Influence of oxidized purine processing on strand directionality of mismatch repair

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Abstract: Replicative DNA polymerases are high-fidelity enzymes that misincorporate nucleotides into nascent DNA with a frequency lower than 1/10^5, and this precision is improved to about 1/10^7 by their proofreading activity. Because this fidelity is insufficient to replicate most genomes without error, nature evolved postreplicative mismatch repair (MMR), which improves the fidelity of DNA replication by up to three orders of magnitude through correcting biosynthetic errors that escaped proofreading. MMR must be able to recognize non-Watson-Crick base pairs and excise the misincorporated nucleotides from the nascent DNA strand, which carries - by definition - the erroneous genetic information. In eukaryotes, MMR is believed to be directed to the nascent strand by pre-existing discontinuities such as gaps between Okazaki fragments in the lagging strand, or breaks in the leading strand generated by the mismatch-activated endonuclease of the MutL homologs PMS1 in yeast or PMS2 in vertebrates. We recently demonstrated that the eukaryotic MMR machinery can make use also of strand breaks arising during excision of uracils or ribonucleotides from DNA. We now show that intermediates of MYH-dependent excision of adenines mispaired with 8-oxoguanine (GO) also act as MMR initiation sites in extracts of human cells or Xenopus laevis eggs. Unexpectedly, GO/C pairs were not processed in these extracts and failed to affect MMR directionality, but extracts supplemented with exogenous OGG1 did so. Because OGG1-mediated excision of GO might misdirect MMR to the template strand, our findings suggest that OGG1 activity might be inhibited during MMR.

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Influence of Oxidized Purine Processing on Strand Directionality of Mismatch Repair*

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Background: We studied the interplay between base excision repair (BER) of 8-oxoguanine (G^O) and mismatch repair (MMR).

Results: BER and MMR interact during the processing of G^O/A but not G^O/C mispairs.

Conclusion: BER of G^O-containing lesions appears to be regulated.

Significance: BER intermediates were believed to be unavailable to other pathways of DNA metabolism. This hypothesis may be incorrect.

Replicative DNA polymerases are high fidelity enzymes that misincorporate nucleotides into nascent DNA with a frequency lower than [1/10^5], and this precision is improved to about [1/10^7] by their proofreading activity. Because this fidelity is insufficient to replicate most genomes without error, nature evolved postreplicative mismatch repair (MMR), which improves the fidelity of DNA replication by up to 3 orders of magnitude through correcting biosynthetic errors that escaped proofreading. MMR must be able to recognize non-Watson-Crick base pairs and excise the misincorporated nucleotides from the nascent DNA strand, which carries by definition the erroneous genetic information. In eukaryotes, MMR is believed to be directed to the nascent strand by preexisting discontinuities such as gaps between Okazaki fragments in the lagging strand or breaks in the leading strand generated by the mismatch-activated endonuclease of the MutL homologs PMS1 in yeast and PMS2 in vertebrates. We recently demonstrated that the eukaryotic MMR machinery can make use also of strand breaks arising during excision of uracils or ribonucleotides from DNA. We now show that intermediates of MutY homolog-dependent excision of adenines mispaired with 8-oxoguanine (G^O) also act as MMR initiation sites in extracts of human cells or Xenopus laevis eggs. Unexpectedly, G^O/C pairs were not processed in these extracts and failed to affect MMR directionality, but extracts supplemented with exogenous 8-oxoguanine DNA glycosylase (OGG1) did so. Because OGG1-mediated excision of G^O might misdirect MMR to the template strand, our findings suggest that OGG1 activity might be inhibited during MMR.

To improve the fidelity of DNA replication, mismatch repair (MMR)^2 must be able to detect base/base mismatches and small insertion/deletion loops that escaped the proofreading activity of the replicative polymerases and direct their excision and resynthesis activity to the nascent strand, which carries by definition the erroneous genetic information. In Gram-negative bacteria such as Escherichia coli, the newly synthesized strand remains transiently unmethylated on adenines within GATC sequences. Mismatch-activated MutS-MutL complex licenses MutH to incise the unmethylated GATC, which allows the loading of the UvrD helicase together with one of several exonucleases. This leads to the degradation of the error-containing strand from the MutH-catalyzed nick toward and some distance past the mismatch to generate a single-stranded gap that is subsequently filled in by polymerase III. The remaining nick is sealed by DNA ligase (1–3).

In eukaryotes, DNA methylation is not used in strand discrimination during MMR. Instead, the nascent strand is distinguished from the template by transient discontinuities such as gaps between Okazaki fragments in the lagging strand. The leading strand contains no such discontinuities other than the 3’ terminus. Because the only MMR-associated exonuclease identified to date, EXO1, has an obligate 5’ to 3’ polarity, it appeared unlikely that MMR would use the 3’ terminus of the primer strand for initiation. However, seminal work from Modrich and co-workers (4, 5) demonstrated that association of the mismatch-activated MutL (a heterodimer of MLH1 and PMS2) with proliferating cell nuclear antigen bound at the 3’ terminus activates a cryptic endonuclease in the PMS2 subunit, which introduces nicks into the newly synthesized strand and thus provides EXO1 with entry sites where 5’ to 3’ degradation of the error-containing strand can initiate.

While studying the involvement of MMR in somatic hypermutation, which is triggered by activation-induced cytidine deaminase that converts cytosines to uracils at the immunoglobulin locus of activated B cells (6), we discovered that the MMR system can hijack strand breaks generated by the base excision repair (BER) system during uracil removal for the purpose of strand discrimination (7). This was unexpected because BER was thought to be a concerted process in which the strand break generated by apurinic endonuclease (APE1) after removal of the aberrant base is not available for other processes of...
DNA metabolism (8). In later experiments, we showed that breaks generated during the RNase H2-catalyzed removal of ribonucleotides misincorporated into DNA during replication can also be utilized by MMR as strand discrimination signals (9).

The above experiments suggested that strand breaks arising during different processes of DNA metabolism may be hijacked by MMR. Although this might lead to improved replication fidelity when breaks in the nascent DNA strand were involved, the opposite would be true if breaks in the template strand were used to direct the MMR process. To gain novel insight into this phenomenon, we set out to examine the interplay between MMR and oxidative DNA damage metabolism because BER-mediated processing of oxidized DNA could potentially take place on both nascent and template strands.

Depending on cell type, human genomes have been estimated to harbor steady-state levels of the major product of DNA oxidation, 2'-deoxy-8-oxoguanosine, ranging between 1000 and 100,000 residues (10–12). This nucleoside can be present in DNA in two distinct contexts: either paired with deoxythymidine or mispaired with deoxyadenosine because the base, 8-oxoguanine (G\textsuperscript{O}), can adopt either an anti or a syn conformation about the glycosidic bond (13, 14). Thus, although oxidation of double-stranded DNA gives rise solely to G\textsuperscript{O}/C pairs in which G\textsuperscript{O} is in the anti conformation, the replicative polymerases α, δ, and ε could insert either a C opposite anti-G\textsuperscript{O} to form a Watson-Crick-like G\textsuperscript{O}/C base pair or an A opposite syn-G\textsuperscript{O} to form a Hoogsteen G\textsuperscript{O}/A mispair (15–17).

