Homologous recombination is required for recovery from oxidative DNA damage

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(Received 8 December 2016, accepted 27 February 2017; J-STAGE Advance published date: 3 April 2017)

We have been studying the genetic events, including chromosome loss, chromosome rearrangements and intragenic point mutations, that are responsible for the deletion of a *URA3* marker in a loss of heterozygosity (LOH) assay in the yeast *Saccharomyces cerevisiae*. With this assay, we previously showed that homologous recombination plays an important role in genome maintenance in response to DNA lesions that occur spontaneously in normally growing cells. Here, to investigate DNA lesions capable of triggering homologous recombination, we examined the effects of oxidative stress, a prominent cause of endogenous DNA damage, on LOH events. Treatment of log-phase cells with H_2O_2 first caused growth arrest and then, during the subsequent recovery, chromosome loss and various chromosome rearrangements were induced more than 10-fold. Further analysis of the rearrangements showed that gene conversion was strongly induced, approximately 100 times more frequently than in untreated cells. Consistent with these results, two diploid strains deficient for homologous recombination, *rad52Δ/rad52Δ* and *rad51Δ/rad51Δ*, were sensitive to H_2O_2 treatment. In addition, chromosome DNA breaks were detected in H_2O_2 -treated cells using pulsed-field gel electrophoresis. Altogether, these results suggest that oxidative stress induced recombinogenic lesions on chromosomes, which then triggered homologous recombination leading to chromosome rearrangements, and that this response contributed to the survival of cells afflicted by oxidative DNA damage. We therefore conclude that homologous recombination is required for the recovery of cells from oxidative stress.

Key words: oxidative stress, loss of heterozygosity (LOH), genome instability

INTRODUCTION

Genetic alterations are more complex in diploid cells than in haploid cells. In particular, alterations at the chromosome level that involve the loss of an essential gene, such as chromosome loss and rearrangement, can only be studied in diploid cells because they are lethal in haploids. In humans, chromosome aberrations are often observed in cancer cells as well as in normal cells of elderly individuals, and are implicated in both cancer development and the aging process (Tischfield, 1997; Lengauer et al., 1998; Rao et al., 2017). Thus, it is clearly important to understand the mechanisms and causes leading to chromosome aberrations.

To gain an overview of chromosome aberrations at the molecular level, we are utilizing the diploid yeast *Saccharomyces cerevisiae* (Hiraoka et al., 2000; Ajima

et al., 2002; Umezu et al., 2002; Watanabe et al., 2002; Yoshida et al., 2003). As shown in Fig. 1, we have developed a loss of heterozygosity (LOH) assay method to analyze the genetic events responsible for loss of a heterozygous *URA3* marker on chromosome III, which makes the cell resistant to 5-fluoroorotic acid (5-FOA). Loss of the *URA3* marker is caused by several genetic alterations including chromosome loss, various chromosome rearrangements and intragenic point mutations, and, to classify these events, two additional markers on the same chromosome are utilized to monitor how chromosomes are rearranged in three different phenotypes: 5-FOA^r Leu⁻ Ade⁻ (Class A), 5-FOA^r Leu⁺ Ade⁻ (Class B) and 5-FOA^r Leu⁺ Ade⁺ (Class C). In addition to determining the frequencies of each class, chromosome structure in LOH clones can be physically analyzed by pulsed-field gel electrophoresis (PFGE) and PCR, in which aberrant-sized chromosome III derivatives are readily detected (Hiraoka et al., 2000). We can define these events at the nucleotide level, when necessary, by determining the fusion points of aberrant-sized chromosomes using a PCR-based

Edited by Hiroshi Iwasaki

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DOI: <http://doi.org/10.1266/ggs.16-00066>

Fig. 1. LOH assay. Classification of genetic alterations leading to functional inactivation of the *URA3* marker in strain RD301. Chromosomes III in parent strain RD301 (top) and their possible alteration in 5-FOA⁺ convertants (bottom) are illustrated with relative positions of the three markers used for the analysis. The 5-FOA⁺ convertants are classified with their indicated phenotypes (Class A–C) and with alteration patterns of chromosome III (a–c). The segments of chromosome III originally harboring the markers are shown by open bars and those of the homologous chromosome III are shown by shaded bars, with their centromeres as circles. The solid bar in Class B-a indicates a chromosome segment translocated to the marked chromosome III from another chromosome. The *URA3* insert at III-205 is indicated by an open triangle, the *ADE2* insert at III-314 is shown by solid triangles, and the positions of intrinsic *LEU2* loci are indicated by vertical lines, which are marked with a cross for the *leu2* allele. A point mutation inactivating the *URA3* insert is shown by a cross on the open triangle.

method to quantify the ploidy at a series of loci along chromosome III (Umezumi et al., 2002).

