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Rad1, rad10 and rad52* Mutations Reduce the Increase of Microhomology Length during Radiation-Induced Microhomology-Mediated Illegitimate Recombination in *Saccharomyces cerevisiae

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Abstract

Illegitimate recombination can repair DNA double-strand breaks in one of two ways, either without sequence homology or by using a few base pairs of homology at the junctions. The second process is known as microhomology-mediated recombination. Previous studies showed that ionizing radiation and restriction enzymes increase the frequency of microhomology-mediated recombination *in trans* during rejoining of unirradiated plasmids or during integration of plasmids into the genome. Here we show that radiation-induced microhomology-mediated recombination is reduced by deletion of *RAD52*, *RAD1* and *RAD10* but is not affected by deletion of *RAD51* and *RAD2*. The *rad52* mutant did not change the frequency of radiation-induced microhomology-mediated recombination but rather reduced the length of microhomology required to undergo repair during radiation-induced recombination. The *rad1* and *rad10* mutants exhibited a smaller increase in the frequency of radiation-induced microhomology-mediated recombination, and the radiation-induced integration junctions from these mutants did not show more than 4 bp of microhomology. These results suggest that Rad52 facilitates annealing of short homologous sequences during integration and that Rad1/Rad10 endonuclease mediates removal of the displaced 3' single-stranded DNA ends after base-pairing of microhomology sequences, when more than 4 bp of microhomology are used. Taken together, these results suggest that radiation-induced microhomology-mediated recombination is under the same genetic control as the single-strand annealing apparatus that requires the *RAD52*, *RAD1* and *RAD10* genes.

INTRODUCTION

Chromosomal DNA double-strand breaks (DSBs) are highly cytotoxic to cells. Homologous recombination (HR) is the main mechanism for repair of DSBs in yeast. In the presence of a homologous sequence, the DSB is repaired by the gene conversion pathway that involves strand invasion and copying of the genetic material from the homologous template (1). When a DSB is flanked by two direct repeats, single-strand annealing occurs and results in deletion of one of the repeats and all of the intervening sequences (2). The HR process is mediated by the *RAD52* epistasis group (3).

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SUPPLEMENTARY INFORMATION

Tables S1–S5. The genomic location, locus and gene of the target sites in the *rad51*, *rad52*, *rad1*, *rad10* and *rad2* mutants. <http://dx.doi.org/10.1667/RR1675.1.S1>

Illegitimate recombination or nonhomologous end joining (NHEJ) joins broken DNA ends without extensive sequence homology (4). It is a minor DSB repair pathway in yeast that is evident in the absence of essential genes in homologous recombination or in the absence of homologous sequences (5–7). The main genetic factors required for NHEJ in yeast include the yKu70/yKu80 heterodimer, Rad50/Mre11/Xrs2 complex and Dnl4/Lif1/Nej1 complex (8).

An alternative pathway of end joining uses a few base pairs of homology at the recombined junctions and is called microhomology-mediated recombination (MHMR) (5,6). MHMR is independent of *YKU70* and *YKU80* and thus is distinct from the classical NHEJ pathway (7,9–12). MHMR is usually associated with deletions and other genomic rearrangements that have been implicated in human cancer and genetic diseases (13–17).

Ionizing radiation and restriction enzymes increase the frequency of nonhomologous integration (NHI) by severalfold in yeast (11,18,19). Previously, we found that ionizing radiation- and restriction enzyme-induced NHI events exhibited an increased frequency of microhomology use at the integration junctions (20). We also showed that restriction enzymes enhance the efficiency of NHI at non-restriction sites and that overexpression of *I-SceI* endonuclease induces MHMR events at random genomic locations rather than being targeted to the proximity of the *I-SceI*-induced DSB. We further demonstrated that exposure of yeast and mammalian cells to radiation before transformation of the linearized plasmid increases the frequency of microhomology-mediated end joining (MMEJ) of the unirradiated plasmid that is *in trans* to radiation-induced damage (21). These previous results suggest that DSBs induce MHMR *in trans* at random non-targeted sites during integration of the plasmid into the genome. However, little is known about the genetic control of the radiation-induced MHMR pathway. Since MHMR is independent of Ku (7,9–12), we investigated some candidate genes other than those involved in the classical NHEJ pathway in the current work. We examined the following genes whose encoded products exhibit homologous pairing or nuclease activities, which we assumed may play a role in the radiation-induced MHMR pathway.