Oxidation also affects the nucleotide pool where it generates dG\textsuperscript{O}TP. However, because this nucleotide is hydrolyzed by MutT homolog 1 (MTH1) protein to dG\textsuperscript{O}MP (18–20), G\textsuperscript{O} should not be incorporated into the nascent DNA strand. Thus, in newly replicated DNA, all G\textsuperscript{O} residues should be in the template strand, and all As mispaired with G\textsuperscript{O} should be in the nascent strand. This point is key to our understanding of the potential interplay between oxidative damage processing and MMR.

G\textsuperscript{O}/C base pairs are addressed primarily by 8-oxoguanine DNA glycosylase (OGG1), a glycosylase/lyase that removes the oxidized base and cleaves the sugar-phosphate backbone to initiate a BER process that ultimately replaces the oxidized nucleotide with a dGMP (13). G\textsuperscript{O}/As are not addressed by OGG1; they are recognized by the MutY homolog (MYH) glycosylase, which removes the mispaired adenines to initiate a BER process that inserts Cs opposite the oxidized guanines. The G\textsuperscript{O}/Cs arising in this way can then be repaired later to G/C by OGG1- and MutY-dependent BER (21, 22). Thus, if MMR were to use breaks generated during MYH-initiated BER of G\textsuperscript{O}/As, these discontinuities would be in the nascent strand, and their hijacking by MMR might improve the efficiency of the latter process. In contrast, incisions made during OGG1-initiated BER of G\textsuperscript{O}/Cs would be in the template strand where they would not only misdirect the mismatch repair process to the wrong strand but where they could also give rise to double strand breaks that could cause replication fork collapse.

In an attempt to learn whether MMR utilized strand breaks arising during MYH- and/or OGG1-initiated BER processes, we generated substrates containing a single nick (a bona fide strand discrimination signal), a G\textsuperscript{O}/A mispair, or a G\textsuperscript{O}/C pair and studied the efficiency and directionality of MMR-catalyzed repair of a G/T mismatch situated in the vicinity. We show that, in extracts of human cells or *Xenopus laevis* eggs, the MMR excision machinery can use strand breaks arising upon MYH-catalyzed removal of adenosines from G\textsuperscript{O}/A mispairs as initiation sites for exonucleolytic degradation of error-containing strands on circular heteroduplex substrates. A similar phenomenon was not observed either on G\textsuperscript{O}/C-containing heteroduplexes or on substrates containing 2-hydroxyadenine (A\textsuperscript{O}), another product of purine oxidation (12), even though A\textsuperscript{O}/C and A\textsuperscript{O}/G mispairs were reported previously to be addressed by MYH (23, 24).

**EXPERIMENTAL PROCEDURES**

**Restriction Enzymes**

All restriction enzymes were purchased from New England Biolabs.

**Recombinant Proteins**

GST-tagged MYH was expressed from the pET41a-MYH expression vector (a gift from Dr. Barbara van Loon) and partially purified using glutathione-Sepharose beads (GE Healthcare) as described previously (25). In a second purification step, eluates were loaded onto a HiTrap™ Heparin HP column (GE Healthcare), which was then washed with 10 column volumes of wash buffer (30 mM Tris-HCl, pH 8, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 50 mM NaCl) at a flow rate of 0.5 ml/min. Elution was performed using a salt gradient (50–600 mM NaCl). MYH-GST was eluted with 400–500 mM NaCl. The active fractions were identified using a MYH nicking assay, pooled, aliquoted, and stored at −80 °C. Recombinant APE1 was purchased from New England Biolabs. Recombinant OGG1 was a kind gift from Barbara van Loon. Recombinant OGG1-GST was purchased from Trevigen (4130-100-EB). Recombinant MutLα and MutSβ were expressed and purified in our laboratory as described previously (26).

The expression constructs for human geminin (geminin-pET28) and p27 (p27-pET21) were a kind gift from Yoshi Hashimoto and Vincenzo Costanzo. Briefly, protein expression was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside in BL21 cells (Invitrogen) grown at 37 °C. The cell pellets were resuspended in 20 mM Tris-HCl, pH 7.5, 500 mM KCl, 10% glycerol, 1 mM β-mercaptoethanol, 0.1% Nonidet P-40, and PMSF for lysis. After cell disruption and centrifugation of cell debris and membranes, the soluble fraction containing 10 mM imidazole was loaded onto a nickel-chelating column, which was then washed with 5–25 mM imidazole. The protein was eluted with a gradient of 50–300 mM imidazole. Fractions containing the desired polypeptides were pooled and dialyzed against EB buffer (100 mM KCl, 2.5 mM MgCl\textsubscript{2}, 50 mM HEPES-KOH, pH 7.5, 10% glycerol).

**Cell Culture**

HCT116 (MutLα-deficient) and HCT116 + chromosome 3 (MutLα-proficient) cells were obtained from Richard Boland (27) and were cultured in McCoy’s 5a medium (Gibco) supplemented...
with 10% bovine calf serum (Gibco). The medium for the chromosome 3-complemented cell line was supplemented with 400 μg/ml G418. LoVo (MutS−/H9251-deficient) cells were grown in DMEM (Gibco) supplemented with 10% bovine calf serum. All media additionally contained 1% penicillin/streptomycin.

Nuclear Extracts of Human Cells

Nuclei were isolated as described previously (28), resuspended in 1⁄3 of their packed volume in cold extraction buffer (25 mM HEPES-KOH, pH 7.5, 292 mM sucrose, 1 mM PMSF, 0.5 mM DTT, 1 μg/ml leupeptin), and transferred to a small beaker fitted with a magnetic stirrer bar. NaCl was added dropwise to a final concentration of 150 mM, and extraction continued for 1 h at 4°C. The nuclei were pelleted by centrifugation at 14,500 g for 20 min at 4°C in a tabletop centrifuge. The supernatant was transferred and dialyzed 1 h at 4°C against 1 liter of cold dialysis buffer (25 mM HEPES-KOH, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 231 mM sucrose, 1 mM PMSF, 2 mM DTT, 1 μg/ml leupeptin). The dialyzed extract was clarified by centrifugation at 20,000 g for 15 min at 4°C. The supernatant was aliquoted, snap frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined with the Bradford assay, and the salt concentration was measured using a conductivity meter.

X. laevis Egg Extracts

S phase extract was prepared as described previously (29). Briefly, eggs were dejellied, activated with calcium ionophore (Sigma-Aldrich), rinsed with S buffer (50 mM HEPES-KOH, pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 250 mM sucrose), transferred to 2-ml Eppendorf tubes, and crushed by centrifugation for 12 min at 13,200 rpm. The cytoplasmic layer was removed and after addition of Cytb (Sigma-Aldrich) cleared by centrifugation for 25 min at 70,000 rpm (Sorvall TL55 swinging bucket rotor). The extract was supplemented with 250 μg/μl cycloheximide, 25 mM phosphocreatine, and 10 μg/ml creatine phosphokinase before use.

Western Blotting and Antibodies

Whole cell extracts of the cell lines were prepared using 2× Laemmlı buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol). Upon determination of the protein concentration by the Lowry assay, proteins were separated by SDS-PAGE. Western blot analyses were carried out using standard procedures. The following antibodies and dilutions were used: MYH (mouse monoclonal, Abcam, ab55551), 1:333; OGG1 (rabbit monoclonal, Abcam, ab124741), 1:10,000; MSH6 (mouse monoclonal, BD Transduction Laboratories, 610919), 1:1000; MSH2 (mouse monoclonal, Calbiochem, NA-27), 1:500; MLH1 (mouse monoclonal, BD Transduction Laboratories, 554073), 1:500; and TF II H (rabbit polyclonal, Santa Cruz Biotechnology, sc-293), 1:1000. The anti-MTH1 antibody was a generous gift from Yusaku Nakabeppu and was used at a dilution of 1:250. Horseradish peroxidase (HRP)-coupled anti-mouse and anti-rabbit secondary antibodies (GE Healthcare) were used at a dilution of 1:5000.