By exploiting these molecular genetic methods, we have determined the factors involved in spontaneous genetic alterations arising in normally growing cells. The total LOH frequency of wild-type cells was $1-2 \times 10^{-4}$ and the analysis of LOH events revealed that homologous recombination is involved in processes leading to LOH in multiple ways, including allelic recombination, chromosome size alteration and a particular kind of chromosome loss (Hiraoka et al., 2000). Analysis of the fusion points of more than 80 aberrant chromosomes revealed that all of them had breakpoints within repetitive sequences scattered over the genome, such as Ty1, indicating that

homologous recombination is a leading process in chromosome size alteration (Umezumi et al., 2002). In recombination-deficient mutants, on the other hand, LOH events were significantly increased up to frequencies of $3-5 \times 10^{-3}$, and mostly involved chromosome loss, indicating that homologous recombination also plays an important role in proper chromosome maintenance (Yoshida et al., 2003). Taken together, these results strongly suggest that spontaneous DNA lesions capable of triggering homologous recombination occur at a strikingly high frequency throughout the genome during normal cell growth; the majority of lesions are repaired through sister chromatid recombination, but occasionally this process leads to LOH events.

To characterize DNA lesions triggering homologous recombination, here we studied the effects of oxidative stress on LOH events. Although previous studies by others have shown that radical oxygen species are involved in chromosome rearrangements in yeast, the assays used can detect a narrower range of rearrangements than our LOH assay. One group analyzed events called gross chromosomal rearrangements in haploid cells (Chen et al., 1998; Ragu et al., 2007), and the other identified a specific deletion between a tandem repeat on a chromosome in diploids (Brennan et al., 1994). We therefore anticipated that our LOH assay would yield an overview of the mutagenic effects of oxidative stress. Yeast cells were treated with H₂O₂, which in the cells generates highly reactive hydroxyl radicals that have direct effects on most cellular constituents including DNA (Dawes, 2006). The effects of oxidative damage on DNA and nucleotides are well defined and cause specific point mutations (Nakatsu and Sekiguchi, 2006; Rasmussen, 2006). We demonstrate that chromosome alterations as well as point mutations were induced by the oxidative treatment, and that gene conversion-type rearrangements, the least erroneous rearrangements among LOH events, were especially increased.

MATERIALS AND METHODS

Media Media for yeast strains included complex glucose (YPD), synthetic complete (SC) and various drop-out media, and were prepared as previously described (Rose et al., 1990). 5-FOA plates were prepared as described (Rose et al., 1990) and depleted of leucine and/or adenine sulfate where indicated.

Strains All yeast strains used in this study are derivatives of YKU23 (*MAT α lys2 Δ 202 leu2 Δ 1 ura3-52 his3 Δ 200 ade2 Δ ::hisG*) and YKU34 (*MAT α lys2 Δ 202 ura3-52 trp1 Δ 63 ade2 Δ ::hisG III-205::URA3 III-314::ADE2*) with the S288c background (Hiraoka et al., 2000). III-205::URA3 signifies that the *URA3* fragment was inserted at a locus 205 kb from the left end of chromosome III. Similarly, III-

314::ADE2 denotes that the ADE2 fragment was inserted at 314 kb. Nucleotide coordinates are as given in the Saccharomyces Genome Database (<http://www.yeastgenome.org>). Derivatives of *rad52Δ* and *rad51Δ* mutations from YKU23 are YMO2 and YMO6, respectively, and those from YKU34 are YMO9 and YMO8, respectively (Yoshida et al., 2003). Diploid cells were constructed by mating between haploid **a** cells and **α** cells, and selection on SC medium depleted of uracil, leucine, adenine, histidine and tryptophan just before use. Diploid strains are designated RD301 (wild-type), RD304 (*rad52Δ/rad52Δ*) and RD305 (*rad51Δ/rad51Δ*) (Hiraoka et al., 2000; Yoshida et al., 2003).