The *RAD51* and *RAD52* genes are involved in DSB repair in yeast. Both gene products promote strand exchange and annealing of complementary DNA strands in homologous recombination (22,23). Rad52 displaces RPA from single-stranded DNA and promotes assembly of Rad51 onto single-stranded DNA to form nucleoprotein filaments for strand invasion (23,24). *RAD52* also promotes *RAD51*-independent single-strand annealing in the presence of two direct repeats flanking a DSB (25).

The Rad1/Rad10 complex in *S. cerevisiae* functions as a structure-specific single-stranded endonuclease that cleaves at the duplex/3' single-strand DNA junction (26). This complex is required for removal of 3' single-strand DNA from the recombining DNA in single-strand annealing (27) and for 5' incision of UV-radiation-induced damage during nucleotide excision repair (28). Rad2, a 5' to 3' exonuclease and a 5' flap endonuclease (29,30), is required for 3' incision of UV-radiation-induced lesions in nucleotide excision repair (31).

In this study, we examined the role of the potential genes *RAD51*, *RAD52*, *RAD1*, *RAD10*, *RAD2* and *DNL4* in MHMR in the absence and presence of γ radiation by sequence analysis of the integration target sites. We examined the effect of these gene mutations on the frequency of spontaneous and radiation-induced NHI, and in particular on the distribution of microhomology use during nonhomologous integration.

MATERIALS AND METHODS

Strains and Media

Experiments were performed in the haploid *Saccharomyces cerevisiae* strain RSY12 (*MATa leu2-3,112 his3-11,15 ura3Δ::HIS3*), in which the entire *URA3* open reading frame and promoter sequence was replaced by the *HIS3* gene (19). *rad51*, *rad52* and *rad1* derivatives of the RSY12 background were described previously (32). The *rad10* derivative was constructed by integration of pDD37 into RSY12 (33). The disruption cassettes of *RAD2* and *DNL4* containing a *LEU2* marker were generated by PCR-based methods using the plasmid YEplac128 as the template and the following primers: RAD2-LEU2-FW 5'-GATGTAATA ACATATACT TATGTATGATTTAC-TATTTATAAACATTACCTACCGTCATCACCG AAACG-3', RAD2-LEU2-RC 5'-GTAAGAGAATTTCAACCGCAACAGG-TAAA CTAAAGAAAAGA AAGATGTAAATAGGCGTATCAC-GAGGC-3', DNL4-LEU2-FW 5'-TAGGTATGATATCAGCAC-TAGATTCTATACCCGAGCCCCAAACTTTGCG ACCGTCA-TCACCGAAACG-3' and DNL4-LEU2-RC 5'-CACCATCAG-TAGTTGA CTACGGGGAAGTC TTCTTCAGGCACTTGA-CAGTTATAGGCGTATCACGAGGC-3'. The disruption cassette was then transformed into RSY12 to generate the *rad2* and *dnl4* derivatives. *E. coli* strain DH5α was used for the maintenance and amplification of plasmid DNA.

Plasmids

YEplac195 contains the *URA3* marker for selection and the 2-μm origin of replication and was used to control for transformation efficiency. Plasmid pM151 was described previously (19) and contains a 1.1-kb *URA3* *HindIII* fragment.

Molecular Techniques

Standard methods were followed except as noted below. pM151 was digested with *Bgl*III and prepared as described (19). For yeast transformation, the "Lithium Acetate/Single Stranded DNA/PEG" transformation method was used (34). The spontaneous and radiation-induced integration target sites were determined by direct genomic sequencing or plasmid rescue that was described previously (20).

