Oxidative Damage and Mutagenesis in S. cerevisiae: Genetic Studies of Pathways Affecting Replication Fidelity of 8-oxoguanine

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ABSTRACT

Oxidative damage to DNA constitutes a major threat to the faithful replication of DNA in all organisms and it is therefore important to understand the various mechanisms that are responsible for repair of such damage, and the consequences of unrepaired damage. In these experiments, we make use of a reporter system in *Saccharomyces cerevisiae* that can measure the specific increase of each type of base pair mutation by measuring reversion to a Trp+ phenotype. We demonstrate that increased oxidative damage due to the absence of the superoxide dismutase gene, *SOD1*, increases all types of base pair mutations and that mismatch repair (MMR) reduces some, but not all, types of mutations. By analyzing various strains that can revert only via a specific CG→AT transversion in backgrounds deficient in Ogg1 (encoding an 8-oxoG glycosylase), we can study mutagenesis due to a known 8-oxoG base. We show as expected that MMR helps prevent mutagenesis due to this damaged base, and that Pol η is important for its accurate replication. In addition we find that its accurate replication is facilitated by template switching, as loss of either *RAD5* or *MMS2* leads to a significant decrease in accurate replication. We observe that these *ogg1* strains accumulate revertants during prolonged incubation on plates, in a process most likely due to retromutagenesis.
INTRODUCTION

Oxidative damage to DNA has long been recognized as an important source of DNA damage and subsequent mutagenesis (Bjelland & Seeberg 2003; D’Errico et al. 2008; Evans et al. 2004; Imlay 2003; Imlay 2008; Kryston et al. 2011; Nakabeppu et al. 2006; Stone et al. 2011). Oxidative damage has been proposed as an important contributor to cancer (Kryston et al. 2011; Maynard et al. 2009; Melis et al. 2011; Nakabeppu et al. 2006; Paz-Elizur et al. 2008; Stone et al. 2011; Tudek et al. 2010), neurological diseases (D’Errico et al. 2008; Fishel et al. 2007; Jeppesen et al. 2011; Liu & Wilson 2012; Lovell & Markesbery 2007; Trushina & McMurray 2007; Ventura et al. 2010), and, somewhat more controversially, aging (Burhans & Weinberger 2007; Gredilla et al. 2010; Maynard et al. 2009; Speakman & Selman 2011; Tudek et al. 2010). Oxidative damage to the DNA bases is a primary concern for mutagenesis due to the propensity of damaged bases to miscode, and a large number of different oxidation products of DNA bases have been analyzed (Evans et al. 2004; Neeley & Essigmann 2006; Stone et al. 2011; Wallace 2002). Of all of the various oxidatively damaged bases, 8-oxoG is perhaps the most significant because of its abundance and frequent mispairings to yield GC→TA transversions (Beard et al. 2010; Evans et al. 2004; Neeley & Essigmann 2006; van Loon et al. 2010; Zahn et al. 2011). The central role of 8-oxoG in mutagenesis is reflected in the fact that there are specific glycosylases that excise 8-oxoG from DNA.