TABLE 1

| Substrate | Oligonucleotide primer (5’ to 3’) |
|-----------|----------------------------------|
| G/T† | CCAGACGT/C/TGACGTGAGGAAAGCGTTAG |
| T/A† | CCAGACGT/C/TGAGGAAAGCGTTAG |
| G/A† | GAATGTAAGACGAAACTATAAGGGCAGAATTGGCGCGCCCGATCTGATCAAGATCCAGACGTGCAAGGAAAGCGTTAG |
| G/C† | GAATGTAAGACGAAACTATAAGGGCAGAATTGGCGCGCCCGATCTGATCAAGATCCAGACGTGCAAGGAAAGCGTTAG |
| G/A-G/T‡ | GAATGTAAGACGAAACTATAAGGGCAGAATTGGCGCGCCCGATCTGATCAAGATCCAGACGTGCAAGGAAAGCGTTAG |
| G/C-G/T‡ | GAATGTAAGACGAAACTATAAGGGCAGAATTGGCGCGCCCGATCTGATCAAGATCCAGACGTGCAAGGAAAGCGTTAG |
| A/G-T/G§ | GGAGGCGGCTGAGGTTGCGGGCCTC and CCAGACGT/C/TGACGTGAGGAAAGCGTTAG |
| A/C-T/G§ | GGAGGCGGCTGAGGTTGCGGGCCTC and CCAGACGT/C/TGACGTGAGGAAAGCGTTAG |
| A/T® | CCAGACGT/C/TGACGTGAGGAAAGCGTTAG |
| G/C® | CCAGACGT/C/TGACGTGAGGAAAGCGTTAG |

a Template was pHRichi-2850topAclI.
b Template was pHRichi-2850botAclI.
c Template was pHRichi-2850topSalI.

Effect of Oxidative Damage Processing on MMR Directionality

Substrate Generation

The detailed procedure was described previously (30). Briefly, hetero- and homoduplexes were constructed by primer extension on single-stranded phagemid templates. The templates differed in the position of the Nt.BstNBI site, which was situated at nucleotide 2850 either in the viral (top) or complementary (bottom) strand. Incubation of the substrates with the nickase yielded substrates in which MMR occurred either 5′ to 3′ or 3′ to 5′, respectively. The mismatches were located within a SalI and/or AclI restriction site, which was restored upon repair. The desired closed-circular heteroduplex substrates were purified on cesium chloride gradients.

Primers

The GO- or AO-containing primers were obtained from Eurogentec (Seraing, Belgium). All other primers were obtained from Microsynth (Balgach, Switzerland). The Sall (GTCGAC) and Acll (AAGCTT) restriction sites are highlighted in bold and gray, respectively. PvuI restriction sites (CGATCG) are italicized, and mispaired residues are underlined. Primer sequences correspond to the outer strand sequence of the substrate. The substrates and the primer/template combinations are listed in Table 1.
Prenicking of Substrates

MYH + APE1—200 ng of the substrates were incubated with 10 ng of purified recombinant MYH-GST and 10 units of APE1 (New England Biolabs) in 1× MMR buffer (20 mM Tris-HCl, pH 7.6, 40 mM KCl, 5 mM MgCl₂, 1 mM glutathione, 50 μg/ml BSA, 0.1 mM dNTPs) for 4 h at 37 °C. The reaction was stopped by heat inactivation, and 50 ng of the prenicked substrate were analyzed on a 1% GelRed-stained agarose gel for nicking efficiency. The remaining 100 ng of the prenicked substrate were used in a MMR assay.

OGG1—1 μg of G⁰/C/G/T substrate was incubated with 0.8 μg of purified recombinant OGG1 in 20 mM Tris-HCl, pH 8, 1 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA for 2.5 h at 37 °C and subsequently purified on a MinElute Spin column (Qiagen). 100 ng of the prenicked substrate were used in a MMR assay.

Nt.BstNBI—Closed-circular DNA substrates (100 ng) were incubated with 1 unit of Nt.BstNBI (New England Biolabs) according to the recommendations of the manufacturer. Subsequently, they were purified on a MinElute Spin column and used in MMR assays.

In Vitro MYH Nicking Assay

100 ng of closed-circular G⁰/A, A⁰/G, A⁰/C, or homoduplex substrate were incubated with 10 ng of purified recombinant MYH-GST, 10 units of APE1, and 1.5 mM ATP in 1× MMR buffer in a total volume of 10 μl. For time course experiments, 5-μl aliquots were withdrawn at the indicated time points. The reactions were stopped by the addition of 2 μl of 6× loading dye (37.5 mg/ml Ficoll 400, 23% glycerol, 0.03% bromphenol blue) and subsequent heat inactivation. The samples were separated on 1% agarose gels and visualized with GelRed. The nicking efficiency was quantified from the ratio of the amount of open-circular DNA product to the total amount of DNA (closed-circular + open-circular).

In Vitro MMR Assay in Nuclear Extracts of Human Cells

The MMR assays were described previously (7, 30, 31). Unless otherwise specified, the reactions were carried out with 100 ng of substrate and 100 μg of nuclear cell extract in a total volume of 25 μl in a buffer containing 20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1 mM glutathione, 50 μg/ml BSA, 0.1 mM dNTPs, 1.5 mM ATP, and 80 mM KCl (in experiments involving MYH-addressed substrates) or 110 mM KCl (in experiments involving OGG1-addressed substrates). 1.8 pmol of purified MutLo or MutSo were added where indicated. The reactions were incubated at 37 °C for 1 h. The reaction was stopped by adding an equal volume of STOP solution (50% Proteinase K, 1 mM EDTA, 3% SDS) and subsequent incubation for 1 h at 50 °C. Substrates were purified on MinElute spin columns and subjected to Sall/Dral or Acl restriction digestion, and the DNA fragments were separated on 1% agarose gels stained with GelRed. The percentage of mismatch repair was quantified from the ratio of the repaired bands (c+d) to the total amount of DNA (c+d+(c+d)) using ImageQuantTL. The band intensities were corrected according to their respective DNA fragment sizes. Some assays were supplemented with 2 μCi of [α-³²P]dATP. To monitor incorporation of the radiolabeled nucleotide, the agarose gels were vacuum-dried, exposed to a Phosphor Screen, and scanned with a Typhoon Scanner (FLA 9500, GE Healthcare).

In Vitro MMR Assay in X. laevis Egg Extracts

Briefly, the reaction mixture containing 150 ng of substrate, 26 μl of S phase extract, and 2 μCi of [α-³²P]dATP in a total volume of 30 μl was incubated at 23 °C for 45 min. To inhibit replication, 500 nm geminin and 40 μg/ml p27 were added. The reaction was stopped by the addition of 70 μl of STOP solution (76 mM EDTA, 1.5% SDS) and 40 μg of RNase (Sigma-Aldrich) and incubated for 30 min at 37 °C. Subsequently, 200 μg of Proteinase K (AppliChem) were added, and incubation continued at 37 °C overnight. The substrates were purified on MinElute Spin columns and subjected to Sall/Dral or Acl restriction digestion in the presence of RNase. The digested DNA was cleaned up again and analyzed on a 1% agarose gel stained with GelRed.