H₂O₂ or methyl methanesulfonate (MMS) treatment H₂O₂ or MMS treatment was performed as previously described (Ragu et al., 2007) with minor modifications. Haploid (YKU34) or diploid cells were grown to mid-logarithmic phase at 30 °C in SC medium depleted of uracil, leucine and adenine, and about 1×10^7 cells were then collected, washed twice with distilled water and suspended in distilled water at 1×10^7 cells/ml, after which H₂O₂ or MMS was added to the indicated concentration. After being incubated for 60 min at 30 °C, the cells were washed twice with distilled water and analyzed. Survival of the H₂O₂-treated cells was determined on YPD plates after incubation for 4 days at 30 °C.

Analysis of cell growth and 5-FOA^r conversion after H₂O₂ treatment H₂O₂-treated cells were inoculated in YPD medium and incubated at 30 °C with vigorous shaking. At the indicated time points, aliquots of cells were removed, diluted, and spread on YPD and 5-FOA plates. Colonies were counted after incubation for 4 days at 30 °C. At least three independent experiments were performed to determine the frequencies of 5-FOA^r clones.

LOH assay LOH assay was performed basically as previously described (Hiraoka et al., 2000; Yoshida et al., 2003) with minor modification. H₂O₂-treated cells were inoculated in YPD medium, and incubated at 30 °C with vigorous shaking for 22 h. After appropriate dilution, the cells were spread on YPD, 5-FOA, 5-FOA leucine-depleted, and 5-FOA leucine- and adenine-depleted plates, and incubated at 30 °C for 3–5 days. At least three independent experiments were performed to determine the frequencies of LOH. The frequency of FOA^r Leu⁻ clones was determined by subtracting the mean frequency of FOA^r Leu⁺ clones from that of FOA^r clones. Similarly, the frequency of FOA^r Leu⁺ Ade⁻ clones was determined by subtracting the mean frequency of FOA^r Leu⁺ Ade⁺ clones from that of FOA^r Leu⁺ cells.

PCR procedures to classify Class C events Yeast genomic DNA was purified using InstaGene Matrix (Bio-

Rad, Hercules, CA, USA) according to the manufacturer's instructions. Two microliters of the DNA preparation was used in a 20-μl PCR reaction. PCR was performed under standard conditions with Ex Taq DNA polymerase (Takara, Tokyo, Japan) to determine genetic events of Class C clones as previously described (Hiraoka et al., 2000). Briefly, intrachromosomal deletion between the *MAT* and *HMR* loci was detected by PCR with primers encompassing these loci. Semi-quantitative PCR using primers encompassing the *URA3*-inserted locus (*III*-205) was performed to distinguish intragenic point mutation and gene conversion-type rearrangements. Genomic DNA of the former generated PCR products from both intact *III*-205 and *URA3*-inserted *III*-205 loci, while that of the latter generated a product only from the intact *III*-205 locus.

PFGE PFGE analysis of chromosomes was performed as previously described (Hiraoka et al., 2000). Agarose plugs of chromosomal DNA were prepared using a CHEF yeast genomic DNA plug kit (Bio-Rad) according to the manufacturer's instructions. The plugs were made using cells from equal volumes of culture. Electrophoresis was carried out with 1% PFGE-certified agarose (Bio-Rad) in $0.5 \times$ TBE buffer at 14 °C, using a CHEF Mapper XA pulsed-field electrophoresis system (Bio-Rad). Chromosomes were separated at 6 V/cm of pulses angled at 120°. Switch time was linearly increased from 24.03 sec to 1 min 33.69 sec for 29 h 57 min for standard analysis (Fig. 6B). Higher resolution around chromosome *XII* was obtained by altering the switch time linearly from 24.03 sec to 3 min 48.48 sec for 28 h 59 min (Fig. 6A). After electrophoresis, the gel was stained with ethidium bromide and destained with water. Gel images were acquired with the CCD camera of a Dolphin-View image system (Wealtec, Sparks, NV, USA).