Much oxidative damage is due to reactive oxygen species (ROS) generated within the cell itself, particularly from the mitochondrion (Karthaikyan & Resnick 2005; Kim et al. 2006; Lambert & Brand 2009; Linford et al. 2006). Most organisms have several enzymes devoted to detoxifying ROS; one important enzyme is superoxide dismutase (SOD), the only enzyme that can detoxify superoxide (Gralla & Valentine 1991; Longo et al. 1996; Serra et al. 2003; Van Raamsdonk & Hekimi 2012). Base excision repair (BER) is the first line of defense againstoxidatively damaged bases in the genome (D’Errico et al. 2008; Dizdaroglu 2005; Maynard et al. 2009; van Loon et al. 2010). Ogg1 is a BER glycosylase that recognizes and removes an 8-oxoG paired with C (Huang & Kolodner 2005; Klungland & Bjelland 2007; Nash et al. 1996; Paz-Elizur et al. 2008; Tsuzuki et al.
Another important repair mechanism for oxidative DNA damage is mismatch repair (MMR). We demonstrated the general effect of MMR on oxidative damage in yeast (Earley & Crouse 1998) and subsequently the specific effect of MMR and 8-oxoG in yeast was demonstrated (Ni et al. 1999). Experiments in many organisms have reinforced the finding that MMR plays an important and conserved role in prevention of mutations due to oxidative damage (Boiteux et al. 2002; Colussi et al. 2002; Gu et al. 2002; Huang & Kolodner 2005; Mazurek et al. 2002; Mure & Rossman 2001; Russo et al. 2004; Shin & Turker 2002; Slupphaug et al. 2003; Wyrzykowski & Volkert 2003; Zlatanou et al. 2011). However, those experiments were not designed to measure the effect of oxidative damage on specific base pair mutations and the effect of MMR on formation of those mutations. Oxidatively damaged bases, such as 8-oxoG, have generally not been thought to affect replication. However, we have recently found (Rodriguez et al. 2013) that 8-oxoG can induce template switching in which the replicating 3’ end invades the sister strand, either by strand invasion or a fork regression, thereby bypassing the damaged base (Li & Heyer 2008). Polyubiquitination of PCNA by a complex of Ubc13-Mms2-Rad5 appears to be necessary for template switching (Chang & Cimprich 2009).

Increased ROS can also have effects through damage to RNA. Although most of the interest in 8-oxoG has understandably been in its direct involvement in DNA mutation, it was also found in vitro that RNA polymerases could frequently misincorporate an A opposite 8-oxoG during the process of transcription (Chen & Bogenhagen 1993; Doetsch 2002; Viswanathan & Doetsch 1998). Later, it was shown that such transcriptional mutagenesis could occur in vivo in E. coli (Brégeon et al. 2003) as well as in mammalian cells (Saxowsky et al. 2008). It has been proposed that mutagenic transcription of damaged DNA could lead to the growth of cells that would otherwise remain quiescent, and thus lead to mutation due to the damaged DNA in a process termed retromutagenesis (Bregeon & Doetsch 2011). Such a process has been difficult to observe experimentally.

Most oxidative damage to DNA is expected to produce single point mutations. We previously developed a reversion assay with six different base substitutions in an essential codon of the S. cerevisiae TRP5 gene, yielding a mutagenesis assay with extremely low background and specificity for point mutations (Williams et
al. 2005). Our interest in developing this assay stemmed from prior results with a similar assay system, using mutations in the CYC1 gene (Hampsey 1991), that demonstrated an extremely high base pair mutation rate in the absence of MMR, due at least in part to oxidative damage (Earley & Crouse 1998). Reversion analysis of cyc1 strains depends on restoration of mitochondrial function, and we found as we continued to use those strains that MMR mutants, in particular, were extremely unstable giving rise to many derivatives that did not revert, presumably due to loss of functional mitochondrial DNA (results not shown). We report here that the trp5 reversion rates of all strains are greatly increased by oxidative damage and that half of the strains demonstrate a synergistic increase in reversion rates with oxidative damage and the loss of MMR, although the absolute values in those cases are much lower than with the cyc1 strains. By employing ogg1 derivatives of the trp5-A149C strains, we are able to study mutagenesis specifically due to the formation of 8-oxoG in DNA. We find that the mutation rates due to 8-oxoG are strongly suppressed by MMR, but substantially decreased in the presence of Pol η, a translesion DNA polymerase known for accurate replication of 8-oxoG (Carlson & Washington 2005; De Padula et al. 2004; Haracska et al. 2000; Yuan et al. 2000). We further find evidence that template switching is important for avoiding mutagenesis due to 8-oxoG, as inactivation of either RAD5 or MMS2 significantly increases reversion rates. In addition, we observe increased numbers of revertants when ogg1 trp5-A149C strains remain on selective plates for extended periods, consistent with 8-oxoG-induced retromutagenesis.
MATERIALS AND METHODS

Yeast Strains

All strains used were derivatives of the previously published collection of *trp5* mutants (Williams *et al.* 2005). Gene deletions were created by one-step disruption with PCR generated fragments. In general, gene deletions were made from a PCR fragment generated from the collection of yeast gene deletions (Winzeler *et al.* 1999). *sod1* strains were grown in anaerobic chambers except for growth immediately before transformation or for reversion analysis. In addition, *sod1* strains were routinely checked for a Lys- phenotype in aerobic growth to test for the presence of suppressors (Gralla & Valentine 1991). A complete list of strains is given in Table S1.

