-oxoG:6G*^{1} oligonucleotide in which the 8-oxoG is at the 3′ end of the 6G run was annealed to complementary strands containing the indicated number of Cs. Duplex molecules were incubated with purified OGG1. After 60 min at 37°C, cleavage products were analysed by denaturing PAGE and autoradiography. Cleavage products are shown arrowed. The extent of OGG1 cleavage of the 8-oxoG duplexes indicated was quantified by the Biorad GelDoc EQ system. Data represent mean of at least two separate experiments; error bars indicate the range of values. G*:C5 (closed square); G*:C6 (open circle); G*:C7 (closed circle). (**B**) Bandshift. The 8-oxoG-containing duplexes indicated were incubated with OGG1 as shown for 30 min at 20°C and products analysed by non-denaturing PAGE. The arrow indicates the position of the OGG1:DNA complex.

also has significant implications. It suggests that some slipped/mispaired substrates of this nature are likely to escape correction by MMR.

Incision and binding by OGG1

The OGG1 DNA glycosylase removes 8-oxoG from 8-oxoG:C pairs and incises the DNA duplex at the resulting apurinic site. To do this, the enzyme recognizes structural features of both partners of its substrate base pair. OGG1-mediated cleavage of duplex substrates, therefore, provides a probe for the interactions of 8-oxoG with a complementary C.

6C:6G*, 5C:6G* and 7C:6G* duplexes end-labelled in the 8-oxoG-containing strand were incubated with a range of OGG1 concentrations, and the incision products were quantified. Figure 4A shows results for the 6G*^{1} set of substrates in which the 8-oxoG is at the 3′ end of the run. Cleavage of 5C:6G*^{1} was reproducibly much less efficient compared with 6C:6G*^{1} and 7C:6G*^{1} which were incised to similar extents. Under conditions that produced ~50% cleavage of 6C:6G*^{1} and 7C:6G*^{1}, there was no significant incision of 5C:6G*^{1} (Figure 4A). A similar cleavage of the 6C and 7C substrates was evident with 6G*^{3} and 6G*^{6} (Figure 4A). In each case, the 8-oxoG:5C duplex was refractory to OGG1, whereas the 6C and 7C duplexes were incised efficiently. Some dependency on the position of the 8-oxoG within the 6G repeat was noted and OGG1 incision was reproducibly more efficient with 6C:6G*^{6} and 7C:6G*^{6} than with the corresponding 6G*^{3} and 6G*^{1} duplexes. However, for each set of substrates, the most striking difference was the inefficient cleavage of 5C:6G*. The anomalous behaviour of the 5C duplexes with respect to OGG1 incision suggests that 8-oxoG cannot form its usual base pairs with C to a significant degree. This problem is not apparent, however, when the complementary strand contains an equal number or one additional C.

An 8-oxoG:C pair stabilizes binding of OGG1 to duplex DNA. This interaction can be observed experimentally by bandshift using OGG1 as a probe. When 8-oxoG duplexes were incubated with increasing amounts of OGG1, an OGG1–DNA complex was formed. An example is shown in Figure 4B. OGG1 bound efficiently to the 6C:6G* and 7C:6G* duplexes, whereas binding to 5C:6G* substrates was barely detectable. The same pattern of poor recognition of the 5C:6G* substrates was repeated with the duplexes containing 8-oxoG at the 1 and 3 positions in the repeat (data not shown). Thus, OGG1 binding to 8-oxoG DNA duplexes mirrored OGG1 cleavage and provided confirmation that the behaviour of 8-oxoG in 5C:6G* substrates is anomalous and inconsistent with the formation of an 8-oxoG:C base pair. When 8-oxoG is present as an extra purine in a G repeat, it is invisible to OGG1 and MutSα.