RESULTS

Diploid cells are more resistant to oxidative stress than haploid cells To examine whether oxidative DNA damage could be a cause of the spontaneous homologous recombination that we detected previously with our LOH analysis (Hiraoka et al., 2000; Umezu et al., 2002), yeast cells were first exposed to oxidative stress using H₂O₂, which is known to be produced endogenously during normal cellular metabolism (Friedberg et al., 2006). Within the cells, H₂O₂ is converted in the Fenton reaction to the hydroxyl radical, which is one of the most reactive oxygen radicals and causes serious damage to most cellular constituents including DNA (Dawes, 2006; Nakatsu and Sekiguchi, 2006). In Fig. 2, the survival curves against H₂O₂ are compared for diploid and haploid cells. The results showed that diploid cells were approximately 10-fold more resistant to H₂O₂ than haploids, suggest-

Fig. 2. Diploid cells are more resistant to H_2O_2 than haploid cells. Log-phase cultures of wild-type diploid or haploid cells were treated with the indicated concentrations of H_2O_2 , and survival was determined as described in Materials and Methods. Viability was determined in experiments repeated at least three times. Error bars represent standard deviation.

ing that some recovery processes available only in diploid cells contributes to cell survival. Another oxidant called menadione, which also generates hydroxyl radicals in the cells (Nutter et al., 1992), yielded similar survival curves to those against H_2O_2 , with diploids again clearly more resistant than haploids (data not shown), suggesting that the difference between diploid and haploid cells was associated with their recovery from damage caused by hydroxyl radicals. Since one repair mechanism that operates only in diploids is recombination between homologous chromosomes, we next tested whether chromosome rearrangements were indeed induced in these cells during their recovery from H_2O_2 treatment.

Oxidative stress causes cell growth arrest and increases LOH emergence During the above experiments, we noticed that H_2O_2 treatment arrested cell growth, presumably by activating DNA damage checkpoints. Figure 3A shows the growth curves of yeast cells after treatment with various concentrations of H_2O_2 . The duration of cell growth arrest was prolonged in a dose-dependent manner: treatment with 32 mM H_2O_2 delayed cell growth for several hours, whereas the delay with 8 mM H_2O_2 was about one hour.

We therefore next examined if and when LOH convertants emerged in the populations after H_2O_2 treatment (Fig. 3B). The total LOH frequency in untreated cells, providing a baseline of spontaneous LOH in normally growing cells, increased depending on cell growth up to nearly 10^{-4} , consistent with our previous results (Hiraoka et al., 2000). LOH frequency in H_2O_2 -treated cells increased in a dose-dependent manner; when the cells were treated with 32 mM H_2O_2 , the emergence of LOH convertants was more than 10-fold higher than that

Fig. 3. H_2O_2 treatment induces cell growth arrest and LOH convertants emerge during the recovery process. Wild-type diploid cells of log-phase cultures were treated with the indicated concentrations of H_2O_2 for 60 min at 30 °C, and then cultured in YPD medium without H_2O_2 at 30 °C. At the indicated time points, the number of viable cells (A) and the frequency of LOH clones (B) in the culture were determined as described in Materials and Methods. Similar experiments were also performed with 2 mM and 16 mM H_2O_2 and the results were consistent with those shown here (data not shown).

in untreated cells. A comparison of the induction of LOH (Fig. 3B) with the population growth curves (Fig. 3A) indicated that LOH clones began to emerge above the basal level of the untreated cells after cells started to grow again. When cells were treated with 32 mM H_2O_2 , it took several hours after the treatment until LOH clones emerged more frequently than the spontaneous level, and afterward the frequency of LOH increased depending on cell growth, suggesting that the recovery process from oxidative damage was accompanied by genetic alterations observed as LOH.

In Table 1 and Fig. 4, LOH clones induced by various concentrations of H_2O_2 were further classified into three groups of genetic events (Classes A, B and C) according to their phenotypes (Fig. 1). For untreated cells, half of the events were Class B interchromosomal rearrangements, and most of the others were Class A chromosome loss, in agreement with our previous findings for spon-