Reversion Analysis

Reversion analysis was performed by growth of parallel cultures inoculated with equal amounts of a dilute culture of cells (Rosche & Foster 2000), followed by plating each culture separately on SD-Trp medium (Sherman 2002). In practice, 3 μl of an overnight culture of a given strain was diluted into 110 ml of YPAD (Sherman 2002), and usually 12 cultures of 5 ml were incubated for 48 hr at 30° and then plated on SD-Trp, with each culture being split between two plates. In addition, before plating, the number of total viable cells was determined in 3 cultures by dilution and plating on YPAD.

Determination of Reversion Rates

Reversion rates were determined by fluctuation analysis using a maximum likelihood method for analysis (Foster 2006; Rosche & Foster 2000). Computations were done using the program Salvador which calculates both *m*, the mean number of mutations occurring in a culture, and 95% confidence intervals from the fluctuation data (Zheng 2002; Zheng 2005; Zheng 2007). The mutation rate was then obtained by dividing *m* by the average number of total cells in the cultures.
RESULTS

Oxidative damage increases mutation of all base pairs

Spontaneous reversion rates of our trp5 strains were measured, and as shown in Figure 1 are extremely low in strains that are otherwise wild type. This low reversion rate is consistent with the very specific nature of the reversion assay in which each strain is able to revert to Trp+ function via only one specific base pair change (Williams et al. 2005). In contrast to results with the cyc1 reversion assay (Earley & Crouse 1998), elimination of MutSα function (msh6 strains) resulted in modest, at best, increases in mutation rates (Figure 1 and Table 1). Oxidative damage in the strains was increased by deleting the SOD1 gene encoding superoxide dismutase (Gralla & Valentine 1991) and the reversion rates of those strains, with and without MMR, were determined (Figure 1 and Table 1).

It is apparent from this analysis that the mutation rates of all base pairs are increased substantially with increased oxidative damage, but that the effect of MMR is quite variable, depending on the particular base pair mutation involved. In some cases, such as the trp5-G148C and trp5-A149G strains, MMR has little effect on sod1-induced mutations, whereas in other strains such as trp5-G148A, trp5-A149C, and trp5-A149T, the effect of MMR is synergistic with oxidative damage.

Mutations due to 8-oxoG

One of the problems with the above analysis is that although the base pair change in each strain is known, the exact insult to the DNA causing the mutation is not. The trp5-A149C mutation reverts via a CG→AT mutation, which is a hallmark of oxidative damage, as an 8-oxoG frequently mispairs with an A, leading to a CG→AT transversion. One could still not ascribe all trp5-A149C reversion events to misreplication of a damaged guanine, but increased mutagenesis in an ogg1 background should all be due to the 8-oxoG, as Ogg1 is quite specific for removal of an 8-oxoG opposite C (Nash et al. 1996; van der Kemp et al. 1996). The impact and specificity of an ogg1 mutation is seen in the reversion analysis of Figure 2. In the trp5-A149C background, the
The reversion rate of an ogg1 mutation is approximately 20X that of wild-type, and an msh6 ogg1 strain is 8-fold greater than the ogg1. In the trp5-A149T background which would revert via a TA→AT transversion, however, neither an ogg1 nor msh6 ogg1 strain has a reversion rate significantly greater than wild-type. Therefore in the trp5-A149C ogg1 background, it is likely that essentially all mutation events can be ascribed to the misreplication of an 8-oxoG.

