The post-replication repair *RAD18* and *RAD6* genes are involved in the prevention of spontaneous mutations caused by 7,8-dihydro-8-oxoguanine in *Saccharomyces cerevisiae*

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ABSTRACT

7,8-Dihydro-8-oxoguanine (8-oxoG) is an abundant and mutagenic lesion produced in DNA exposed to free radicals and reactive oxygen species. In *Saccharomyces cerevisiae*, the *OGG1* gene encodes the 8-oxoG DNA N-glycosylase/AP lyase (Ogg1), which is the functional homologue of the bacterial Fpg. Ogg1-deficient strains are spontaneous mutators that accumulate GC to TA transversions due to unrepaired 8-oxoG in DNA. In yeast, DNA mismatch repair (MMR) and translesion synthesis (TLS) by DNA polymerase η also play a role in the prevention of the mutagenic effect of 8-oxoG. In the present study, we show the *RAD18* and *RAD6* genes that are required to initiate post-replication repair (PRR) are also involved in the prevention of mutations by 8-oxoG. Consistently, a synergistic increase in spontaneous Can^R and Lys^+ mutation rates is observed in the absence of Rad6 or Rad18 proteins in *ogg1* mutant strains. Spectra of Can^R mutations in *ogg1 rad18* and *ogg1 rad6* double mutants show a strong bias in the favor of GC to TA transversions, which are 137- and 189-fold higher than in the wild-type, respectively. The results also show that Pol η (*RAD30* gene product) plays a critical role on the prevention of mutations at 8-oxoG, whereas Pol ζ (*REV3* gene product) does not. Our current model suggests that the Rad6–Rad18 complex targets Pol η at DNA gaps that result from the MMR-mediated excision of adenine mispaired with 8-oxoG, allowing error-free dCMP incorporation opposite to this lesion.

INTRODUCTION

The integrity of DNA in the cell is under constant threat from physical and chemical agents of endogenous and exogenous

origin (1–4). Reactive oxygen species (ROS) that escape the cellular metabolism are an important source of DNA damage, which has been involved in pathological processes such as carcinogenesis or aging (1–6). ROS can attack base and sugar moieties in DNA yielding a variety of lesions such as damaged bases, apurinic and apyrimidinic (AP) sites or DNA strand breaks (7). An oxidized guanine, 7,8-dihydro-8-oxoguanine (8-oxoG), is an abundant and potentially mutagenic lesion in DNA (8–12). Several *in vitro* studies show that 8-oxoG can be bypassed by eukaryotic DNA polymerases or RNA polymerases (9,13–18). However, the ability to incorporate nucleotides opposite 8-oxoG greatly depends upon the enzyme used and the assay conditions (13–18). These studies point to the incorporation of dCMP or dAMP opposite 8-oxoG in the template strand (13–18). Therefore, 8-oxoG is thought to be mutagenic, yielding GC to TA transversions after two rounds of replication (8–12). To counteract the deleterious effects of 8-oxoG, living organisms have evolved robust DNA repair mechanisms. In *Escherichia coli*, the repair of 8-oxoG mostly relies on two DNA glycosylases, Fpg and MutY, whose simultaneous inactivation results in a strong and specific (GC to TA) spontaneous mutator phenotype (8–10). In *Saccharomyces cerevisiae*, the main defense against the mutagenic effect of 8-oxoG is also the base excision repair (BER) pathway that is initiated by the Ogg1 protein (12). Yeast Ogg1 is a DNA glycosylase/AP lyase that excises 8-oxoG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) from γ -irradiated DNA (19–22). In *S.cerevisiae*, the inactivation of the Ogg1 protein results in a specific GC to TA spontaneous mutator phenotype (23). Indeed, the yeast Ogg1 is the functional homologue of the bacterial Fpg, although they do not belong to the same structural family (24,25).

In *S.cerevisiae*, general DNA mismatch repair (MMR) and error-free translesion DNA synthesis (TLS) cooperate with Ogg1 to prevent the mutagenic effect of 8-oxoG (26,27). The data suggest that MMR acts as a functional homologue of the MutY protein, catalyzing the excision of adenine residues incorporated by DNA polymerase δ opposite to 8-oxoG

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(26). It should be noted that MutY is absent in *S.cerevisiae* but present in mammals (12). On the other hand, DNA polymerase η (the *RAD30* gene product) is thought to efficiently promote the error-free incorporation of cytosine opposite 8-oxoG (27). It has been proposed (26,27) that 8-oxoG residues escaping repair by Ogg1 are bypassed by DNA Pol δ , which inserts an adenine across from the lesion yielding 8-oxoG/A. Afterwards, 8-oxoG/A is recognized by MMR that excises the DNA fragment containing adenine opposite the lesion. The resulting DNA gap is filled in by DNA Pol η , which inserts a cytosine opposite 8-oxoG thus regenerating the 8-oxoG/C. Together, these data point to a complex cellular network that prevents the mutagenic effect of 8-oxoG in *S.cerevisiae*. From this point of view, the spontaneous mutator phenotype of Rad18-deficient strains was challenging. Indeed, *rad18* mutant strains exhibit a spectrum of mutations that indicates an accumulation of GC to TA transversions, like Ogg1-deficient cells (28,29). In addition, genetic studies place *RAD30* and *RAD18* in the *RAD6* group of epistasis (30). These observations led us to hypothesize a role of Rad18 and Rad6 in the cellular network that prevents the mutagenic effect of 8-oxoG.