Incorporation of 8-oxodGMP—formation of 8-oxoG:A

In addition to C, 8-oxoG can base pair with A. Like 8-oxoG:C, 8-oxoG:A pairs are recognized poorly by MutSα (23,24). We, therefore, investigated the efficiency of 8-oxoG incorporation opposite A in a repetitive sequence and examined whether the replication product might trigger mismatch recognition. We tested the ability of Kf^{exo-} and pol α to incorporate 8-oxodGMP opposite a 6A repeat of a 36mer template (Figure 5A and B). Since the incorporation of 8-oxodGMP opposite C was largely independent of the position of C in the run, formation of 8-oxoG:A mismatch was only examined at a single position, the beginning of the A run. Two-stage incorporation/extension experiments were carried out as previously described for the 6C template and the results are shown in Figure 5. Incorporation of 8-oxoG opposite A by Kf^{exo-} was efficient and occurred to a measurable extent even at the lowest 8-oxodGTP concentration (0.01 μM). At the highest 8-oxodGTP concentration, the infrequent addition of a second 8-oxodGMP was again observed. As expected, there was minimal use of the incorrect dGTP opposite the template A and even at the highest concentration (0.4 μM) there was no detectable incorporation. Elongation from the terminal 8-oxoG:A mismatch by Kf^{exo-} was also highly efficient. Compared with incorporation opposite C (Figure 1A), Kf^{exo-} exhibits an approximately 40-fold preference for incorporation of 8-oxoG opposite A. Both the preferred 8-oxoG:A and the less favoured 8-oxoG:C base pairs are nevertheless easily elongated by this DNA polymerase.

Human pol α also incorporated 8-oxoG opposite A. At a triphosphate concentration of 10 μM, addition of 8-oxoG occurred significantly more frequently than the control G (Figure 5B). As with Kf^{exo-}, further extension of the 8-oxoG:A pair by pol α was efficient, although less processive, and there was a similar extent of dTMP addition to the terminal 8-oxoG and to the remaining unmodified primer. Table 1 shows the kinetic parameters for the incorporation of 8-oxoG and T opposite A by Kf^{exo-} and pol α. The selectivity indices for dTTP over 8-oxodGTP were 67 and 83 for Kf^{exo-} and pol α, respectively, corresponding to misincorporation frequencies greater than 10^{-2} for both enzymes. Steady-state kinetic analysis showed that the overall frequency of misincorporation by pol α with normal dNTPs under similar *in vitro* conditions is of the order of 10^{-3} (34). Thus, although