**Factors affecting 8-oxoG mutagenesis**

A series of reversion experiments were performed with the trp5-A149C mutant gene in both orientations with respect to the ARS306 origin of replication in a variety of different genotypes. The results are shown graphically in Figure 3, the reversion rates given numerically in Table S2, and comparisons of the reversion rates of various genotypes in Table 2. Consistent with the results in Figure 2 with the trp5-A149C F strain, in both orientations of the TRP5 gene, we find substantial increases in reversion rates in ogg1 and msh6 ogg1 genotypes. Pol η, the product of the RAD30 gene, is responsible for accurate bypass of 8-oxoG (Carlson & Washington 2005; De Padula *et al*. 2004; Silverstein *et al*. 2010) and when RAD30 is deleted, we see a 2 to 4-fold increase in reversion rate in both ogg1 and msh6 ogg1 strains (Figure 3 and Table 2). Inactivation of MMR and Pol η show a synergistic effect in an ogg1 background, as would be expected for the independent activities of MMR and Pol η in accurate replication of 8-oxoG (Mudrak *et al*. 2009).

In general, 8-oxoG has not been thought to affect replication, but we have found in work with oligonucleotides containing an 8-oxoG that this lesion can induce template switching (Rodriguez *et al*. 2013). Therefore we tested the role of template switching by deleting the RAD5 or MMS2 gene, either of which should inactivate template switching (Branzei *et al*. 2008; Minca & Kowalski 2010; Zhang & Lawrence 2005). In both cases, reversion rates were increased by approximately 2-fold in an ogg1 background, and 4 to 5-fold in an ogg1 rad30 background (Figure 3 and Table 2).
**ogg1 and transcriptional mutagenesis**

In working with *ogg1* derivatives of the *trp5-A149C* mutation, we noticed that new colonies arose when those strains were left on selection plates (SD-Trp) over extended periods of time. An example with *ogg1* and *msh6* derivatives of *trp5-A149C F* is shown in Figure 4. We examined other *trp5-A149C ogg1* genotypes, including several with considerably higher reversion rates than the *msh6 ogg1* background, and all showed a similar pattern of colonies arising on plates over time (Figure S1, A-C). We wanted to determine if the phenomenon of increasing numbers of revertants on plates was a function of the *ogg1* mutation. We had tested various *msh6* strains, but the reversion rate in *msh6* strains is so low that one cannot obtain a reasonable number of revertants on one plate. We therefore treated *msh6* strains with H$_2$O$_2$ in order to increase the initial mutation rate and observed the resulting revertants over time. In contrast to the *ogg1* strains, no increase in revertants over time was seen (Figure S1 D). One concern was that the late-arising phenotype of some revertants could be due to petite formation or an inherent slow growth phenotype. On a plate containing *trp5-A149C ogg1* revertants that had appeared at various times, 28 of the smallest colonies were picked and assayed for respiratory competence; only 25% were petites. Colonies that arise late on a plate will in general be smaller than colonies that arise earlier, due to a shorter growth time. In order to determine if colony size were related to inherent growth rate, rather than time of appearance, we picked colonies of various sizes and then determined their rate of growth in rich medium. The results, shown in Figure S2, indicate that the growth rate of small versus large colonies was not different.

When plated on such selection plates lacking tryptophan, Trp- cells remain viable for weeks, but do not grow for even one cell cycle in the absence of tryptophan (Rodriguez et al. 2012). Thus colonies that appear late must have gained the ability to produce tryptophan in order to form a colony, in a process that appears to be “adaptive mutation” (Foster 2004; Rosenberg 2001). As we discuss below, these late-arising colonies are likely the result of transcriptional mutagenesis followed by mutation, or retromutagenesis (Brégeon et al. 2003; Saxowsky & Doetsch 2006; Saxowsky et al. 2008).
Oxidative damage, mutagenesis and mismatch repair