In *S.cerevisiae*, the *RAD18* and *RAD6* genes play a prominent role in the post-replication repair (PRR) process thought to come into play when the replication machinery stalls at DNA damage (31–33). Yeast strains lacking Rad6 or Rad18 proteins are highly sensitive to a wide variety of DNA damaging agents such as γ -radiation, UVC-light and alkylating agents (31–36). Biochemical studies show that Rad6 and Rad18 form a heterodimer that binds single-stranded DNA (37,38), and promotes the PRR process (32,33). The Rad6–Rad18 complex is an ubiquitin-conjugating enzyme that catalyzes the mono-ubiquitination of proliferating cell nuclear antigen (PCNA) at Lys-164, allowing the recruitment of DNA polymerases involved in TLS such as Rev1, Pol ζ or Pol η (39–42).

To explore the impact of the *RAD18* and *RAD6* genes and PRR on the processing of 8-oxoG in *S.cerevisiae*, we have constructed and characterized *ogg1 rad18* and *ogg1 rad6* double mutants. The results show a synergistic increase in the rates of spontaneous Can^R and Lys⁺ mutations in the *ogg1 rad18* and *ogg1 rad6* double mutants, compared to those in the *ogg1*,

rad18 or *rad6* single mutants. The analyses of spontaneous Can^R mutation spectra reveal that *ogg1 rad18* and *ogg1 rad6* double mutants accumulate GC to TA transversions, which is the hallmark of 8-oxoG. Our genetic data indicate a role for the *RAD18* and *RAD6* genes in the cellular ‘replication–repair network’ that prevents mutations at 8-oxoG. Our current model suggests that the role of Rad18–Rad6 relies on the specific recruitment of Pol η to perform incorporation of dCMP opposite 8-oxoG after the MMR-dependent removal of adenine from 8-oxoG/A.

MATERIALS AND METHODS

Media and growth conditions

Yeast strains were grown at 30°C in YPD medium (1% yeast extract, 1% bacto-peptone, 2% glucose, with 2% agar for plates) or yeast nitrogen base dextrose (YNBD) medium (0.17% yeast nitrogen base without amino acids, 2% glucose, with 2% agar for plates) supplemented with appropriate amino acids and bases. All media, including agar, were purchased from Difco. Supplemented YNBD medium lacking arginine but containing canavanine (Sigma) at 60 mg/l was used for the selective growth of canavanine-resistant (Can^R) mutants. Pre-sporulation and sporulation media have been described previously (43).

Yeast strains, plasmids and microbiological methods

Saccharomyces cerevisiae strains used in the present study are listed in Table 1. All yeast strains are isogenic to wild-type strain FF18733 (*MATa*, *leu2-3,112*, *trp1-289*, *his7-2*, *ura3-52*, *lys1-1*). The *RAD6*, *RAD18*, *RAD30* and *MSH6* gene deletions were performed by a PCR-mediated one-step replacement technique using plasmid pFA6a-kanMX6 (44,45). Mutants were selected on YPD medium containing geneticin (Gibco-BRL) at 200 mg/l. All disruptions were confirmed by PCR on genomic DNA. The primer sequences used for all disruptions are available upon request. Pre-sporulation and sporulation procedures were performed as described previously (43). Micromanipulation and dissection of asci were