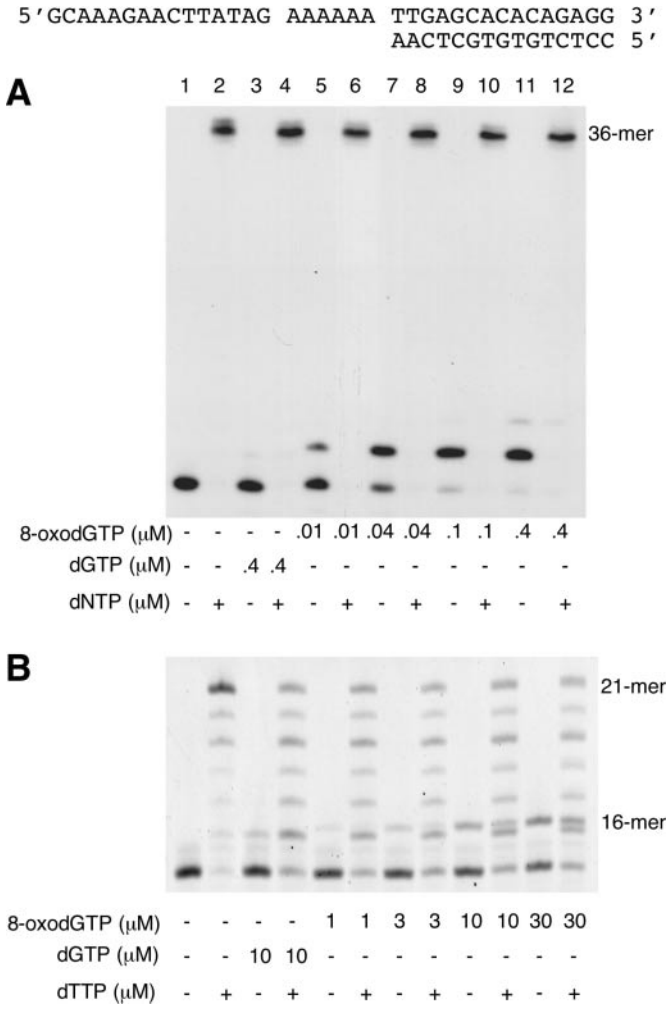

Figure 5. 8-oxodGMP incorporation into a 6A repeat and extension of 8-oxoG:A mismatch. The sequence of the template/primer was as indicated. Experimental conditions for first and second stage reactions were as described in the legends to Figures 1 and 2. (**A**) Kf^{exo-} DNA polymerase. (**B**) pol α DNA polymerase.

the incorporation of the oxidized base is relatively inefficient, the misincorporation frequency of 8-oxodGMP opposite a template A is 10 times higher than for a normal nucleotide.

These data indicate that members of two different DNA polymerase families, bacterial Kf^{exo-} (an A family polymerase) and human DNA pol α (B family) can incorporate 8-oxoG into A repeats. Although incorporation of the oxidized purine by either polymerase is somewhat less efficient than incorporation of a correct T, once it has been incorporated a terminal 8-oxoG does not significantly impede further extension of the growing DNA chain.

8-OxoG:A base pairs and recognition by MutSα

We examined whether an 8-oxoG:A base pair in a repetitive sequence had the same impact as 8-oxoG:C on recognition by MutSα. Since MutSα binding to 8-oxoG:C was affected dramatically by the number of bases in the complementary strand rather than the position of 8-oxoG, we limited the investigation to 8-oxoG at one position. In the generic duplex, 6T replaced

Table 1. Kinetic parameters for 8-oxodGTP or dTTP incorporation opposite A by Kf^{-exo} and DNA polymerase α

	K_m[a] (μM)	V_{max} (pmol × min^{-1})	V_{max}/K_m (pmol × min^{-1} × μM^{-1})	f_{inc}[b]	Selectivity index[c]
DNA polymerase Kf^{exo-}					
8-OxodGTP	0.08 (±0.01)	0.05 (±0.01)	0.6		
dTTP	0.03 (±0.01)	1.5 (±0.1)	50	1.2×10^{-2}	83.3
DNA polymerase α					
8-OxodGTP	8 (±1)	0.06 (±0.01)	7.5×10^{-3}		
dTTP	1 (±0.3)	0.5 (±0.08)	0.5	1.5×10^{-2}	66.7

[a]K_m and V_{max} values were determined as described in Materials and Methods. Numbers in brackets represent ±SD.
[b]8-OxodGTP incorporation frequency (f_{inc}) has been calculated as $(V_{max}/K_m)_{8\text{-oxodGTP}}/(V_{max}/K_m)_{dTTP}$.
[c]Selectivity index is defined as $1/f_{inc}$.