Table 1. Classification of LOH induced by H₂O₂ treatment

H ₂ O ₂ (mM)	5-FOA ^r frequency ($\times 10^{-5}$) (fold increase)					
	Class A		Class B		Class C	Total
0	3.9 $\pm$ 0.7	($\times 1.0$)	5.4 $\pm$ 1.1	($\times 1.0$)	1.1 $\pm$ 0.2 ($\times 1.0$)	10.4 $\pm$ 0.9 ($\times 1.0$)
0.5	24.5 $\pm$ 6.0	($\times 6.3$)	27.9 $\pm$ 3.2	($\times 5.2$)	10.2 $\pm$ 1.6 ($\times 9.3$)	62.6 $\pm$ 10.0 ($\times 6.0$)
2	28.4 $\pm$ 7.5	($\times 7.3$)	33.3 $\pm$ 3.5	($\times 6.2$)	12.9 $\pm$ 1.4 ($\times 11.7$)	74.6 $\pm$ 12.3 ($\times 7.2$)
8	29.5 $\pm$ 5.7	($\times 7.6$)	39.8 $\pm$ 3.1	($\times 7.4$)	17.4 $\pm$ 1.2 ($\times 15.8$)	86.8 $\pm$ 8.7 ($\times 8.3$)
16	33.9 $\pm$ 13.6	($\times 8.7$)	44.5 $\pm$ 11.1	($\times 8.2$)	24.1 $\pm$ 4.0 ($\times 21.9$)	102.6 $\pm$ 9.2 ($\times 9.7$)
32	29.8 $\pm$ 3.3	($\times 7.6$)	58.6 $\pm$ 17.1	($\times 10.8$)	32.3 $\pm$ 7.6 ($\times 29.4$)	120.7 $\pm$ 16.4 ($\times 11.6$)

LOH clones were classified into the indicated three classes according to their phenotypes as shown in Fig. 1. Indicated are the mean frequencies with standard deviations determined from at least three independent experiments. In parenthesis, fold increase relative to untreated cells is indicated.

Fig. 4. Oxidative stress induces all three classes of LOH events, but especially Class C. The cumulative bar graphs plot the results shown in Table 1. Frequencies of LOH Classes A–C in cells treated with the indicated concentration of H₂O₂ are incorporated into each bar, whose height consequently indicates the total LOH frequency.

taneous LOH (Hiraoka et al., 2000). For LOH events induced by H₂O₂, although the frequencies of all three classes increased, the degree of induction and the dose dependency of frequencies varied among classes. Class C was most highly induced among the three and the frequency increased in a dose-dependent manner. While Class C events that occurred spontaneously without H₂O₂ treatment accounted for about 10% of total LOH, treatment with 32 mM H₂O₂ increased their proportion to around 25%, and the frequency was 30-fold higher than that in untreated cells. The induction of Class B interchromosomal recombination events was also dose-dependent. When the cells were treated with 32 mM H₂O₂, the frequency of Class B was 11-fold higher than that in untreated cells, about one-third of the induction

level of Class C. On the other hand, Class A chromosome loss events were induced 6- to 9-fold by H₂O₂ and the dose dependency of their induction was unclear. These results led us to consider whether one specific genetic event within Class C might be particularly induced by H₂O₂.

We therefore further defined Class C clones and classified them into the following three genetic events by PCR-based analysis: a) an intrachromosomal 94-kb deletion between the *MATa* and *HMR* loci encompassing the *URA3* marker, known as Hawthorne deletion (Hawthorne, 1963); b) allelic interchromosomal rearrangements in which only *URA3* among the three markers was lost (gene conversion-type); and c) point mutations within the *URA3* marker (Fig. 1). Among spontaneous Class C LOH events analyzed in our previous work, *MATa-HMR* deletion was the most common, followed by gene conversion-type rearrangements, while point mutations were rare (Hiraoka et al., 2000). H₂O₂ treatment significantly changed the distribution of these events within Class C. Table 2 shows the classification of Class C clones obtained from untreated and 32 mM H₂O₂-treated cells. Although all three events were induced by H₂O₂ treatment, the contributions of individual events differed from those in spontaneous LOH. The vast majority of the events induced by H₂O₂ were gene conversion-type rearrangements and their frequency was almost 100 times that in untreated cells. The magnitude of this increase indicated that gene conversion was the most highly induced type of LOH event in all three Classes. This allelic interchromosomal recombination results in the restoration of a normal-sized chromosome with internal exchange between homologous chromosomes, and hence could be defined as the least erroneous type of rearrangement among LOH events. It is notable that spontaneous gene conversion in our assay depends on both *RAD51* and *RAD52* genes, and hence presumably resulted from a conventional type of homologous recombination reaction involving both of these genes (Yoshida et al., 2003).

Table 2. Determination of genetic events giving rise to Class C LOH clones

Class C event	0 mM H ₂ O ₂		32 mM H ₂ O ₂		Fold increase in frequency
	Ratio (%)	Frequency (× 10 ⁻⁵)	Ratio (%)	Frequency (× 10 ⁻⁵)	
Point mutation	6.6	0.07	6.7	2.2	31
Gene conversion	26.7	0.29	86.6	27.9	97
Deletion	66.7	0.74	6.7	2.2	3
Total	100	1.1	100	32.3	29

LOH clones of Class C obtained from untreated and 32 mM H₂O₂-treated cells were analyzed by PCR-based methods as described in Materials and Methods to determine genetic causes of LOH. Thirty clones were analyzed for H₂O₂-treated cells and 15 for untreated cells.