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
FF18733	<i>MATa</i> , <i>leu2-3-112</i> , <i>trp1-289</i> , <i>his7-2</i> , <i>ura3-52</i> , <i>lys1-1</i>	F. Fabre (Fontenay aux Roses, France)
FF18734	<i>MATa</i> , <i>leu2-3-112</i> , <i>trp1-289</i> , <i>his7-2</i> , <i>ura3-52</i> , <i>lys1-1</i>	F. Fabre (Fontenay aux Roses, France)
CD138	FF18733 with <i>ogg1Δ::TRP1</i>	D. Thomas (CNRS, Gif/Yvette, France)
BPS1002	FF18733 with <i>rad18Δ::kanMX6</i>	This study
BPS1000	FF18734 with <i>rad6Δ::kanMX6</i>	This study
BPS1003	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad18Δ::kanMX6</i>	This study
BPS1021	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad6Δ::kanMX6</i>	This study
BPS1013	FF18733 with <i>rad6Δ::kanMX6</i> , <i>rad18Δ::kanMX6</i>	This study
BPS1016	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad6Δ::kanMX6</i> , <i>rad18Δ::kanMX6</i>	This study
BPS1011	FF18733 with <i>rad30Δ::kanMX6</i>	This study
BPS1001	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad30Δ::kanMX6</i>	This study
BPS1006	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad18Δ::kanMX6</i> , <i>rad30Δ::kanMX6</i>	This study
FF181134	FF18733 with <i>rev3Δ::URA3</i>	F. Fabre
BPS1005	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rev3Δ::URA3</i>	This study
BPS1007	FF18733 with <i>ogg1Δ::TRP1</i> , <i>rad18Δ::kanMX6</i> , <i>rev3Δ::URA3</i>	This study
BPS1031	FF18733 with <i>msh6Δ::kanMX6</i>	This study
BPS1050	FF18733 with <i>msh6Δ::kanMX6</i> , <i>ogg1Δ::TRP1</i>	This study
BPS1053	FF18733 with <i>msh6Δ::kanMX6</i> , <i>ogg1Δ::TRP1</i> , <i>rad18Δ::kanMX6</i>	This study

performed using a Singer MSM System (46). The genotypes of strains were inferred from the segregation patterns and PCR analysis when two genes possessed the same marker. Plasmid pYSB210 contains the *OGG1* gene placed under the control of the *TPI* promoter in the pYX212 plasmid (47). Plasmid pYCP11 contains the *RAD18* gene placed under the control of its own promoter in the pYCP50 plasmid, and is a gift by Dr F. Fabre (Fontenay aux Roses, France).

Spontaneous mutation rates

Yeast strains were grown in 2 ml of YPD or YNBD medium with the appropriate amino acids and bases for 2–3 days at 30°C. For each strain, 10 independent cultures were inoculated with about 10^2 cells and grown at 30°C for 2–3 days. Cell density was measured by plating dilutions on YPD or YNBD agar plates and counting the colonies after 3 days at 30°C. The quantification of canavanine-resistant mutants (Can^R) was determined after plating on selective medium (YNBD containing canavanine) (48). The quantification of Lys^+ revertants was determined after plating on selective medium (YNBD without lysine). This assay measured the reversion of the suppressible ‘ochre’ nonsense allele *lys1-1* (49). Colonies were counted after 4–5 days at 30°C. All experiments were carried out independently 3–5 times. Mutation rates were determined from the number of Can^R and Lys^+ colonies by the method of the median (50).

Spontaneous mutation spectra

For each strain tested, at least 30 independent cultures were inoculated with 10^2 cells, grown at 30°C in YPD and spread onto YNBD canavanine-containing plates. From each culture, a single mutant colony was selected and streaked onto selective canavanine-containing plates and grown for 3 days at 30°C. Genomic DNA was extracted from saturated 1.5 ml YPD cultures obtained from individual colonies isolated from the canavanine-containing plates using ZymolyaseR (ICN) and the DNeasy Tissue Kit (Qiagen). *CAN1* gene was amplified by PCR from genomic DNA with 5CAN-40 (5'-CAGAC-TTCTTAACCTCCTG-3') and 3CAN1880 (5'-GAAATGTGATCAAAGGTAATAAAACG-3'). Sequencing of the *CAN1* gene (1773 bp) was performed using three primers: 3CAN577 (5'-GGAACCTTTGTACGTCCAAATTTG-3'), 5CAN650 (5'-GGAACCTTAGTGTAGTTGG-3') and 3CAN1880 (5'-GAAATGTGATCAAAGGTAATAAAACG-3'). Sequence alignment and analysis were performed using the DNA-STRIDER program.

RESULTS

Synergistic increase of spontaneous mutation rates in *ogg1 rad18* and *ogg1 rad6* double mutant strains of *S.cerevisiae*

To investigate the capacity of the *RAD18* and *RAD6* genes and more generally of the PRR pathway in reducing the mutagenic effect of 8-oxoG in *S.cerevisiae*, we constructed the *ogg1 rad18* and *ogg1 rad6* double mutants and the *ogg1 rad6 rad18* triple mutant, and examined spontaneous mutation rates in these strains. Table 2 shows that in terms of forward Can^R mutation rates, *ogg1*, *rad18* and *rad6* single mutants are

Table 2. Rates of spontaneous Can^R and Lys^+ mutations in *ogg1*, *rad6* and *rad18* mutants of *S.cerevisiae*