Immunodepletions

20 μl of Protein A/G PLUS-agarose beads (sc-2003, Santa Cruz Biotechnology) were washed twice with 750 μl of binding buffer (30 mM HEPES-KOH, pH 7.6, 7 mM MgCl₂) and spun down at 2700 × g for 2 min at 4 °C. The beads were resuspended in binding buffer, and 1 μg anti-MYH or anti-MSH6 antibody was added. The mixture was then incubated for 3 h at 4 °C, the beads were washed three times with 750 μl of binding buffer, and subsequently used to immunodeplete 100 μg of nuclear cell extracts for 30 min at 4 °C. MMR assays were performed immediately after depletion. Mock-depleted nuclear cell extracts were obtained by incubation with beads only.

MMR- and/or BER-dependent Incorporation Assays

This assay was used to test MMR or BER activities. It was performed similarly to the MMR assay except for the following modifications. To track MMR-, MYH-, or OGG1-dependent nucleotide incorporation, the reactions were supplemented with 2 μCi of [α-³²P]dATP, [α-³²P]dCTP, or [α-³²P]dGTP, respectively. Finally, the substrates were digested with NotI/BsaI and analyzed on a GelRed-stained 1% agarose gel. Repair tracts of up to 330 bp in Nt.BstNBI-nicked substrates, up to 29 bp in the G⁰/A substrates, and up to 22 bp in the G⁰/C substrates are seen in the 1808-bp fragment a. Longer repair tracts appear in the 1387-bp fragment b (see Fig. 1A). Although MMR-dependent [³²P]dAMP incorporation gave rise to a strong radioactive signal in both bands, MYH-dependent [³²P]dCMP or OGG1-dependent [³²P]dGMP incorporation occurred only in DNA fragment a. Quantification of [³²P]dCMP or [³²P]dGMP incorporation in MYH- or OGG1-induced BER was determined from the ratio between the band intensities of fragment a on the autoradiographs and on the GelRed-stained agarose gels.

BER Assay

To determine A⁰/G to C/G or A⁰/C to G/C repair by MYH-dependent BER, the substrates were incubated with the extracts as described for the MMR assay. After the reaction, 50% of the purified, eluted substrate was digested with Sall/Dral (MMR
assay), whereas the other half was used for Pvul digestion (BER assay).

RESULTS

**G°/A and G°/C Mispairs Are Not Addressed by Canonical MMR—BER and MMR are mechanistically distinct biochemical pathways. They differ principally in their substrate specificity and in repair patch size.** The specificity of BER is dictated by the enzymes that initiate the process, DNA glycosylases, which recognize and excise a limited number of damaged or modified bases (32). The repair process is thus initiated at the site of the base modification and involves the replacement of one (short patch BER) or two (long patch BER) nucleotides (34, 35). Canonical MMR addresses non-Watson-Crick base pairs that arise during replication, but in contrast to BER, the excision process initiates at a site distal to the mispair at a strand discontinuity that marks the nascent DNA strand. The repair tracts can thus be several hundred nucleotides long (36, 37). Because we wished to study the potential interplay of BER and MMR in the processing of substrates that could conceivably be addressed by both repair systems, we first needed to learn whether both repair pathways were active in our cell extracts. To achieve this, we generated covalently closed circular plasmid substrates containing a single G/T, G°/C, or G°/A base pair or a control substrate containing a G/C at the same site. By subsequent nicking of the substrates at their Nt.Bst.NBI sites, we generated an initiation site for MMR (Fig. 1A). We then incubated the nicked substrates with extracts of MutLα-deficient HCT116 human cells (Fig. 1B) that were supplemented with purified recombinant MutLα and [α-32P]dATP. Following recovery of the phagemids, restriction digestion with NotI and BsaI, and separation of the fragments on agarose gels, we anticipated that long patch repair events (MMR) would give rise to heteroduplexes labeled in both fragments a and b (Fig. 1A).

As shown in Fig. 1C, intense radiolabeling was seen only in the **bona fide** MMR substrate G/T (lane 2). That both fragments a and b were labeled with similar intensity indicates that the repair patch spanned at least the distance of 361 nucleotides between the nick and the mispair. Only background amounts of [32P]dAMP were detected in fragments a and b of the control homoduplex, G°/A, or G°/C substrates (lanes 1, 3, and 4), which confirmed that the G°/C and G°/A pairs failed to activate nick-dependent long patch excision and resynthesis characteristic of MMR (38, 39) and exemplified in lane 2.

**MYH-dependent BER in Human Nuclear Cell Extracts—** Having shown that MMR was active in the extracts, but that it did not address the G°/A and G°/C substrates, we wanted to see whether they were processed by BER. G°/A repair should be initiated by MYH, which should remove the mispaired adenine. The resulting apurinic site should then be incised by APE1. We therefore asked whether incubation of the supercoiled G°/A substrate with purified, recombinant MYH-GST and APE1 gave rise to nicked circular molecules. As shown in Fig. 2A (lanes 3–6), the G°/A substrate was efficiently converted to the open-circular form upon incubation of the plasmid with the recombinant proteins.

MYH has been reported to excise (in addition to adenine from mispairs with G°) also the oxidation product of adenine, A°, from mispairs with guanine or cytosine (23, 24). We therefore included the covalently closed A°/G and A°/C substrates in this assay. As shown in Fig. 2A, only limited nicking was detected on the A°/G (lanes 7–10) and A°/C (lanes 11–14)
substrates with only 42 and 38% of the plasmids having been nicked after 8 h, respectively. This was only slightly above the nonspecific nicking levels observed on the homoduplex (26%).

We next set out to test BER activity in the extracts. MYH-initiated BER of G^0/A should give rise to G^0/C. Thus, upon incubation of the covalently closed G^0/A substrate with extracts supplemented with [α-^32P]dCTP, specific incorporation of [^32P]dCMP into fragment a of the substrate (Fig. 2B) should be detectable. This was indeed the case (Fig. 2C, lanes 4–6). As in the case of the purified enzymes, after incubation of the A^0/G (Fig. 2C, lanes 7–9) or A^0/C (Fig. 2D, lanes 4–6) substrate with [α-^32P]dTTP- or [α-^32P]dGTP-supplemented HCT116 extracts, respectively, the amount of radioactivity incorporated into the A^0-containing fragment a of the two substrates was only slightly greater than that detected in the homoduplex substrate.

MYH-dependent Mismatch Repair in Human Nuclear Cell Extracts—Having obtained preliminary evidence that both MYH-dependent BER and MMR were active in the extracts, we next wanted to learn whether there was a cross-talk between these processes. To address this question, we deployed a phagemid substrate containing a G/T mismatch within the unique SalI restriction site (G/T; covalently closed or nicked with Nt.BstNBI), molecular size marker.

Effect of Oxidative Damage Processing on MMR Directionality

When the above substrates were incubated with MMR-deficient HCT116 nuclear cell extracts supplemented with [α-^32P]dATP, only background levels of repair and[^32P]dAMP incorporation were detected in the bona fide MMR substrate, nicked G/T (Fig. 3B, lane 2). However, the same substrate was repaired with high efficiency when the extracts were supplemented with purified recombinant MutLα (lane 3), particularly when compared with the unnicked control G/T plasmid (lane 1), which was processed to a limited extent by non-canonical MMR (42). Strikingly, although only low levels of[^32P]dAMP incorporation were detected in this system when the closed-circular G^0/A-G/T control substrate was used (lanes 6 and 7), around 60% of the covalently closed G^0/A-G/T phagemid were repaired in the extract supplemented with recombinant MutLα (lane 5). This result implied that A opposite G^0 can serve as a cryptic strand discrimination signal for MMR.