γ Irradiation

After transformation with the integrating DNA, yeast cells were plated onto synthetic complete medium lacking uracil and exposed to 50 Gy of γ rays using a Mark-I irradiator (J. L. Shepherd and Associates, San Fernando, CA), with a ¹³⁷Cs source and a dose rate of 5.29 Gy/min.

Statistical Analysis

The frequency of NHI before irradiation was compared to that after irradiation by the Student's *t* test. The frequencies of microhomology use during spontaneous and radiation-induced NHI were analyzed by a χ^2 test, Fisher's exact test or Mann-Whitney *U* test. A χ^2 test with Yates' correction was used when one of the expected frequencies was less than 5 in a 2 by 2 table.

RESULTS

Radiation-Induced Increase of Microhomology Length during Nonhomologous Integration is Reduced by *rad52* but not *rad51* Deletions

We determined the roles of the *RAD51* and *RAD52* genes, which are known to be involved in homologous pairing (22,23), in spontaneous and radiation-induced NHI. We transformed the *Bgl*III-linearized *URA3*-containing plasmid pM151 and the circularized episomal plasmid YEplac195 in parallel, as the transformation efficiency control, into the mutant cells in the absence and presence of 50 Gy of γ radiation and selected for stable *Ura*⁺ transformants. Genomic DNA was subsequently isolated from stable transformants. The junctions between the integrated plasmid and the flanking genomic sequences were recovered by plasmid rescue and sequenced. The sequences obtained were compared with the yeast chromosomal DNA in the *Saccharomyces* Genome Database (SGD) to identify the integration target sites. The *rad51* and *rad52* mutations caused a 2.0- and 1.8-fold decrease, respectively, in the frequency of spontaneous NHI events relative to wild-type cells ($P < 0.005$ for *rad51*, not significant for *rad52*; Table 1). Radiation increased the frequency of NHI events by 4.7-fold in the wild-type, 14-fold in the *rad51* mutant ($P < 0.01$), and 70-fold in the *rad52* mutant ($P < 0.01$) (Table 1). In the *rad51* mutant, six out of 16 junctions (38%) displayed 2 to 3 bp of microhomology in spontaneous integration (Figs. 1B and 2A), whereas after irradiation 21 out of 28 NHI events (75%) involved 2 to 5 bp of microhomology, showing an induction of microhomology use after irradiation ($P < 0.05$) (Fig. 2B). Nine out of 28 junctions in irradiated *rad51* mutant displayed 4 bp or more of microhomology, which is not significantly different from the distribution of microhomology use in irradiated wild-type cells (20), namely nine out of 22 junctions with 4 bp or more by a χ^2 test (Fig. 1A). There was also no significant difference in the overall distribution of microhomology use between irradiated *rad51* mutant and irradiated wild-type cells by the Mann-Whitney test. These results indicate that Rad51 does not play any role in radiation-induced MHMR.

Similarly, in the *rad52* mutant, two out of 18 spontaneous NHI events (11%) used 2 bp of microhomology (Figs. 1C and 3A) whereas 10 of 22 NHI events (45%) displayed 2 to 5 bp of microhomology in mutant cells irradiated with 50 Gy (Fig. 3B), showing an induction of microhomology use after irradiation ($P < 0.05$). Noticeably, irradiated *rad52* mutant cells exhibited fewer MHMR events with ≥ 4 bp of microhomology compared to irradiated wild-type cells, namely only two out of 22 junctions (9%) in irradiated *rad52* mutant cells compared to nine of 22 junctions (41%) in irradiated wild-type cells ($P < 0.05$, χ^2 test). Furthermore, there was a significant difference in the overall distribution of microhomology use, in which no cut-off value for microhomology is required, between the *rad52* mutant irradiated with 50 Gy and the irradiated wild-type cells ($P < 0.05$, Mann-Whitney test).