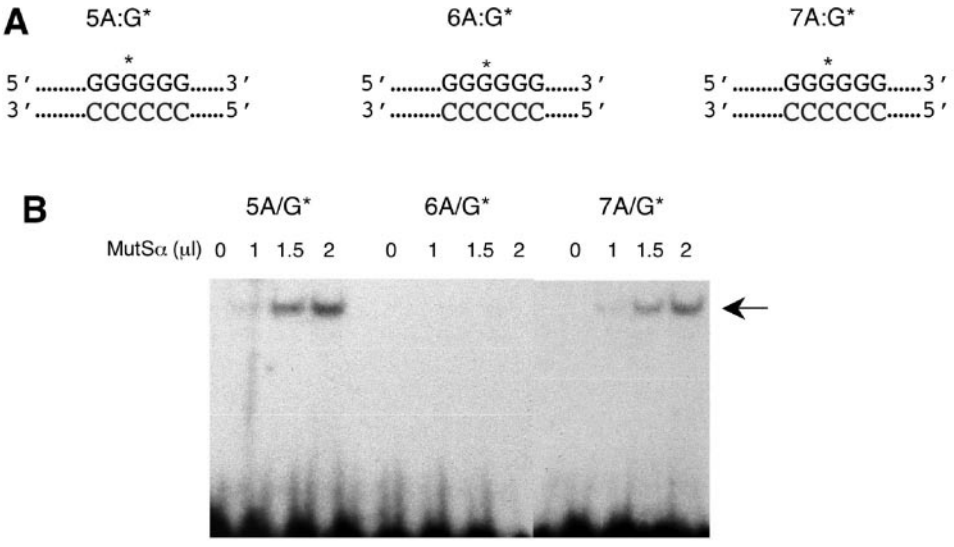

Figure 6. MutSα binding to 8-oxoG/A duplexes. (**A**) Sequences of repeated region. (**B**) Bandshift of 8-oxoG:5A, 6A and 7A duplexes. The indicated 8-oxoG repeat duplexes and substrates containing an A:8-oxoG or C:8-oxoG in a non-repeat sequence were incubated with MutSα as shown. Products were analysed by non-denaturing PAGE as for Figure 3.

the 6G repeat with 8-oxoG in place of T[3] (Figure 6A). Complementary strands contained 5A, 6A or 7A. Sequences flanking the repeats were the same as those shown in Figure 1A.

MutSα binds poorly to 8-oxoG:A in non-repetitive sequences (23,24). This was confirmed and binding to both A/G* in a random sequence was almost undetectable and was comparable with C/G* binding (data not shown). Recognition of the 8-oxoG:A pair was not significantly improved when it was placed in a repeat (Figure 6B, 6A/G*). However, MutSα binding to substrates that contained an extra base in the 8-oxoG strand or in the complementary strand was extensive, and recognition of the 5A/G* and 7A/G* duplexes was comparable with that for a 7C/6G* duplex (Figures 6B and 3C).

In summary, MutSα recognition of an 8-oxoG:A base pair is poor and is not improved by placing the mismatch in a repetitive sequence. In this, it resembles the 8-oxoG:C base pair which is also poorly bound in both repeated and random sequences. However, in the context of a putative unpaired base, 8-oxoG is efficiently recognized in T:A repeats. Unlike 8-oxoG in the G repeat, putative slipped/mispaired frameshift intermediates in the A repeat are recognized efficiently when the extra base is in either the 8-oxoG-containing or the complementary strand. 8-oxoG:A base pairs can only be formed during replication and 8-oxoG is implicated in instability of A:T microsatellites in an MMR-deficient background. The facile recognition of 8-oxoG-containing slipped/mispaired intermediates in repetitive A:T tracts suggests how MMR might counteract the effects of oxidation-induced mutation in A:T repeat microsatellites.

DISCUSSION

The experiments we have described were designed to investigate the relationship between MMR and DNA 8-oxoG, in particular the involvement of the oxidized purine in frameshift mutagenesis. Our findings reveal previously unrecognized properties of DNA 8-oxoG that have a bearing on this problem. Two different and unrelated DNA polymerases were able to use 8-oxodGTP and to incorporate the oxidized base opposite C and A in the repetitive DNA sequences that are associated with replication slippage and frameshifts. Incorporation was invariably followed by efficient elongation. MutSα recognized 8-oxoG in the context of a model slipped/mispaired intermediate in runs of either G or T. This provides the first evidence that human MMR might occur at 8-oxoG residues in a biologically relevant DNA structure. Previously, only 8-oxoG:T and 8-oxoG:G mispairs had been identified as effective substrates for MutSα recognition (23,24). Our

observations are compatible with a contribution of incorporated 8-oxoG to frameshifts and indicate how MMR might influence the steady-state level of DNA 8-oxoG and how oxidative stress might influence microsatellite stability. The observed inability of MutSα and OGG1 to recognize some slipped/mispaired structures involving the oxidized base is also significant and suggests that 8-oxoG can adopt configurations that render it undetectable by DNA repair.