Genotype	Can^R mutation rate (10^{-7})	Lys^+ mutation rate (10^{-8})
Wild-type	3.6 ± 1.4 [1]	3.8 ± 2.2 [1]
$\Delta ogg1$	18.0 ± 3.1 [5]	33.3 ± 6.0 [9]
$\Delta rad6$	7.6 ± 1.4 [2]	4.2 ± 1.2 [1]
$\Delta rad18$	12.9 ± 1.9 [4]	9.7 ± 2.3 [3]
$\Delta ogg1 \Delta rad6$	126.3 ± 11.5 [35]	85.6 ± 13.6 [23]
$\Delta ogg1 \Delta rad18$	109.4 ± 11.1 [30]	126.6 ± 19.8 [33]
$\Delta ogg1 \Delta rad6 \Delta rad18$	105.6 ± 14.9 [29]	ND

Can^R and Lys^+ mutation rates were determined using the method of the median (50). Numbers in brackets are the fold induction relative to the wild-type value. ND, not determined.

moderate spontaneous mutator strains: the rank order being *ogg1* > *rad18* > *rad6*. Table 2 also shows that reversion rates of the suppressible ochre nonsense allele *lys1-1* are also higher in *ogg1* and *rad18* single mutants than in the wild-type strain. Importantly, the *ogg1 rad18* and *ogg1 rad6* double mutants exhibit a synergistic (greater than additive) increase in Can^R and Lys^+ mutation rates, compared with those of the *ogg1*, *rad18* or *rad6* single mutants (Table 2). Indeed, the *ogg1 rad18* and *ogg1 rad6* double mutants exhibit 30- and 35-fold enhanced Can^R mutation rates, respectively, whereas additive effects would result in 9- and 7-fold increases in Can^R mutation rates, respectively (Table 2). It should be noted that the *ogg1 rad6* and *ogg1 rad18* double mutants present about the same spontaneous Can^R and Lys^+ mutation rates (Table 2). Furthermore, the rate of Can^R mutations in the *ogg1 rad6 rad18* triple mutant is very similar to those of the *ogg1 rad6* and *ogg1 rad18* double mutants (Table 2).

Since, *ogg1*, *rad6* and *rad18* single mutants are spontaneous mutators, the enhanced mutagenesis observed in *ogg1 rad6* or *ogg1 rad18* double mutants could be due to secondary mutations in unidentified genes. To exclude this possibility, we have measured Can^R mutation rates in *ogg1* and *rad18* single mutants and *ogg1 rad18* double mutant harboring plasmids that express either Ogg1 or Rad18 proteins. The results show that Ogg1 and Rad18 suppress the spontaneous mutator phenotype of *ogg1* and *rad18* single mutants, respectively (Figure 1). Figure 1 also shows that both Ogg1 and Rad18 greatly reduce the Can^R mutation rates in the *ogg1 rad18* double mutant. Therefore, the synergistic effect on spontaneous mutagenesis observed in the *ogg1 rad18* double mutant strain is most likely due to the inactivation of *OGG1* and *RAD18* genes. Taken together, these results strongly suggest that *RAD18* and *RAD6* cooperate with *OGG1* to prevent spontaneous mutations in yeast. The critical role played by *OGG1* in this process points to endogenous 8-oxoG at the origin of the mutations.

Spectrum of Can^R mutations in *ogg1 rad18* and in *ogg1 rad6* double mutants

If 8-oxoG was at the origin of the spontaneous mutator phenotype of *ogg1 rad6* and *ogg1 rad18* double mutants, the spectrum of spontaneous Can^R mutations in these strains should be strongly biased in favor of the GC to TA transversions. To test this hypothesis, sequence analysis of a number of

Figure 1. Complementation of the *ogg1 rad18* double mutant by *OGG1* or *RAD18*. Plasmids pYCP11 and pYSB210 contain the *RAD18* and *OGG1* genes placed under the control of constitutively expressed promoters. The mutation rates are determined as described in Table 2.

independent Can^R mutations was performed in wild-type and mutant yeast strains (Table 3). The results show that the spectrum of Can^R mutations in the wild-type strain is mostly composed of base pair substitutions (BPSs) (84%) with an excess of mutations at G/C base pairs (76% of BPS) (Table 3). It should be noted that the spectrum of Can^R mutations in the wild-type strain used in the present study was very similar to that obtained with another wild-type strain (26). Furthermore, spectra of Can^R mutations in *ogg1*, *rad6* and *rad18* single mutants are consistent with previous studies (26,28,29,51). Interestingly, the Can^R mutation spectra derived in *rad6* and *rad18* single mutants are significantly different, since the level of BPS is reduced in *rad6* (64%) compared to *rad18* (94%). Relevant to this study, *rad18* and *ogg1* single mutants present 11- and 24-fold increase in GC to TA transversions compared to the wild-type strain, respectively (Table 3). Moreover, Can^R mutation spectra in *ogg1 rad6* and *ogg1 rad18* double mutants exhibit a strong bias in favor of GC to TA transversion events that represent by far the major class of mutations (Table 3). Indeed, GC to TA events are 189- and 137-fold higher in *ogg1 rad6* and *ogg1 rad18* double mutants, respectively, than in the wild-type strain (Table 3). Therefore, the synergistic increase in spontaneous Can^R mutations in *ogg1 rad6* and *ogg1 rad18* double mutants is mostly due to high levels of GC to TA transversions, which convincingly points to 8-oxoG as the origin of the mutations.