Oxidative damage of DNA has long been known to be highly mutagenic, and as we demonstrated indirectly in yeast (Earley & Crouse 1998) and was shown specifically for the *ogg1* mutation (Ni et al. 1999), MMR is very important in preventing mutation due to oxidative damage in yeast. Our original analysis of the effect of MMR on specific point mutations had measured reversion of mutations in one codon of the *CYC1* gene (Earley & Crouse 1998), whereas in the experiments here we measure reversion of mutations in one codon of the *TRP5* gene. Removal of MMR had drastically different effects in the two sets of strains, increasing reversion rates by up to orders of magnitude in the *cyc1* strains and only modestly in the *trp5* strains. We have subsequently found that the *cyc1* strains have extremely high levels of reactive oxygen species, likely accounting for much of the levels of mutagenesis seen in the absence of MMR (results not shown). In contrast, the *trp5* mutations are in an S288c background, which has a defect in Hap1, a transcription factor regulating response to oxygen and whose loss impacts mitochondrial function, and alleles in other genes found to affect mitochondrial function (*MIP1, MRM1, ADE2*), likely resulting in levels of ROS below those of other strain backgrounds (Gaisne et al. 1999; Young & Court 2008). Therefore much of the observed differences in reversion rates between the two sets of strains is likely due to endogenous levels of oxidative damage and the nature of the ROS.

In the experiments reported here, levels of endogenous ROS were substantially increased by deletion of the *SOD1* gene and those ROS levels were found to substantially increase the reversion rates of all six different mutations (Figure 1). However, for any of the strains, it is difficult to know what types of base damages are the ones likely causing the increased reversion rates. That is particularly true in considering that increased levels of ROS could also damage free nucleotides, leading to misincorporation during replication (Kamiya 2010). There are a variety of different structures known to be caused by oxidative damage for all four DNA bases; their ultimate effect on mutation is a complicated process depending on their frequency of formation, miscoding potential, stability in the cell, and endogenous repair processes (Dizdaroglu 2005). The increase in reversion
rate in the \textit{trp5-A149C} strains due to oxidative damage is most likely a result of the formation of 8-oxoG, in
spite of the presence of Ogg1 in the cell, although other oxidative products of G could also contribute to the
same mutation (Dizdaroglu 2012; Neeley & Essigmann 2006). The increased reversion rates in the \textit{trp5-A149G}
strains (GC→AT transition) is likely due to oxidized cytosines resulting in C→T transitions (Kreutzer &
Essigmann 1998). The increase in CG→GC transversions in the \textit{trp5-G148C} strains could be due to oxidized
guanines (Neeley & Essigmann 2006) or 5-OH-C (Dizdaroglu 2012). The mutations observed in the
\textit{trp5-G148A}, \textit{trp5-G148T}, and \textit{trp5-A149T} strains could be due to oxidized A (Dizdaroglu 2012); another
possibility for the \textit{trp5-G148A} would be a T→C transition due to a thymine glycol perhaps further converted to
a urea species (McNulty \textit{et al.} 1998). Thus all of the mutations we observe can be explained by known
oxidative products, but which of the possible products are most important in leading to the mutations is for the
most part not known. These experiments do demonstrate that increased oxidative damage can, in fact, lead to
significant numbers of all types of mutations. We find here that MMR has a variable role in preventing
mutations due to the oxidative damage, with loss of MMR showing synergistic increases in half of the strains,
and little increased effect over the \textit{sod1} mutation in the others (Figure 1 and Table 1).

The advantage of a reversion assay such as the one used here is that it is possible to observe events that would
be relatively rare in an entire spectrum of mutations. For example, the CG→GC transversions observed in the
\textit{msh6 trp5-G148C} strain are relatively infrequent compared to the reversion rates observed in other \textit{msh6}
strains, but can still be reliably measured. A disadvantage of reversion assays is that one observes mutation
within only one sequence context. The dependence of mutation rate on sequence context is well known; the
effect of sequence surrounding a mispair on MMR recognition has been well studied, for example (Marsischky
& Kolodner 1999). In spite of that concern, our results clearly demonstrate two major points: increased levels
of ROS can lead to all types of base pair changes, and the effect of MMR in preventing mutation due to
oxidatively damaged bases is quite variable. It is important to realize that the reversion rates only measure the
mispairs that escape cellular repair pathways. The reversion rates in \textit{sod1} strains are increased over wild-type
strains, but the increase in reversion rates in the absence of MMR demonstrates that in many cases much of the
potential mutagenesis due to increased ROS was concealed by the presence of MMR. This point becomes even clearer with the study of mutagenesis due to 8-oxoG.