Because the *rad52* mutant is highly sensitive to ionizing radiation (35), the phenotype of the *rad52* mutant cells after exposure to 50 Gy of radiation could be confounded by selection bias of the mutant cells that survived after irradiation. To exclude this possibility, we transformed the integrating DNA into the *rad52* mutant and exposed cells to 10 Gy of γ rays, a dose equitoxic to 50 Gy in the wild-type cells that results in about 45% survival (data not shown). Exposure to 10 Gy of γ rays increased the frequency of NHI by 15-fold in the *rad52* mutant ($P < 0.01$) (Table 1). In this instance, eight out of 16 (50%) radiation-induced NHI events contained 2 to 4 bp of microhomology (Fig. 1C and 3C), showing a significant induction of microhomology use after irradiation with 10 Gy ($P < 0.05$). In particular, one out of 16 NHI events with ≥ 4 bp of microhomology were observed in the *rad52* mutant cells irradiated with 10 Gy. This is significantly different from nine out of 22 events in the irradiated wild-type cells ($P < 0.05$, χ^2 test), indicating that the NHI events in the *rad52* mutant irradiated with 10 Gy exhibited less extensive microhomology. The overall distribution of microhomology use in the *rad52* mutant irradiated with 0 Gy was also

significantly different from that of the irradiated wild-type cells ($P < 0.05$, Mann-Whitney test). The fraction of MHMR events in the *rad52* mutant cells irradiated with 10 Gy was similar to that of the mutant cells irradiated with 50 Gy (50% for 10 Gy compared to 45% for 50 Gy). These observations eliminate the possibility that the *rad52* phenotype was due to selection of a small population of *rad52* mutant cells that survived after irradiation. These results indicate that radiation-induced MHMR with 4 bp or more is required by *RAD52*.

Human Rad52 has been shown to bind DSBs and to protect DNA ends from exonuclease attack *in vitro* (36). Our previous results have shown that radiation protects the single-stranded termini of the integrating DNA from degradation and that the single-stranded termini are required for MHMR (20). We determined whether degradation of the PSS DNA ends may have caused the lower extent of microhomology use in irradiated *rad52* mutant cells. In the unirradiated *rad52* mutant, 18 out of 72 nucleotides were deleted at the PSS ends of the integrating DNA whereas only nine out of 88 were deleted in the *rad52* mutant cells after 50 Gy of radiation ($P < 0.05$), indicating that there is a radiation-induced protection of DNA termini in the *rad52* mutant cells as in wild-type cells (20). This rules out the possibility that the lower extent of microhomology use in irradiated *rad52* mutant than in the wild-type cells was caused by degradation of the DNA ends that provide the microhomologies prior to integration.

Radiation-Induced Recombination with Microhomology of more than 4 bp is Reduced by Mutation of the RAD1 and RAD10 Genes

We examined the roles of the *RAD1* and *RAD10* genes in spontaneous and radiation-induced NHI and found that the *rad1* and *rad10* mutations did not have any effect on the frequency of spontaneous NHI events (Table 1). Radiation increased the frequency of NHI by an average of 2.55-fold in the *rad1* mutant ($P < 0.05$) and by 2.61-fold in the *rad10* mutant cells ($P < 0.005$) (Table 1). In the *rad1* mutant, four out of 14 (29%) spontaneous NHI events displayed 2 to 3 bp of microhomology whereas 17 out of 24 (71%) radiation-induced NHI events involved 2 to 4 bp of microhomology (Figs. 4A and 5). This demonstrates an increase of microhomology use after irradiation ($P < 0.05$, χ^2 test). Of note, the extent of microhomology use in the irradiated *rad1* mutant was significantly lower than that observed in the wild-type cells, where none of 24 junctions in the irradiated *rad1* mutant displayed more than 4 bp, compared to four out of 22 radiation-induced events in the wild-type cells ($P < 0.05$, Fisher's exact test). In the *rad10* mutant, four out of 20 (20%) junctions in spontaneous NHI events displayed 2 to 3 bp of microhomology whereas 13 out of 24 (54%) radiation-induced NHI events in the wild-type cells used 2 to 4 bp of microhomology ($P < 0.05$, χ^2 test) (Figs. 4B and 6). Similar to the irradiated *rad1* mutant, none of 24 junctions in the irradiated *rad10* mutant used more than 4 bp of microhomology, which again is significantly different from that of irradiated wild-type cells ($P < 0.05$, Fisher's exact test). These results led us to conclude that the Rad1/Rad10 complex is required for radiation-induced MHMR involving more than 4 bp of microhomology.