DNA polymerase η (Rad30) plays a major role in the prevention of mutation at 8-oxoG by Rad18 and Rad6, whereas DNA polymerase ζ (Rev3) does not

Genetic and biochemical studies argue against a direct role of Rad18 and Rad6 in the removal of 8-oxoG in DNA and rather suggest an indirect intervention (32,33). In the current model of PRR, Rad6 and Rad18 form a complex that binds

Table 3. Spectrum of Can^R mutations

Genotype	Mutation	Occurrence	Mutation rate ^a (10 ⁻⁷)
Wild-type	GC to TA	11/61 (18%)	0.6 [1] ^b
	GC to AT	20/61 (33%)	1.2
	GC to CG	8/61 (13%)	0.5
	AT to TA	2/61 (3%)	0.1
	AT to CG	7/61 (9%)	0.3
	AT to GC	3/61 (5%)	0.2
	(-1) deletion	5/61 (8%)	0.3
	Insertions	2/61 (3%)	0.1
	Complex ^c	3/61 (5%)	0.3
	GC to TA	45/56 (80%)	14.4 [24]
$\Delta ogg1$	GC to AT	4/56 (7%)	1.3
	AT to TA	2/56 (4%)	0.7
	AT to GC	2/56 (4%)	0.7
	(+1) addition	2/56 (4%)	0.7
	Insertion	1/56 (2%)	0.4
$\Delta rad6$	GC to TA	7/31 (23%)	1.7 [3]
	GC to AT	12/31 (39%)	3.0
	GC to CG	1/31 (3%)	0.2
	AT to TA	1/31 (3%)	0.2
	(-1) deletion	4/31 (13%)	1.0
	(+1) addition	4/31 (13%)	1.0
	Insertion	1/31 (3%)	0.2
	Complex	1/31 (3%)	0.2
	GC to TA	16/30 (53%)	6.8 [11]
	GC to AT	8/30 (27%)	3.5
$\Delta rad18$	AT to TA	3/30 (10%)	1.3
	AT to CG	1/30 (3%)	0.4
	(-1) deletion	1/30 (3%)	0.4
	(+1) addition	1/30 (3%)	0.4
	GC to TA	7/21 (33%)	1.1 [2]
	GC to AT	3/21 (14%)	0.4
	AT to TA	4/21 (19%)	0.6
	AT to CG	1/21 (5%)	0.2
	AT to GC	1/21 (5%)	0.2
	(-1) deletion	4/21 (19%)	0.6
$\Delta ogg1 \Delta rad6$	Complex	1/21 (5%)	0.2
	GC to TA	27/30 (90%)	113.7 [189]
	GC to CG	1/30 (3%)	3.8
	(-1) deletion	2/30 (7%)	8.8
$\Delta ogg1 \Delta rad18$	GC to TA	24/32 (75%)	82.1 [137]
	GC to AT	5/32 (16%)	17.5
	AT to TA	1/32 (3%)	3.3
	AT to CG	1/32 (3%)	3.3
	AT to GC	1/32 (3%)	3.3
$\Delta ogg1 \Delta rad30$	GC to TA	29/30 (97%)	88.7 [148]
	AT to TA	1/30 (3%)	2.7
$\Delta ogg1 \Delta rad18 \Delta rad30$	GC to TA	28/30 (95%)	169.4 [282]
	AT to TA	1/30 (3%)	5.3
	(-1) deletion	1/30 (3%)	5.3

^aCan^R mutation rates are the product of the proportion of a specific class of mutation and the total mutation rate for each strain (Tables 2 and 4).

^bNumber in brackets is the fold induction of GC to TA relative to the wild-type value.