Fig. 5. Recombination-deficient diploid cells are sensitive to H₂O₂. Survival curves without or after H₂O₂ treatment were determined for wild-type (wt/wt), *rad51Δ/rad51Δ* and *rad52Δ/rad52Δ* diploid strains, as described in Materials and Methods. The strains used were RD301 (wt/wt), RD305 (*rad51Δ/rad51Δ*) and RD304 (*rad52Δ/rad52Δ*). Viability was determined in experiments repeated at least three times. Error bars represent standard deviation.

Mutant strains defective in homologous recombination are more sensitive to oxidative stress Next, we examined the effects of the recombination genes *RAD51* and *RAD52* on survival after H₂O₂ treatment (Fig. 5). *rad52Δ/rad52Δ* mutant cells, known to be totally deficient in homologous recombination (Symington, 2002; Mehta and Haber, 2014), were sensitive to H₂O₂, displaying 30–40% survival relative to wild-type cells. The homozygous deletion mutant of *RAD51*, which encodes the key enzyme responsible for homology search and strand exchange in yeast (Symington, 2002; Mehta and Haber, 2014), was also more sensitive to H₂O₂ than wild type. These strains were also treated with menadione and showed patterns of sensitivity similar to those obtained with H₂O₂ (data not shown). These results suggest that homologous recombination plays an important

Fig. 6. H₂O₂ treatment induces DNA strand breaks on chromosomes. DNA within cells treated with H₂O₂ or MMS was analyzed by PFGE as described in Materials and Methods. Cells from equal volumes of culture were used to make PFGE plugs for each experiment shown in (A) and (B). The ethidium bromide-stained gels are shown. The identity of the chromosome (Ch) bands is indicated to the right. (A) Cells were treated with H₂O₂ or MMS at the indicated concentration for 60 min at 30 °C. (B) Cells were treated with the indicated concentration of H₂O₂ or MMS for 60 min at 30 °C, and, for 64 mM H₂O₂-treated cells, subsequently incubated in fresh rich medium for the indicated time period.

part in the recovery process and consequent survival of cells damaged by hydroxyl radicals.

Oxidative stress causes strand breaks on chromosomal DNA Finally, we directly analyzed chromosomal DNA within H₂O₂-treated cells by PFGE to examine if visible DNA lesions were induced by the treatment (Fig. 6). As a control agent, cells were also treated with MMS, which is known to cause strand breaks in DNA (Schwartz, 1989). As shown in Fig. 6A, when the cells were treated with H₂O₂, chromosome bands became smeared in a dose-dependent manner and the intact bands of longer chromosomes became faint at the higher concentrations. The longest chromosome, Ch XII, became almost invisible at the highest concentration 500 mM. This result is consistent with the notion that a longer chromosome has a higher likelihood of acquiring a DNA lesion. These results indicate that DNA double-strand breaks were introduced by the treatment. In the time-course experiments shown in Fig. 6B, smeared chromosome bands were observed up to six hours after H₂O₂ treatment, corresponding to the growth-arrested cell population (Fig. 3A). The extent of degradation of chromosomal DNA proceeded up to four hours after treatment, presumably reflecting the occurrence of double-strand breaks within cells entering S phase (see Discussion). On the other hand, within the cells surviving growth arrest that were sampled at 22 h after treatment, chromosomal DNA appeared to have become intact again. DNA double-strand breaks are

well-known recombinogenic lesions (Symington, 2002; Mehta and Haber, 2014), and the breaks detected here may represent the trigger events for the observed chromosome rearrangements induced by H_2O_2 treatment.