**Analysis of defined oxidative damage: 8-oxoG**

The base pair specificity of our trp5 reversion assay allowed us to monitor only reversion events due to CG→AT mutations in the trp5-A149C strains. Such mutations are a signature of oxidative damage due to mutagenic replication of 8-oxoG. Elimination of OGG1 would be expected to increase only CG→AT mutations and in addition only those CG→AT mutations due to formation of 8-oxoG; as expected we observed a large increase in the reversion rate of the trp5-A149C strain, but not of another oggl strain (Figure 2).

Additionally, there was a synergistic increase in the reversion rate of the msh6 oggl strain compared to either single mutant (Table 2). The reversion rate of the trp5-A149C oggl strain is approximately the same as the trp5-A149C sod1 strain (Figure 2). Given the sensitivity of guanine to oxidative damage, there should be much more 8-oxoG formed in a sod1 strain than in an oggl strain; the high reversion rate observed in the oggl strain is an indication that much of the 8-oxoG in the sod1 strain is being repaired by the Ogg1 present in the cell, and a reminder, as noted above, that the reversion rates we measure are due to damage that has escaped the various repair mechanisms active in the cell. Similarly, the reversion rate of the trp5-A149C oggl strain is much lower than that of the trp5-A149C oggl msh6 strain, an indication that many of the 8-oxoG-A mispairs formed in the oggl strain are repaired by MMR. Because we expect nearly all of the increased reversion events in the trp5-A149C oggl strain to be due to 8-oxoG-A mispairs, which is a substrate for MMR, the increased reversion rate of the oggl strain must be due to overwhelming the MMR system.

The specificity of the trp5 reversion assay allows us to examine other components of pathways dealing with oxidative damage. One example is the translesion DNA polymerase Pol η, a product of the RAD30 gene. Pol η is necessary for accurate bypass of 8-oxoG (Carlson & Washington 2005; De Padula et al. 2004; Haracska et al. 2000; Yuan et al. 2000) and thus one would expect to observe an increased reversion rate in the trp5-A149C strain in the absence of Pol η. However, the importance of overlapping repair functions is observed in the
Trp5-A149C rad30 strains, as the reversion rates are not significantly different from wild-type rates (Figure 3). The increase in reversion rates of ogg1 strains over wild-type and of ogg1 msh6 strains over msh6 strains is an indication that there is insufficient Pol η present for replication of all 8-oxoG present in the absence of Ogg1 protein. Table 2 shows that deletion of RAD30 in ogg1 strains increases reversion rates by 2.7- and 2.3-fold in F and R strains, deleting MSH6 increases reversion rates by 8.1- and 9.2-fold, and deletion of both increases reversion rates by 30- and 32-fold. This synergistic increase in reversion rates is consistent with the results of Mudrak et al., who showed synergism of Pol η and MMR (Mudrak et al. 2009).

As mentioned above, we found evidence in other experiments that 8-oxoG could induce template switching (Rodriguez et al. 2013). Both the RAD5 and MMS2 genes are necessary for template switching (Branzei et al. 2008; Minca & Kowalski 2010; Zhang & Lawrence 2005). As can be seen both in Figure 3 and Table 2, loss of either MMS2 or RAD5 in either ogg1 or ogg1 rad30 strains leads to significant increases in reversion rates. Template switching is an error-free method of bypassing lesions, and these experiments indicate that the absence of template switching leads to increased mutagenesis due to the presence of 8-oxoG. Template switching appears to be more important for fidelity in ogg1 rad30 strains, in which 8-oxoG replication is less accurate, as the increase due to loss of either MMS2 or RAD5 is 2-fold greater than the loss in ogg1 strains.