8-OxodGTP is a substrate for several DNA polymerases which exhibit the same preferential formation of C:8-oxoG and A:8-oxoG base pairs (35–39). The ability of 8-oxodGTP to assume a *syn* conformation and the shape of the polymerase active site have been proposed as the main determinants of miscoding (40,41). Some Y-family DNA polymerases and polymerase β preferentially incorporate 8-oxoG opposite A, whereas replicative polymerases do not show this preference (39,42). The prevailing mutations in plasmids replicated *in vitro* in the presence of 8-oxodGTP are the AT→CG transversions expected from 8-oxoG incorporation opposite A and a low frequency of GT→TA transversions consistent with incorporation opposite C and miscoding during subsequent replication (43–45). Our previous findings implicated oxidized purines in frameshift mutation in MMR-defective cells. Here, we show that at the level of dNTP selection, there appears to be no insurmountable obstacle to a low level incorporation of the oxidized base into the repeated sequences that comprise frameshift targets. Representatives of the A family (Klenow fragment) and B family (DNA pol α) DNA polymerases both incorporated 8-oxoG throughout A or C homo-polymeric runs and both were capable of efficiently extending a terminal 8-oxoG base paired with either A or C.

The preferential base pairing of 8-oxoG was considered in designing substrates for MutSα binding. We confirmed that MutSα efficiently recognized duplex substrates based on a 6G:6C sequence and designed to resemble slipped/mispaired frameshift intermediates. However, when an 8-oxoG was introduced, recognition by MutSα was highly selective (Figure 7A). For 8-oxoG/C combinations, duplexes with a supernumerary C opposite the 8-oxoG were good substrates for binding by MutSα, consistent with efficient recognition of an unpaired C. However, when the extra base was in the 8-oxoG/G repeat, recognition was poor. This behaviour contrasted with 8-oxoG in a T repeat. One more base in either the 8-oxoG-containing strand or in the complementary A strand produced a good substrate for MutSα binding (Figure 7A summarizes these data).

MutSα recognition of 8-oxoG-containing duplexes with an unpaired C or T within a repeat defines a class of oxidized DNA substrates for MMR. The consequences of MMR in this type of sequence would be 2-fold: it would help prevent frameshifts and, depending on the orientation of the slippage event, it would remove 8-oxoG from DNA. This is consistent with our previous observations implicating MMR in reducing frameshifts generated from incorporated 8-oxoG (19). Inefficient