DISCUSSION

In living cells, genomic DNA is continuously assaulted, with various DNA lesions occurring endogenously even in normal growth, and the efficient repair of these lesions is essential to protect genome integrity (Friedberg et al., 2006; Rasmussen, 2006). In this context, homologous recombination serves as an important pathway, as shown by the fact that its inactivation results in loss of chromosomes at a high frequency (Yoshida et al., 2003). Although the majority of recombination is precise and accompanied by no genetic changes, it is occasionally inaccurate, leading to chromosome rearrangements (Kuzminov, 1999; Mehta and Haber, 2014). These observations indicate that DNA lesions triggering recombination occur at a high frequency during mitotic growth. Several lines of evidence suggest that, in our assay, most LOH events occur in S phase, implying that recombinogenic lesions arise during DNA replication (Watanabe et al., 2002; Yoshida et al., 2003). When the replication machinery encounters DNA damage in regions lacking coding information, the machinery collapses or stalls, leaving replication-induced lesions on both strands, namely two-stranded lesions (Kuzminov, 1999; Symington, 2002; Mehta and Haber, 2014). These lesions can be repaired using an undamaged sister chromatid as a donor for homologous recombination, and this kind of role for recombination in replication restart is widely accepted (Kuzminov, 1999; Symington, 2002; Mehta and Haber, 2014). However, the nature of the original DNA damage that collapses or stalls the replication machinery has not yet been clarified. Here, we focused on oxidative stress as a trigger of homologous recombination, as it is one of the leading causes of DNA damage in normally growing cells (Friedberg et al., 2006).

Among the reactive oxygen species produced in normal cellular metabolism, hydroxyl radicals are the most prominent and active, and are known to directly react with DNA and nucleotides, which could lead to recombinogenic lesions on chromosomal DNA (Friedberg et al., 2006; Nakatsu and Sekiguchi, 2006; Rasmussen, 2006). To generate hydroxyl radicals in yeast cells, we utilized H_2O_2 and also, in some cases, menadione. H_2O_2 treatment of cells caused growth arrest, presumably by activating DNA damage checkpoints, and the process of recovery from the arrest was accompanied by genetic alterations which were isolated as LOH events. Point mutations were induced by H_2O_2 treatment about 30-fold more frequently than in untreated cells (Table 2), indicating that H_2O_2 treatment directly caused DNA and/or nucleotide

damage within the treated cells. In addition, physical analysis of chromosomal DNA within H_2O_2 -treated cells revealed that many double-strand breaks were generated in the arrested cells, as previously shown by others (Ribeiro et al., 2006; Azevedo et al., 2011). These double-strand breaks could either have been caused directly by hydroxyl radicals or have resulted from replication-induced strand breaks triggered by oxidative DNA damage generated by the hydroxyl radicals. In either case, the resulting DNA double-strand breaks are well known to induce homologous recombination reactions (Symington, 2002; Mehta and Haber, 2014).

Consistent with the idea that double-strand breaks are recombinogenic lesions, various chromosome rearrangements were observed at high frequency within H_2O_2 -treated cells (Table 1 and 2), among which gene conversion-type rearrangements were most highly increased, to about 100-fold their frequency in untreated cells. As the gene conversion-type rearrangements of our LOH assay could result from several mechanisms of homologous recombination, the reason for this particular induction of gene conversion is, as yet, unclear. However, it cannot be explained simply by two crossing-over events across the *URA3* marker on the observed chromosome, which may be triggered when multiple DNA lesions are generated by H_2O_2 treatment, because allelic interchromosomal recombination of Class B (crossing-over type) was not increased to a level comparable to that of the gene conversion type. It is noteworthy that, in our previous studies of spontaneous LOH events, gene conversion-type rearrangements were absolutely dependent on both *RAD51* and *RAD52* genes (Yoshida et al., 2003). The requirement of *RAD51* strongly suggests that gene conversion-type rearrangements in our LOH assay, at least spontaneous ones, are produced by a conventional type of homologous recombination reaction involving homology search and strand exchange by Rad51 protein. In the case of gene conversion-type rearrangements, the resulting rearranged chromosome maintains its overall original structure, other than in the region around the *URA3* marker, and hence may be defined as the least erroneous rearrangement within LOH events, compared both with more drastic rearrangements such as translocations and also with allelic crossing over. Cells may possess a regulatory mechanism to control homologous recombination in such a way as to suppress genome instability if possible, even when responding to a massive dose of recombinogenic DNA lesions, as in the case of the H_2O_2 treatment in this study. In the absence of such a mechanism, the same repair process might result in more extensive genetic alterations, such as aberrant chromosomes and chromosome loss, and then lead to devastating outcomes including cancer and various genetic diseases.

This work was supported by the MEXT-Supported Program

for the Strategic Research Foundation at Private Universities, 2008–2012 and 2011–2016. We thank Dr. Mutsuo Sekiguchi and Dr. Hiroaki Nakayama for discussions and advice.

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