Retromutagenesis in ogg1 strains

When we plated trp5-A149C ogg1 and ogg1 msh6 strains on Trp- medium, we were surprised to see colonies continue to arise even after 10 days on plates (Figure 4). Late arising revertants were also observed in other ogg1 backgrounds of the trp5-A149C strains (Figure S1), but were not inherently slow growing (Figure S2). If the late-arising colonies were due to some inherent property of the ogg1 mutation, one would expect other ogg1 strains to also display late arising colonies, but that was not seen in other of our trp5 strains such as the trp5-A149T ogg1 and trp5-A149T ogg1 msh6 strains for example. The late arising phenotype appeared to be associated with the persistence of the 8-oxoG, for strains treated with H2O2 displayed increased initial reversion rates, but did not show any delayed appearance on plates (Figure S1 D). Another possible explanation would
be that the late arising colonies are the result of low levels of replication and resulting mutation. As we have demonstrated previously (Rodriguez et al. 2012) the Trp- selection for these strains is extremely tight and cells plated on Trp- medium do not even undergo one round of replication although they remain viable on the selection plates for up to two weeks. Although Ogg1 is the main repair mechanism for 8-oxoG-C pairs, any type of repair mechanism would presumably target the removal of the damaged base and thus prevent reversion. We have shown that MMR acting outside of the context of replication can result in non-directed repair of mismatches (Rodriguez et al. 2012), but in this case MMR is not responsible for the late arising colonies as there are substantially more late arising revertants in the absence of MMR (Figure 4). The presence of the 8-oxoG on the transcribed strand also appears to be important. We demonstrated that we could induce reversion of *trp5-G148T* strains by electroporating 8-oxoGTP into cells (Rodriguez et al. 2012); in that case reversion was due to incorporation of the 8-oxoG into the nontranscribed strand. MMR-defective cells plated on Trp-plates that were not given sufficient time for a subsequent cell division after electroporation with 8-oxoGTP showed few revertants and did not accumulate any revertants over time on the plates (Rodriguez et al. 2012).

It has previously been shown that 8-oxoG can be mis-transcribed by RNA polymerase resulting in a phenomenon known as transcriptional mutagenesis (Brégeon et al. 2003; Saxowsky & Doetsch 2006; Saxowsky et al. 2008). As illustrated in Figure 5, the target G in the *trp5-A149C* strain is on the transcribed strand; if the G at position 149 is an 8-oxoG and is transcribed with an A, the resulting mRNA will be Trp+ and when enough Trp+ mRNA accumulates, the strain would be able to begin growth. Such growth itself would not be sufficient to cause a mutation. However, misreplication of the 8-oxoG in a subsequent round of replication would produce the needed mutation. Given that these late arising mutations depend on mis-transcription before replication, the process has been called retromutagenesis (Bregeon & Doetsch 2011; Doetsch 2002). In this case, transcriptional mutagenesis would lead to the production of some wild-type Trp mRNA, which could then allow sufficient cell growth to allow a round of replication, leading to a permanent DNA change. The particular attributes of our assay have allowed us to demonstrate that retromutagenesis can be a significant cause of mutation in certain circumstances.
The advantage of a reversion assay compared to a forward mutation assay is that specific types of mutational events can be studied. In the experiments reported here, we increased ROS in the cell by inactivating \textit{SOD1}, thus indicating both the importance of Sod1 in removing superoxide and the large increase in cellular oxidative damage in its absence. The reversion rates of all possible base-base mutations were increased in \textit{sod1} and \textit{sod1 msh6} strains, but the reversion rates differed by over an order of magnitude (Figure 1). Although the experiments shown in Figure 1 confirmed that MMR is important in preventing mutation due to oxidative damage, it is not possible to say exactly what damage is causing the increased reversion rates even when the base pair change is known. Elimination of \textit{OGG1} specifically increases only the incidence of 8-oxoG opposite C, and when replicated, that mispair leads to CG\textrightarrow\textit{AT} transversions. Our studies confirm not only that Pol \textit{\eta} is important for accurate replication, but that it is independent of MMR. In addition, a role for template switching in accurate bypass has been observed. The particular characteristics of our assay system allow us to observe one of the first examples of retromutagenesis in a natural system.