We postulated that the MMR machinery might be hijacking intermediates of G^0/A processing for EXO1 loading. We therefore set out to confirm that the above described phenomenon was dependent on MYH by immunodepleting it from the extract (Fig. 3C) prior to incubation with the substrates. This reduced MMR efficiency on the G^0/A-G/T substrate from 52 (lane 7) to ~20% (lane 8). The latter level was comparable with
Effect of Oxidative Damage Processing on MMR Directionality

**A**

```
5'-AGCGAACNTGCGAGGGTGG-3'
3'-TTCGACCATTGATGACG-5'
```

Incubation with nuclear cell extracts
Restriction digest with SalI and Dral

---

**B**

- **MutLa**
  - +
  - +
  - +
  - +

- **GelRed**
  - +
  - +

- **[³²P]dAMP**
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7

---

**C**

- **MYH depletion**
  - -
  - +
  - +
  - -
  - +
  - +

- **MYH**
  - -
  - +
  - +

- **GelRed**
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7
  - 8
  - 9

---

**D**

- **HCT116 NE MYH-GST**
  - -
  - +

- **MYH depletion**
  - -
  - +

- **MYH-GST**
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7
  - 8
  - 9

---

**E**

- **Nicking enzyme**
  - -
  - +

- **MYH**
  - -
  - +

- **GelRed**
  - 1
  - 2
  - 3
  - 4

---

**F**

- **A⁰**
  - 3197 bp

- **PvuI**
  - (3059)

---

**G**

- **MutLa**
  - +
  - -

- **SalI/Dral**
  - +
  - -

---

**Legend**

- Black = no BER
- Blue = BER

---

**Note**

- α = 2-hydroxyadenine
- θ = guanine or cytosine
the efficiency of non-canonical MMR on the covalently closed G/T substrates (~30%) (lanes 1–3). That the observed inhibition was due to MYH depletion was confirmed by complementation of the depleted extracts with purified, recombinant MYH-GST (Fig. 3D), which restored the MMR efficiency on the G/O-A-G/T substrate to 43% (Fig. 3C, lane 9). As anticipated, MMR efficiencies on the G/T (lanes 1–3) and nicked G/T (lanes 4–6) substrates were unaffected by the amount of MYH-GST in the extracts.

To confirm that processing of G/O/A intermediates by the BER machinery indeed generates DNA termini that can be utilized by EXO1 in the strand degradation step of MMR, we pre-incubated the closed-circular G/O-A-G/T substrate with purified, recombinant MYH-GST and APE1 (Fig. 3E, upper panel, lane 3), which cleaves the sugar-phosphate backbone at the MYH-generated abasic site. We then incubated the MYH/APE1-nicked substrate with HCT116 extracts supplemented with MutLα. As shown in Fig. 3E (lower panel), the MYH/APE1-generated nick in the G/O-A-G/T substrate (lane 3) was efficiently recognized by MMR factors. That the latter substrate was even more efficiently repaired than the Nt.BstNBI-nicked G/T phagemid (lane 2) is most likely indicative of the shorter distance between the nick and the mispair in the G/O-A-G/T substrate (54 nucleotides) as compared with the Nt.BstNBI-nicked G/T phagemid (361 nucleotides).

We extended the in vitro MMR assays also to substrates containing an A/O/G or an A/O/C pair in the vicinity of a T/G mismatch. These substrates differed from those described above inasmuch as the A/O residues were located within a PvuI site (see “Experimental Procedures”). Successful repair of A/O/G to C/G or of A/O/C to G/C by BER would restore the restriction site such that PvuI digestion of the recovered DNA would allow quantification of BER activity at the A/O sites (Fig. 3F). As anticipated from the absence of detectable BER activity on these substrates, incubation of the A/O/G-T/G and A/O/C-T/G substrates with MMR-proficient HCT116 extracts supplemented with recombinant MutLα yielded only 15 and 12% PvuI-sensitive products, respectively (Fig. 3G, lower panel, lanes 4 and 5), whereas the G/T phagemid was completely digested under identical conditions (lane 3). These results were reflected in MMR efficiency determined from the same assays by digestion of the recovered phagemids with SalI/DraI (Fig. 3G, upper panel). Repair of the T/G mismatch in the A/O/G-T/G and A/O/C-T/G substrates was very inefficient (20 and 18%, respectively), whereas the nicked G/T (lane 3) and the covalently closed G/O-A-G/T (lane 1) substrates were efficiently repaired (93 and 59%, respectively). Taken together, these data show that A/O/G or A/O/C pairs are inefficiently processed by MYH in cell extracts and therefore fail to act as entry sites for mismatch-activated excision.

G/O/A Mispairs Act as MMR Initiation Sites also in X. laevis Egg Extracts—Because G/O/A mispairs arise during replication, MYH should be able to act on newly replicated DNA, and indeed, available experimental evidence shows that this glycosylase is more abundant during S phase (43). In an attempt to learn whether MYH is also more efficient during S phase, we decided to make use of X. laevis egg extracts, which are enriched in S phase proteins (44).

In the first experiment, we wanted to learn whether the extracts were proficient in MYH-initiated BER. We decided to make use of the assay described above (Fig. 2B) in which the homoduplex or G/O/A closed-circular substrates were incubated with X. laevis egg extracts supplemented with [α-32P]dCTP. Digestion of the recovered phagemid DNA with NotI/Bsal revealed a 3-fold greater incorporation of [32P]dCMP into the G/O-containing fragment of the G/O/A substrate than into the corresponding fragment of the homoduplex phagemid (Fig. 4A). This demonstrated that MYH-dependent BER was active in the X. laevis extracts.