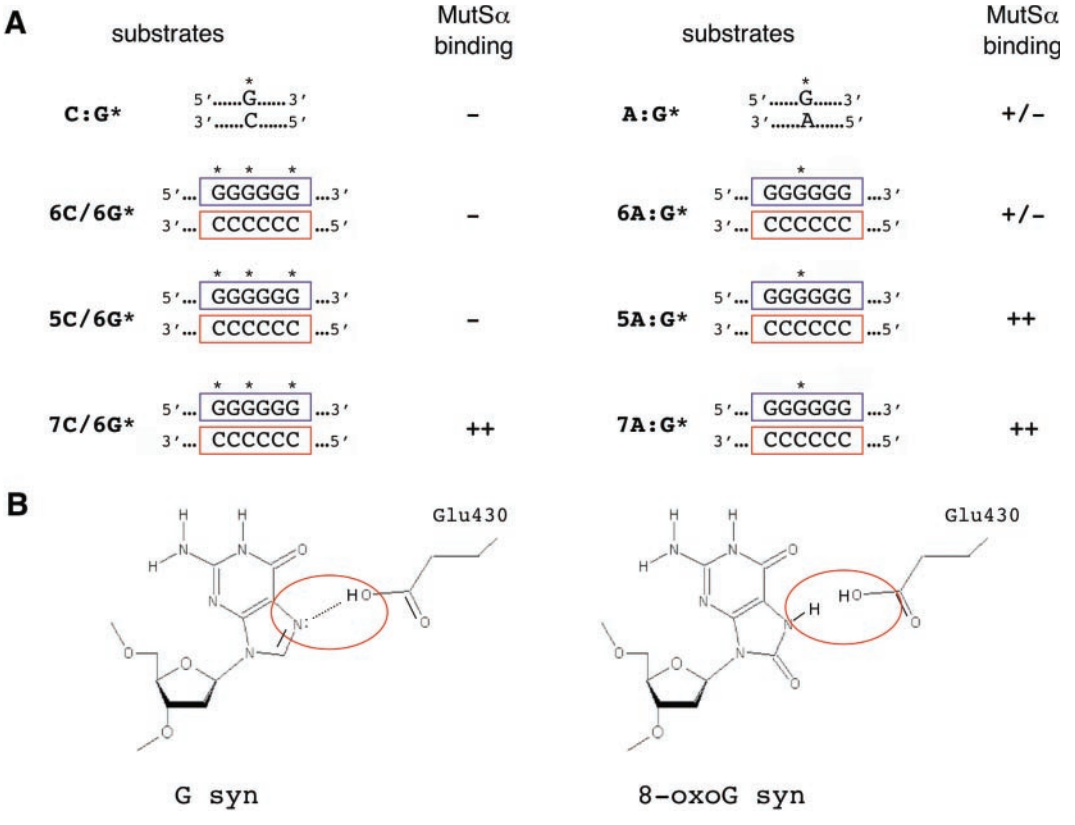

Figure 7. Summary of MutSα recognition of 8-oxoG-containing substrates. (**A**) Summary of the MutSα binding to various 8-oxoG:C and 8-oxoG:A-containing substrates (G* = 8-oxoG). (**B**) Possible interactions between MutSα and an unpaired G (left) or 8-oxoG (right) in the *syn* conformation. The G_{syn} conformation and H bonding to Glu430 in hMSH6 are taken from ref. (54,55). Formation of the Glu430 H-bond may be compromised by the protonated N7 of 8-oxoG (right panel).

MutSα binding to other slipped/unpaired substrates also has implications for oxidation-related frameshifts. Intermediates of this type would most likely escape correction, and a class of frameshifts would be independent of MMR status. Our data, therefore, suggest that MMR might introduce a bias in the formation of frameshifts resulting from incorporated 8-oxoG. This, together with other acknowledged sources of bias, such as the more efficient repair of mismatches during lagging strand replication (46,47), might contribute to the overrepresentation of −1 frameshifts related to oxidation in MMR-deficient cells (19).

How do 8-oxoG in 6G*/5C duplexes escape recognition by MutSα? If each purine were equivalent, a G would be unpaired five times out of six. Binding would be efficient because unpaired Gs in repeats (e.g. the 6G:5C and G4 control duplexes we used) are excellent substrates. The most likely explanation that reconciles the data from MutSα binding and OGG1 binding and incision is that the purines in the G run are not equivalent and that 8-oxoG is preferentially unpaired. OGG1 scans the helix and, possible alerted to the presence of its substrate by an altered torsional rigidity in the DNA (48,49), extrudes the oxidized base from the helix and accommodates it deep within the active site (50,51). An important feature of this mechanism is the contacts OGG1 makes with the orphaned complementary C (50). These contacts are essential for enzymatic activity and recognition of DNA with 8-oxoG:A and 8-oxoG:G pairs (52), or in which the lesion is opposite an abasic site is very inefficient (49). The preferential unpairing of 8-oxoG may have a thermodynamic basis. G:C base pairs are more stable than 8-oxoG:C base pairs and their formation would be favoured (48,53). Consistent with this possibility, the thermal denaturation profiles of the 6G/5C and 6G*/5C duplexes are superimposable (F. Mazzei, unpublished data) suggesting the formation of an equivalent number of G:C hydrogen bonds, together with an unpaired 8-oxoG in the 6G*/5C duplex. In the T repeat, the behaviour of 8-oxoG is likely to be different. In its adopted *syn* conformation, 8-oxoG forms a stable Hoogsteen pair with A. The changes of free energy associated with the formation of 8-oxoG:A and 8-oxoG:C base pairs suggest that T:A pairs are thermodynamically less stable than 8-oxoG:A (47). This would tend to favour the formation of duplexes with an unpaired T or an unpaired A both of which would be conventional substrates for MutSα and MMR.