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Table 1  Relative increases in trp5 reversion rates

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For each trp5 mutant strain, the reversion rate of wild-type is set to 1.0 and the reversion rates of the indicated genotypes are compared to the wild-type rate. Differences are considered to be significant (*) if the 95% confidence intervals do not overlap.

Table 2 Relative increases in reversion rates for selected trp5-A149C genotypes

<table>
<thead>
<tr>
<th>Genotypes Compared</th>
<th>Reversion Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>msh6/wt</td>
<td>3.7*</td>
</tr>
<tr>
<td>ogg1/wt</td>
<td>19. *</td>
</tr>
<tr>
<td>msh6 ogg1/wt</td>
<td>150 *</td>
</tr>
<tr>
<td>ogg1 rad30/ogg1</td>
<td>2.7*</td>
</tr>
<tr>
<td>msh6 ogg1/ogg1</td>
<td>8.1*</td>
</tr>
<tr>
<td>msh6 ogg1 rad30/ogg1</td>
<td>30. *</td>
</tr>
<tr>
<td>msh6 ogg1 rad30/msh6 ogg1</td>
<td>3.8*</td>
</tr>
<tr>
<td>ogg1 rad5/ogg1</td>
<td>2.7*</td>
</tr>
<tr>
<td>ogg1 mms2/ogg1</td>
<td>1.3</td>
</tr>
<tr>
<td>ogg1 rad5/ogg1 mms2</td>
<td>2.1*</td>
</tr>
<tr>
<td>ogg1 rad30 rad5/ogg1 rad30</td>
<td>5.2*</td>
</tr>
<tr>
<td>ogg1 rad30 mms2/ogg1 rad30</td>
<td>4.1*</td>
</tr>
<tr>
<td>ogg1 rad30 rad5/ogg1 rad30 mms2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

For the indicated genotypes and TRP5 orientation, the ratio of reversion rates given in Table S1 are shown. The difference is judged to be significant (*) if the 95% confidence intervals do not overlap.
Figure 1  Spontaneous reversion rates of trp5 strains. Reversion rates and 95% confidence intervals for strains of the indicated trp5 mutation and genotype, all in the F orientation, are shown. The particular base pair change required for reversion of each strain is also shown.

Figure 2  Spontaneous reversion analysis of trp5 strains containing ogg1 mutations. Reversion rates of ogg1 strains of the indicated genotypes were determined; remaining data are from Figure 1. All strains are in F orientation; revertants were counted after 3 days on plates.
Figure 3  Spontaneous reversion analysis of trp5-A149C strains and the effect of 8-oxoG on reversion. Reversion rates of trp5 strains of the indicated orientation relative to the ARS306 origin of replication and with the indicated genotypes were determined and plotted as in Figure 1. In the F orientation, the presumed 8-oxoG would be replicated on the lagging strand.

Figure 4  Trp+ revertants as a function of time on selection plates. ogg1 and msh6 ogg1 derivatives of trp5-A149C F strains were plated on SD-Trp media and colonies counted on the indicated days after plating.
Figure 5 Model for 8-oxoG induced retromutagenesis. The mutant codon in the trp5-A149C strain is shown. If the indicated G is oxidized while cells are in a non-growth phase on SD-Trp plates, cells cannot grow due to lack of tryptophan. However, transcriptional mutagenesis of the 8-oxoG will result in Trp+ mRNA being produced, and after sufficient tryptophan is made, the cells will be able to undergo replication. If, during replication, an A is replicated opposite the 8-oxoG, a mutant, Trp+ strain, will be formed in a process known as retromutagenesis.