**FIGURE 3.** A single G/O/A base pair in a DNA heteroduplex can act as an initiation site for MMR in human nuclear cell extracts. A, schematic representation of the G/O-A-G/T substrate and the in vitro MMR assay. The substrate carries a G/O/A mispair 57 nucleotides away from a G/T mismatch, which is located in a SalI recognition site. The presence of the mismatch makes the phagemid refractory to cleavage by the enzyme such that incubation with Sall and DraI yields fragments c + d (2484 bp) and e (692 bp). (The third, 19-bp-long fragment is not detectable on 1% agarose gels stained with GelRed.) The presence of a nick in the inner strand, introduced either by Nt.BstNBI or through BER-catalyzed incision of the A strand results in a repair of the G/T mismatch to G/C in human nuclear cell extracts that regenerates the Sall site. Upon incubation with Sall and DraI, the repaired phagemid gives rise to fragments c (1328 bp), d (1156 bp), and e (692 bp). B, G/O/A mispairs serve as initiation sites for MMR. The MMR assay shows the efficiency of repair of a G/T mismatch in closed-circular G/T (lane 1), nicked G/T (lanes 2 and 3), closed-circular G/O-A-G/T (lanes 4 and 5), and closed-circular G/O-A-G/C (lanes 6 and 7) substrates that were incubated with nuclear extracts of MutLα-deficient HCT116 cells supplemented with purified MutLα (+) where indicated. The autoradiograph shows MMR-dependent [32P]dAMP incorporation into the different substrate fragments. C, G/T repair in the G/O-A-G/T substrate is dependent on MYH. The closed-circular G/O-A-G/T (lanes 1–3), nicked G/T (lanes 4–6), and closed-circular G/O-A-G/C (lanes 7–9) substrates were incubated with MutLα-supplemented extracts of HCT116 cells, which were either mock-depleted (lanes 1, 4, and 7), MYH-depleted (lanes 2, 5, and 8), or MYH-depleted and supplemented with recombinant MYH-GST (lanes 3, 6, and 9). The efficiency of the repair reactions shown in B and C was estimated by ImageQuant from scans of GelRed-stained agarose gels. The indicated MMR efficiencies (%) represent an average of three independent experiments. Error bars represent S.D. D, Western blot showing MYH immunodepletion efficiency of HCT116 nuclear extracts. Subsequently, they were digested with Sall/DraI, and repair efficiencies were analyzed on a GelRed-stained agarose gel and quantified. The G/T mismatch in the MYH/APE1-preincubated G/O-A-G/T substrate was efficiently repaired. The panel shows a representative image of two independent experiments. E, schematic representation of a BER assay using phagemid substrates containing A/O mispaired with guanine or cytosine. The A/O residue is located within a PvuI restriction site, which makes the phagemid refractory to cleavage by this enzyme. A/O/G to C/G or A/O/C to G/C repair by BER restores the PvuI site. Incubation with the enzyme gives rise to 1846-bp fragment f and 1351-bp fragment g. Positions are as follows: A/O/G, 3060; A/O/C, 3057. G, A/O/G and A/O/C mispairs do not serve as MMR initiation sites. The indicated substrates were incubated with MMR-proficient HCT116 extracts supplemented (+) or not (−) with purified MutLα. Upper panel, the efficiency of G/T to G/C (lanes 1–3) or T/G to C/G (lanes 4 and 5) repair mediated by MMR was estimated by Sall/DraI restriction digestion of the following recovered substrates: closed-circular G/O-A-G/T (lane 1), closed-circular G/T (lane 2), nicked G/T (lane 3), closed-circular A/O-G/T-G (lane 4), and closed-circular A/O-C-T/G (lane 5). Lower panel, an aliquot of the recovered phagemids was digested with PvuI, which detects MYH-dependent BER of A/O to C in the A/O/G substrate or of A/O to G in the A/O/C substrate. The nicked G/T substrate (lane 3) served as the positive control for PvuI digestion. A/O-G/T and A/O/C-T/G substrates that were not incubated with the extracts (lanes 6 and 7) contain defective PvuI sites and thus served as negative controls. MMR and BER efficiencies (%) are indicated below the panels. M, molecular size marker.
We also had to show that the extracts supported nick-directed MMR because an earlier report indicated that mismatch processing in *X. laevis* oocyte extracts was efficient but not nick-directed (45). We therefore incubated the covalently closed and the nicked isoforms of the G/T phagemid with the extracts supplemented with [α-^32P]dATP. As shown in Fig. 4B, we were able to detect nick-directed MMR on the G/T substrate (lane 2), whereas the covalently closed phagemid was only inefficiently processed (lane 1). Under these conditions, the covalently closed G^O/A-G/T substrate (lane 3) was almost as efficiently repaired as the nicked G/T phagemid (lane 2). Because extracts of *X. laevis* eggs support plasmid replication under certain conditions, which would result in considerable[^32P]dAMP incorporation and conversion of 50% of the G/T mismatches to G/C in the absence of MMR, we carried out a control experiment in extracts supplemented with the DNA replication inhibitors p27 and geminin. No detectable differences between the levels of radionucleotide incorporation in the presence and absence of these inhibitors were observed, indicating that the plasmid did not replicate in the assay (cf. lanes 4 and 5).

OGG1-dependent Mismatch Repair Is Inefficient in Human Nuclear Cell and *X. laevis* Egg Extracts—Assuming that MTH1 hydrolyzes oxidized dGTP in the nucleotide precursor pool with a 100% efficiency, all G^O residues in oxidized DNA should be in the template strand following replication irrespective of whether they are paired with A or C. As discussed above, MYH-initiated BER of G^O/A mispairs arising during replication would be directed to the A strand that is also the nascent DNA strand. In contrast, BER-mediated repair of G^O/C pairs arising during replication would have the opposite effect on MMR because OGG1-dependent G^O/C repair would introduce breaks in the template DNA strand and thus provide MMR with an incorrect strand bias.

We set out to test the above hypothesis by studying the effect on MMR efficiency and directionality of a G^O/C pair situated in the vicinity of a G/T mismatch. We generated a G^O/C-G/T substrate in which the G^O was positioned 54 nucleotides 5’ from the mispaired G. OGG1-initiated BER of the G^O would give rise to a break that should activate MMR to correct the G/T mismatch to A/T and thus regenerate an Accl site in the substrate. This repair bias should be identical to that introduced by a Nt.Bst.NBI-generated nick in the G/T substrate (Fig. 5A).

When we incubated the nicked G/T substrate or the covalently closed G/T, G^O/C-G/T, or G^O/C-A/T phagemid heteroduplexes with HCT116 nuclear cell extracts supplemented with [α-^32P]dATP, no significant repair or[^32P]dAMP incorporation was observed (Fig. 5B, lanes 1, 3, 5, and 7), but when the extract was supplemented with purified, recombinant MutLoA, the Nt.BstNBI-nicked G/T substrate was repaired (lane 4). In contrast to what was observed with the G^O/A-G/T substrate (Fig. 3B, lane 5), only background levels of repair and[^32P]dAMP incorporation were detected with the G^O/C-G/T substrate (Fig. 5B, lane 6) and with the control, covalently closed G/T phagemid (lane 2). Similar results were obtained also with MutSo-deficient LoVo extracts supplemented with purified recombinant MutSo (Fig. 5C, lanes 1–3). Given that both extracts contained the key MMR and BER factors as ascen-
Effect of Oxidative Damage Processing on MMR Directionality

were detected in the GO/C-G/T substrate (lane 2) or nicked G/T phagemid (Fig. 5C, cf. lanes 2 and 4). This shows that MMR can use OGG1-generated strand breaks as initiation sites.

The second possibility was that OGG1 in the test cell extracts was inactive or that it was present in insufficient amounts. We therefore incubated the G9/C and homoduplex substrates with LoVo extracts supplemented with [α-32P]dGTP to test for OGG1-dependent BER, which should result in [32P]dGMP incorporation into fragment a (Fig. 5F). As shown in Fig. 5G, only background levels of the radiolabeled nucleotide were incorporated into fragment a of the phagemid heteroduplex, suggesting that G9/C repair was indeed inefficient despite the fact that the extracts contained readily detectable amounts of OGG1 (Figs. 1B and 5D). Similar results were obtained with [32P]-labeled G9/C-containing oligomers, which were inefficiently processed in LoVo extracts, whereas processing of U/G-containing oligomers was very efficient. Moreover, purified, recombinant OGG1 was able to process the G9/C oligonucleotide substrate with ~50% cleavage of the G9/O strand seen after only 10 min (data not shown).

To test whether the LoVo extracts contained an inhibitor of OGG1, we supplemented them with an amount of purified recombinant OGG1-GST that was comparable with that of the endogenous protein present in the nuclear extracts (Fig. 5H). Under these conditions, the G9/C-G/T substrate (Fig. 5I, lane 3) was repaired with efficiency similar to that of the nicked G/T phagemid (lane 1).