^cComplex mutations are the mutations that involve multiple molecular events such as base substitutions and frameshifts.

lesion-containing single-stranded DNA at stalled replication forks, Rad18 being the DNA binding protein (32,33). The Rad6–Rad18 complex catalyzes the mono-ubiquitination of target proteins such as PCNA, and promotes TLS, a process that involves DNA polymerases such as Pol η (*RAD30*) or Pol ζ (*REV3*) (32,33). Therefore, the role of Rad18 and Rad6 in the prevention of spontaneous mutations at 8-oxoG could rely on the recruitment of DNA polymerase(s). To explore this possibility, we have constructed the *rev3* and *rad30* single mutants, the *ogg1 rev3* and *ogg1 rad30* double mutants and

Table 4. Rates of spontaneous Can^R and Lys⁺ mutations in *ogg1*, *rad18*, *rev3* and *rad30* mutants of *S.cerevisiae*

Genotype	Can ^R mutation rate (10 ⁻⁷)	Lys ⁺ mutation rate (10 ⁻⁸)
Wild-type	3.6 ± 1.4 [1]	3.8 ± 2.2 [1]
Δ <i>ogg1</i>	18.0 ± 3.1 [5]	33.3 ± 6.0 [9]
Δ <i>rev3</i>	0.7 ± 0.3 [0.2]	1.3 ± 0.4 [0.3]
Δ <i>rad30</i>	3.2 ± 0.6 [1]	6.4 ± 1.5 [2]
Δ <i>ogg1</i> Δ <i>rad18</i>	109.4 ± 11.1 [30]	126.6 ± 19.8 [33]
Δ <i>ogg1</i> Δ <i>rev3</i>	12.8 ± 2.4 [4]	28.1 ± 4.4 [7]
Δ <i>ogg1</i> Δ <i>rad30</i>	91.4 ± 6.7 [25]	135.9 ± 19.5 [36]
Δ <i>ogg1</i> Δ <i>rad18</i> Δ <i>rev3</i>	128.9 ± 16.7 [36]	ND
Δ <i>ogg1</i> Δ <i>rad18</i> Δ <i>rad30</i>	178.3 ± 18.7 [50]	190.7 ± 17.2 [50]

The mutation rates are determined as described in Table 2. Numbers in brackets are the fold induction relative to the wild-type values. ND, not determined.

the *ogg1 rev3 rad18* and *ogg1 rad30 rad18* triple mutants. Table 4 shows that the Can^R and Lys⁺ mutation rates in the *ogg1 rev3* double mutant are very similar to those in the *ogg1* single mutant. Therefore, the inactivation of Polζ does not affect the Can^R or Lys⁺ mutation rates in Ogg1-deficient strains. Furthermore, the *ogg1 rad18* double mutant and the *ogg1 rad18 rev3* triple mutant exhibit similar Can^R mutation rates (Table 4). These results strongly suggest that Polζ is not required either to generate, or to prevent mutations at 8-oxoG. Consequently, the role of Rad18 and Rad6 in the prevention of 8-oxoG-induced mutations is presumably not to recruit Polζ at the site of this lesion.

In contrast, the *ogg1 rad30* double mutant exhibits a synergistic increase in Can^R and Lys⁺ mutation rates, compared to those in the *rad30* and *ogg1* single mutants, which confirms the role for Polη in the prevention of mutations at 8-oxoG [Table 4 and (27)]. It should be noted that the *rad30* single mutant does not exhibit a spontaneous mutator phenotype (Table 4). Furthermore, the results show that the *ogg1 rad18* and *ogg1 rad30* double mutants exhibit similar rates of Can^R and Lys⁺ mutation rates (Table 4). Finally, Can^R and Lys⁺ mutation rates in the *ogg1 rad18 rad30* triple mutant are only slightly (~1.5-fold) higher than those in the *ogg1 rad18* double mutant (Table 4). Spectrum of Can^R mutations was determined in the *rad30* single mutant, the *ogg1 rad30* double mutant and the *ogg1 rad18 rad30* triple mutant (Table 3). Spectrum of Can^R mutations in the *rad30* single mutant and in the wild-type are not significantly different (Table 3). Furthermore, spectrum of Can^R mutations in the *ogg1 rad30* double mutant exhibits a strong bias in favor of GC to TA events, which are 148-fold higher than in the wild-type (Table 3). Spectrum of Can^R mutations in the triple mutant is nearly exclusively composed of GC to TA transversions, which are 282-fold higher than in the wild-type (Table 3). These results suggest that Rad18, Rad6 and Polη act in the same pathway to prevent mutations at 8-oxoG. However, the action of Polη is probably not strictly dependent upon the functional Rad18–Rad6 complex, which could explain the high mutations rates in the *ogg1 rad18 rad30* triple mutant.