Obviously, MutSα recognition of slipped/mispaired intermediates cannot require interaction with a complementary base. Recognition of different base:base mispairs and single unpaired bases by bacterial MutS mismatch binding proteins (and MutSα) may be accomplished by scanning DNA for flexible joints or deformable sites rather than for specific structures. Crystal structures (54,55) and atomic force microscopy (56) of MutS bound to a mismatch indicate that DNA bending is an important factor. Mismatch recognition involves extensive minor groove interactions causing kinking and widening of the minor groove relative to the major groove. The overall structural and dynamic properties of the 6G/5C and 6G*/5C duplexes are closely similar. Fluorescence polarization anisotropy measurements show that the 6G/5C and 6G*/5C duplexes have a similar torsional stiffness (F. Mazzei, unpublished data) and indicate that the extra-helical purine is stacked into the helix in both cases, in agreement with NMR studies of a DNA duplex containing an unpaired G (57). Since both the normal and the oxidized purine are likely to be intrahelical and the respective duplexes have similar flexibility, these factors cannot explain the inability of MutSα to recognize 6G*/5C. Although there are no structural studies of duplexes with an unpaired 8-oxoG, the *syn* conformation is known to be favoured because of steric hindrance between the base and the deoxyribose. Structural studies indicate that a mispaired G in the *syn* conformation makes important contacts with internal amino acids of MutS proteins (54,55). This appears to be a common strategy, most likely conserved in human MutSα, to stabilize binding to mispaired and unpaired bases (Figure 7B). Guanine in a G:G or G:T mispair is involved in stacking interactions with conserved phenylalanine (Phe36 in *E.coli* MutS, Phe337 in *S.cerevisiae* MSH6, Phe432 in human MSH6). An important hydrogen bond is formed between the guanine N7 and an adjacent glutamic acid (Glu38, Glu339 and Glu430 in bacteria, yeast and human, respectively) (54,55). These residues are major determinants of efficient mismatch recognition and repair (58,59). Protonation of N7 in a *syn* 8-oxoG may impede the formation of the crucial glutamic acid H-bond (Figure 7B). Ultimately, all these interactions appear important in kinking the DNA towards the major groove. An inappropriate conformation, the presence of the 8-hydroxy group and resulting N7 protonation could all impose steric constraints on the DNA bending essential for stable MutSα interaction.

In summary, two different DNA polymerases can use 8-oxodGTP to incorporate the oxidized base into repetitive DNA regions. Extension of a terminal 8-oxoG-containing base pair in these sequences is efficient. A subsequent slippage event in the same repeat can generate structures that are good substrates for recognition by MutSα and are therefore likely to provoke MMR. In certain cases, this would result in removal of DNA 8-oxoG. Other 8-oxoG-containing slipped/mispaired intermediates would, however, escape correction, possibly because, unlike a normal DNA base, a preferentially unpaired 8-oxoG is in an inappropriate conformation or is unable to participate in the stabilizing interactions with MSH6 amino acids. The presence of the oxidized purine in these slippage intermediates would, therefore, render them invisible to MMR thereby increasing the probability of a frameshift.

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