Importantly, the above described phenomenon was not limited to extracts of human cells as identical results were obtained with the X. laevis MMR system (Fig. 6). The reason underlying the low OGG1 activity in the extracts is currently unknown, but we were able to eliminate inappropriate salt concentration, lack of an activator protein in the cytoplasmic fraction, and short half-life of OGG1 in our assay as possible causes (data not shown).

DISCUSSION

The postreplicative mismatch repair system improves the fidelity of DNA replication by several orders of magnitude through removing from nascent DNA nucleotides that fail to form Watson-Crick base pairs. To fulfill this function, it has to satisfy two key criteria: it has to (i) recognize base-base mismatches and small insertion/deletion loops generated by the replicative polymerases during DNA synthesis and (ii) direct the repair process to the newly synthesized DNA strand. How the major mismatch recognition factor MutSα recognizes the different helical distortions that are caused by purine/purine, purine/pyrimidine, and pyrimidine/pyrimidine mispairs as well as by insertion/deletion loops is still poorly understood despite the fact that several structures of protein-DNA complexes exist (48). However, because MMR deficiency leads to transition, transversion, and frameshift mutations, MutSα clearly has broad substrate specificity. Although this characteristic of MutSα is beneficial as far as its role in the maintenance of replication fidelity is concerned, it might also be deleterious should MutSα bend to lesions that ought to be processed by other repair systems. One such example is mispairs containing G9/C base pair does not act as an initiation site for MMR in nuclear extracts of human cells. A, schematic representation of the G9/C-G/T substrate used in the in vitro MMR assay. The circular heteroduplex substrate carries a G9/C base pair 54 nucleotides from a G/T mismatch in the recognition site of AcI endonuclease. The positions of two further AcI cleavage sites and the Nt.BstNBI site where a nick can be introduced selectively into the outer strand are indicated. In the absence of repair, digestion of the phagemid with AclI gives rise to fragments of 2824 (fragments c + d) and 373 bp (fragment e). Repair of the G/T mismatch to A/T regenerates a third AcI restriction site such that the phagemid DNA is cleaved into three fragments of 1518 (fragment c), 1306 (fragment d), and 373 bp (fragment e). B, substrates G/T (lanes 1 and 2), nicked G/T (lanes 3 and 4), G9/C-G/T (lanes 5 and 6), and G9/C-A/T (lanes 7 and 8) were incubated with extracts of HCT116 cells supplemented (+) or not (−) with purified recombinant MutLα and analyzed on a GelRed-stained agarose gel. The autoradiograph visualizes [32P]dAMP incorporation into the different substrate fragments. The indicated MMR efficiencies (%) were estimated by ImageQuant from scans of GelRed-stained agarose gels and represent an average of three independent experiments. Error bars represent S.D. Only background levels of repair were detected in the G9/C-G/T substrate (lane 6). C, OGG1-generated DNA termini at G9/C sites act as MMR initiation sites. The G9/C phagemid substrate was preincubated with Nt.BstNBI (lane 2), and the G9/C-G/T phagemid was preincubated with recombinant, purified OGG1 (lane 4) to generate nicked, open-circular substrates (see E). The substrates were then purified and incubated with LoVo nuclear cell extracts supplemented with purified MutSα (lanes 1–4). The closed-circular G/T (lane 1) and G9/C-G/T (lane 3) phagemid substrates served as controls. The OGG1 preincubated G9/C-G/T substrate (lane 4) was at least as efficiently repaired as the positive control, nicked G/T (lane 2). D, Western blot analysis of the relative abundance of MMR (MSH6, MSH2, and MLH1) and BER (MYH, OGG1, and MTH1) proteins in nuclear extracts of HCT116 and LoVo cells. The absence of MutSα in HCT116 cells and of MutSα in LoVo cells is evident. Where required, these extracts were complemented with the purified recombinant proteins. E, the G9/C-G/T phagemid substrate was preincubated with purified recombinant OGG1 (lane 2) to give rise to an open-circular (oc) substrate, which migrates in the 1% agarose gel in the same position as the Nt.BstNBI-nicked G/T substrate (lane 1). These preincubated substrates were purified and used in the MMR assay shown in C,F, schematic representation of the OGG1-dependent nucleotide incorporation assay. Short patch BER should replace the oxidized G with a radiolabeled G (asterisk). This should result in detectable radioactivity in band a of the NotI/Bsal digest. G, absence of OGG1 activity in human nuclear cell extracts. Upper panel, homoduplex (lanes 1–3) or G9/C (lanes 4–6) substrate was incubated with LoVo extracts supplemented with [α-32P]dGTP for the indicated times. As seen in the autoradiograph, both substrates incorporated similar amounts of [32P]dGMP that were close to background. H, Western blot showing the amount of OGG1 in 100 μg of LoVo nuclear extract used in one MMR reaction (lane 1) and the amount of recombinant purified OGG1-GST required to supplement the reaction (lane 2). I, a G9/C base pair activates MMR in LoVo extracts supplemented with purified, recombinant OGG1-GST. The nicked G/T (lane 1) and the G9/C-G/T (lanes 2–3) substrates were incubated with LoVo nuclear cell extracts and purified MutSα supplemented (+) or not (−) with an amount of purified recombinant (Rec.) OGG1-GST shown in H, lane 2. H and I show representative images and quantifications of three independent experiments. M, molecular size marker.