Role of MMR in the prevention of mutation at 8-oxoG by Rad18 and Rad6

Several lines of evidence indicate that Msh2–Msh6-dependent long patch MMR is the major pathway by which adenine

Table 5. Rates of spontaneous Can^R mutations in *ogg1*, *rad18* and *msh6* mutants of *S.cerevisiae*

Genotype	Can ^R mutation rate (10 ⁻⁷)
Wild-type	3.6 ± 1.4 [1]
Δ <i>ogg1</i>	18.0 ± 3.1 [5]
Δ <i>msh6</i>	20.7 ± 2.6 [6]
Δ <i>rad18</i>	12.9 ± 1.9 [4]
Δ <i>ogg1</i> Δ <i>msh6</i>	161.8 ± 20.9 [45]
Δ <i>ogg1</i> Δ <i>rad18</i>	109.4 ± 11.1 [30]
Δ <i>ogg1</i> Δ <i>rad18</i> Δ <i>msh6</i>	171.1 ± 22.1 [48]

The mutation rates are determined as described in Table 2. Numbers in brackets are the fold induction relative to the wild-type values. ND, not determined.

incorporated opposite 8-oxoG by DNA polymerase δ is corrected in *S.cerevisiae* (26). In this study, we show that the *RAD6* and *RAD18* genes also play a critical role in the prevention of mutations at 8-oxoG. To further explore the role of the Rad18–Rad6 complex, we compared the Can^R mutation rates in yeast strains whose *OGG1*, *RAD18* or *MSH6* genes have been deleted. The results show a synergistic increase in Can^R mutations in the *ogg1 msh6* double mutant, compared to those of *ogg1* and *msh6* single mutants [Table 5 and (26)]. The results also show that the *ogg1 msh6* double mutant and the *ogg1 rad18 msh6* triple mutant present about the same Can^R mutation rate, which is the highest one observed in this study and similar to that of the *ogg1 rad18 rad30* triple mutant (Tables 4 and 5). These results indicate that the *msh6* mutation is epistatic to the *rad18* mutation. Therefore, Rad6 and Rad18 presumably act after the MMR-dependent excision of the DNA fragment that contained adenine mispaired with 8-oxoG.

DISCUSSION

Recent studies point to the evolution of an efficient and complex ‘Replication–repair’ cellular network that prevents the mutagenic action of endogenous 8-oxoG in bacteria and eukaryotes (8–12). In *S.cerevisiae*, this network involves at least three components: the 8-oxoG DNA glycosylase Ogg1, the Msh2–Msh6-dependent MMR and the DNA polymerase η. It should be noted that Ogg1 is the essential player, because it is the only one able to remove 8-oxoG from DNA (12). As a consequence, the role of the other members of the network is unambiguously revealed only in Ogg1-deficient cells. Based on genetic and biochemical evidences, a model of repair of 8-oxoG has been proposed. It suggests that DNA Polδ bypasses 8-oxoG during the replication process, mostly incorporating an adenine opposite this lesion yielding 8-oxoG/A. This is in agreement with biochemical studies showing that 8-oxoG is not a strong block for RNA and DNA polymerases (13–18). Although yeast Polδ replicates through 8-oxoG inefficiently *in vitro*, additional factors such as PCNA may promote bypass of this lesion as described for the human enzyme (13). Afterwards, MMR promotes the removal of the adenine mispaired with 8-oxoG. Finally, Polη incorporates a cytosine across from the lesion (12,26,27).

In the present study, we show that Rad6 and Rad18 are also involved in the ‘replication–repair’ network that prevents mutation at 8-oxoG. How can Rad18 and Rad6 prevent the mutagenic action of endogenous 8-oxoG in DNA?

The antimutagenic action of *RAD18* and *RAD6* could be due to a higher rate of the formation of 8-oxoG in Rad18- or Rad6-deficient cells. Several data argue against this possibility. The over-expression of Ogg1 in the *rad18* single mutant does not suppress its spontaneous mutator phenotype, which is not consistent with an enhanced level of 8-oxoG in DNA saturating the wild-type level of Ogg1 (Figure 1). In fact, the over-expression of Ogg1 in the *rad18* mutant does not significantly reduce its mutator phenotype, which also argues against the idea that 8-oxoG is at the origin of GC to TA transversions in *rad18* single mutant strains (28). The results also show that the *msh6* mutation is epistatic to *rad18* for the spontaneous mutator phenotype in Ogg1-deficient strains, which is not expected if the role of Rad18 and Rad6 was to prevent the formation of 8-oxoG in DNA. Alternatively, Rad6 and Rad18 could be involved in the removal of 8-oxoG, like Ogg1 or in the removal of adenine incorporated opposite 8-oxoG, like MMR or MutY. Two facts argue against this hypothesis. First, the 8-oxoG DNA glycosylase activity in cell-free extracts of the *rad18* and *rad6* simple mutants is similar to that observed in wild-type extracts, whereas it is abolished in *ogg1* extracts (data not shown). Second, the *msh6* mutation is epistatic to the *rad18* mutation, which strongly argues against a direct involvement of Rad6 and Rad18 in the removal of 8-oxoG in 8-oxoG/C or that of adenine in 8-oxoG/A. Finally, these hypotheses do not fit well with the known properties of the Rad18–Rad6 complex, which primarily acts as a mono-ubiquitin-conjugating enzyme (32,33). Therefore, we favor an indirect role of Rad18 and Rad6 downstream of MMR-dependent excision of adenine in 8-oxoG/A.

Our data are consistent with models where Rad6 and Rad18 prevent the mutagenic effect of 8-oxoG via the modulation of

the activity of a DNA polymerase such as Pol η . Figure 2 summarizes our interpretation of the antimutator role of Rad6 and Rad18 in *S.cerevisiae*. The novelty of the model relies on the targeting of Pol η by the Rad6–Rad18 complex at the 3'-OH primer end of single-stranded DNA gaps that result from the excision of DNA fragments containing adenine opposite 8-oxoG by MMR. The 3'-OH end can be immediately upstream of 8-oxoG in the template strand, allowing error-free dCMP incorporation opposite this lesion and minimal incorporation at undamaged DNA by Pol η . This model is consistent with the apparent lack of contribution of Pol ζ to the mutagenic process at 8-oxoG and the very limited capacity of Pol ζ to incorporate opposite 8-oxoG (15). In addition, Pol ζ is not required to extend the primer following Pol η -mediated nucleotide incorporation opposite 8-oxoG (27). The model is also consistent with the fact that *msh6* or *msh2* mutations are epistatic to *rad18* or *rad30* mutations, for spontaneous mutagenesis in Ogg1-deficient cells. This model allows some speculation at the molecular level concerning the enzymatic properties of the Rad6–Rad18 complex. Rad6–Rad18 could mono-ubiquitinate PCNA at Lys-164 promoting the recruitment of Pol η (39–41). It also implies that Rad18 and Rad6 are not only recruited at stalled replication forks but also at single-stranded DNA gaps resulting from the action of the long-patch MMR. The binding of Rad18 at single-stranded DNA gaps may or may not require the presence of DNA damage in the template strand.

The complexity of the network orchestrated by Ogg1 points to 8-oxoG as a major cellular threat. Indeed, Ogg1 is highly conserved in the course of evolution and it has been characterized in *S.cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster* and mammals (52). In mammals, the inactivation

Figure 2. Role of Rad18 and Rad6 in the prevention of spontaneous mutations by 8-oxoG in *S.cerevisiae*. Endogenous oxidative stress generates 8-oxoG in DNA yielding (G⁰/C). 8-OxoG mispaired with cytosine (G⁰/C) is removed by the Ogg1 glycosylase in the course of a BER process restoring G/C (WT). If Ogg1 is absent or not available, Polδ replicates past 8-oxoG by inserting an adenine across from the lesion. The resulting 8-oxoG/A (G⁰/A) is recognized and a DNA fragment containing adenine in G⁰/A is excised by MMR. In the absence of MMR, cells accumulate GC to TA transversions (MUTANT). In MMR-proficient cells, the removal of adenine opposite 8-oxoG generates single-stranded DNA gaps that contain the lesions in the template strand nearby the 3'-OH end for DNA repair synthesis. Such DNA structures could be recognized by PCNA (red buoy). Due to its affinity for single-stranded DNA, Rad18 (black rectangle) binds the gap, interacts with PCNA and recruits Rad6 (violet oval). The Rad18–Rad6 complex mono-ubiquitinates PCNA (small yellow buoy), allowing the recruitment of Polη (green cube). Then, Polη undergoes error-free TLS across from 8-oxoG, restoring G⁰/C, i.e. the substrate of Ogg1. In the absence of Rad18 or Rad6, unmodified PCNA preferentially recruits Polδ at excision gaps and promotes the mutagenic incorporation of adenine. This model does not exclude minor pathway(s), with recruitment of Polη at unmodified PCNA or that of another DNA polymerase (Polx) at modified PCNA yielding MUTANT and WT.

of two major components of the network that prevents mutation at 8-oxoG, namely Ogg1 and Myh1, results in a high incidence of lung cancer (53). In contrast, the *ogg1*^{-/-} and *mhy1*^{-/-} single mutant mice are viable, and they do not show gross pathological changes compared to wild-type mice (53–55). Dysfunction of the human Rad18 results in hypersensitivity to several carcinogens, like in the yeast Rad18-deficient cells (56). Our results suggest there could be enhanced instability and cancer predisposition in *ogg1*^{-/-} *rad18*^{-/-} double mutant mice. Intriguingly, human *RAD18* and *OGG1* map on chromosome 3p24–25 (56) and 3p25–26 (57), respectively, where deletions are often found in many types of cancer in human (58). Therefore, many of the deletions found in cancers would encompass both *OGG1* and *RAD18*. This could lead to hypersensitivity following the mutagenic and carcinogenic action of the endogenous oxidative stress.

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