FIGURE 5. A G9/C base pair does not act as an initiation site for MMR in nuclear extracts of human cells. A, schematic representation of the G9/C-G/T substrate used in the in vitro MMR assay. The circular heteroduplex substrate carries a G9/C base pair 54 nucleotides from a G/T mismatch in the recognition site of AcI endonuclease. The positions of two further AcI cleavage sites and the Nt.BstNBI site where a nick can be introduced selectively into the outer strand are indicated. In the absence of repair, digestion of the phagemid with AcI gives rise to fragments of 2824 (fragments c + d) and 373 bp (fragment e). Repair of the G/T mismatch to A/T regenerates a third AcI restriction site such that the phagemid DNA is cleaved into three fragments of 1518 (fragment c), 1306 (fragment d), and 373 bp (fragment e). B, substrates G/T (lanes 1 and 2), nicked G/T (lanes 3 and 4), G9/C-G/T (lanes 5 and 6), and G9/C-A/T (lanes 7 and 8) were incubated with extracts of HCT116 cells supplemented (+) or not (−) with purified recombinant MutLα and analyzed on a GelRed-stained agarose gel. The autoradiograph visualizes [32P]dAMP incorporation into the different substrate fragments. The indicated MMR efficiencies (%) were estimated by ImageQuant from scans of GelRed-stained agarose gels and represent an average of three independent experiments. Error bars represent S.D. Only background levels of repair were detected in the G9/C-G/T substrate (lane 6). C, OGG1-generated DNA termini at G9/C sites act as MMR initiation sites. The G9/C phagemid substrate was preincubated with Nt.BstNBI (lane 2), and the G9/C-G/T phagemid was preincubated with recombinant, purified OGG1 (lane 4) to generate nicked, open-circular substrates (see E). The substrates were then purified and incubated with LoVo nuclear cell extracts supplemented with purified MutSα (lanes 1–4). The closed-circular G/T (lane 1) and G9/C-G/T (lane 3) phagemid substrates served as controls. The OGG1 preincubated G9/C-G/T substrate (lane 4) was at least as efficiently repaired as the positive control, nicked G/T (lane 2). D, Western blot analysis of the relative abundance of MMR (MSH6, MSH2, and MLH1) and BER (MYH, OGG1, and MTH1) proteins in nuclear extracts of HCT116 and LoVo cells. The absence of MutSα in HCT116 cells and of MutSα in LoVo cells is evident. Where required, these extracts were complemented with the purified recombinant proteins. E, the G9/C-G/T phagemid substrate was preincubated with purified recombinant OGG1 (lane 2) to give rise to an open-circular (oc) substrate, which migrates in the 1% agarose gel in the same position as the Nt.BstNBI-nicked G/T substrate (lane 1). These preincubated substrates were purified and used in the MMR assay shown in C,F, schematic representation of the OGG1-dependent nucleotide incorporation assay. Short patch BER should replace the oxidized G with a radiolabeled G (asterisk). This should result in detectable radioactivity in band a of the NotI/Bsal digest. G, absence of OGG1 activity in human nuclear cell extracts. Upper panel, homoduplex (lanes 1–3) or G9/C (lanes 4–6) substrate was incubated with LoVo extracts supplemented with [α-32P]dGTP for the indicated times. As seen in the autoradiograph, both substrates incorporated similar amounts of [32P]dGMP that were close to background. H, Western blot showing the amount of OGG1 in 100 μg of LoVo nuclear extract used in one MMR reaction (lane 1) and the amount of recombinant purified OGG1-GST required to supplement the reaction (lane 2). I, a G9/C base pair activates MMR in LoVo extracts supplemented with purified, recombinant OGG1-GST. The nicked G/T (lane 1) and the G9/C-G/T (lanes 2–3) substrates were incubated with LoVo nuclear cell extracts and purified MutSα supplemented (+) or not (−) with an amount of purified recombinant (Rec.) OGG1-GST shown in H, lane 2. H and I show representative images and quantifications of three independent experiments. M, molecular size marker.
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that eventually leads to cell death (1). Processing of G/O/A mismatches arising through the incorporation of dAMP opposite G/O in the template strand might have also been expected to trigger futile MMR, but the in vivo evidence for such a process is currently lacking, and in vitro data showing that G/O/A mismatches are very poorly addressed by the MMR system in human cell extracts (this study and Ref. 39) would appear to argue against it. However, in vivo, the MMR system appears to remove from the nascent strand G/O misincorporated opposite template A (49, 50) and to increase the efficiency of MYH-dependent BER at least in vitro (51). Thus, available experimental evidence suggests that MMR supports BER during oxidative DNA damage processing. In the present study, we asked whether the reverse might also be true, namely whether BER-dependent processing of G/O affects MMR efficiency.

In our earlier studies, we showed that the MMR system could use breaks generated during the BER-mediated processing of uracil residues (7, 42) or during the RNase H2-mediated excision of ribonucleotides misincorporated into DNA during replication (9) as strand discrimination signals. We postulated that MMR might also use breaks generated during the BER-dependent processing of oxidative damage. However, the outcome of such interference would be positive only in the case that the breaks were generated in the nascent DNA strand. We show here that MYH-dependent processing of a G/O/A mispair in extracts of human cells or of Xenopus laevis eggs directs MMR to the A strand (Figs. 3 and 4). In vivo, the ability of the MMR system to use breaks generated during the MYH-directed processing of G/O/A mismatches arising through the incorporation of dAMP opposite G/O would direct mismatch correction to the A strand (Figs. 3 and 4).

Breaks introduced into the nascent DNA strand by OGG1 during the processing of C/G/O pairs arising through the incorporation of dG/O/MP opposite template C would also help improve MMR efficiency and replication fidelity. However, due to the sanitization of dNTP pools by MTH1, dG/O/MP concentrations should be extremely low such that dAMP incorporation should be minimal in vivo (51). Thus, available experimental evidence suggests that MMR supports BER during oxidative DNA damage processing. In the present study, we asked whether the reverse might also be true, namely whether BER-dependent processing of G/O affects MMR efficiency.

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Breaks introduced into the nascent DNA strand by OGG1 during the processing of C/G/O pairs arising through the incorporation of dG/O/MP opposite template C would also help improve MMR efficiency and replication fidelity. However, due to the sanitization of dNTP pools by MTH1, dG/O/MP concentrations should be extremely low such that dAMP incorporation should be minimal in vivo (51). Thus, available experimental evidence suggests that MMR supports BER during oxidative DNA damage processing. In the present study, we asked whether the reverse might also be true, namely whether BER-dependent processing of G/O affects MMR efficiency.

In our earlier studies, we showed that the MMR system could use breaks generated during the BER-mediated processing of uracil residues (7, 42) or during the RNase H2-mediated excision of ribonucleotides misincorporated into DNA during replication (9) as strand discrimination signals. We postulated that MMR might also use breaks generated during the BER-dependent processing of oxidative damage. However, the outcome of such interference would be positive only in the case that the breaks were generated in the nascent DNA strand. We show here that MYH-dependent processing of a G/O/A mispair in extracts of human cells or of Xenopus laevis eggs directs MMR to the A strand (Figs. 3 and 4). In vivo, the ability of the MMR system to use breaks generated during the MYH-directed processing of G/O/A mismatches arising through the incorporation of dAMP opposite G/O would direct mismatch correction to the A strand (Figs. 3 and 4).
FIGURE 7. Schematic representation of the BER and MMR interplay in G⁰ metabolism. Left panel, should dG⁰MP be incorporated into the nascent strand (red) opposite C, it could be removed by OGG1-dependent BER or by MMR without deleterious consequences. In contrast, should A-template/G⁰-nascent mispairs arise during replication, MYH-mediated BER to C/G⁰ would give rise to A to C mutations, whereas MMR would process the G⁰-containing strand and prevent mutagenesis. In cases where the oxidized base was in the vicinity of a replication error (e.g., an A/G mispair), BER intermediates of C-template/G⁰-nascent processing would direct MMR to the correct strand, whereas MYH-initiated BER of A-template/G⁰-nascent would misdirect MMR to the parental template strand (black). However, the likelihood of incorporation of dG⁰MP into the nascent DNA strand is minimized by hydrolysis of dG⁰TP in the nucleotide pool by MTH1. Most G⁰ residues in the DNA should therefore be in the template strand as shown in the right panel. Right panel, BER of G⁰/C pairs would lead to the removal of the oxidized base, but during S phase, the OGG1-generated strand break might result in replication fork collapse or in the misdirection of MMR to the template strand. Processing of G⁰-template/A-nascent mispairs by MYH-dependent BER or by MMR would have no deleterious consequences. Moreover, in cases where the oxidized base was in the vicinity of a replication error (e.g., a G/T mispair), BER intermediates of G⁰-template/A-nascent processing would direct MMR to the correct strand.

onset of S phase and that OGG1 should be inactivated during this cell cycle stage. Interestingly, we found that G⁰/C processing in both in vitro systems was inefficient and that the presence of a G⁰/C pair in the heteroduplex substrate failed to activate MMR of the G/T mismatch (Figs. 5 and 6) despite the fact that OGG1 was present in considerable quantities (Figs. 1B and 5, D and H). Because addition of the recombinant polypeptide to the extracts resulted in G⁰-dependent G/T repair (Fig. 5I), we postulate that OGG1 present in the cell extracts is inactive either as a result of post-translational modifications or through complexation with an inhibitor. We are currently attempting to identify the underlying cause of this inhibition as well as carrying out a series of in vivo experiments that should show whether the observations described above correspond to the situation in living cells. Our current findings indicate that the repair of oxidative damage in vertebrate cells is highly regulated to prevent genomic instability, and future experiments should show how this regulation is mediated at the molecular level.

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