Radiation-Induced MHMR is not Reduced by Mutation in the RAD2 Gene

To determine the effect of a nucleotide excision repair function in addition to the Rad1/Rad10 endonuclease complex in radiation-induced MHMR, we examined whether Rad2, a structure-specific endonuclease required for nucleotide excision repair that cleaves 5' flap structures (30), was involved. The *rad2* mutation did not have any effect on the frequency of spontaneous NHI (Table 1). Radiation increased the frequency of NHI events by 6.6-fold in the *rad2* mutant ($P < 0.005$) (Table 1). Six out of 18 (33%) spontaneous NHI events in the *rad2* mutant involved 2 and 3 bp of microhomology whereas 11 out of 16 (69%) of radiation-induced NHI events contained 2, 3, 4 and 7 bp of microhomology (Figs. 4C and 7), showing a significant induction of microhomology use after irradiation ($P < 0.05$). In

particular, four out of 16 radiation-induced MHMR events in the *rad2* mutant used 4 bp or more whereas none of 18 spontaneous events used more than 3 bp, showing a significant increase in the length of microhomology used in the irradiated *rad2* mutant ($P < 0.05$, Fisher's exact test), similar to the irradiated wild-type cells (Fig. 1A). In addition, four out of 16 junctions in irradiated *rad2* mutant displayed 4 bp or more of microhomology compared to nine out of 22 junctions in irradiated wild-type cells, which is not significantly different (by χ^2 test). There was also no significant difference in the distribution of microhomology use between the irradiated *rad2* mutant and irradiated wild-type cells by the Mann-Whitney test. These results showed that the *rad2* mutation does not reduce radiation-induced MHMR, in contrast to the *rad1/rad10* mutants.

The *dnl4* Mutation Reduces Spontaneous and Radiation-Induced Nonhomologous Integration

To determine whether radiation-induced NHI events are dependent on *DNL4*, which is required for religation during NHEJ (8), we examined the effect of the *dnl4* mutation on NHI in the absence and presence of ionizing radiation. The *dnl4* mutant caused an 8.6-fold decrease in the frequency of spontaneous NHI compared to the wild-type cells ($P < 0.01$) (Table 1). Radiation caused a 4.5-fold increase in the NHI frequency in the *dnl4* mutant ($P < 0.01$), similar to wild-type cells. These results indicate that the radiation-induced increase in the NHI frequency is independent of *DNL4*. Out of 11 spontaneous NHI events and eight radiation-induced NHI events in the *dnl4* mutant, only two junctions from the same NHI transformant, in the irradiated *dnl4* mutant, could be sequenced by the plasmid rescue method. These junctions did not involve any microhomology, exhibited deletions from the ends of the integrating plasmid, and involved a 950-bp deletion at the integration target site (data not shown).

DISCUSSION

We showed previously that ionizing radiation and restriction enzymes increase the frequency of MHMR events that are probably *in trans* at non-targeted sites (20). We report here that radiation-induced MHMR partly requires the *RAD52*, *RAD1* and *RAD10* genes but not the *RAD51* and *RAD2* genes, which is the same for single-strand annealing, suggesting that MHMR resembles the single-strand annealing pathway in its genetic control.

Radiation Increases the Normalized Frequency of both MHMR and Non-MHMR NHI Events in *rad51* and *rad52* Mutants

Since radiation increased the relative frequency of NHI in all of the mutants we studied (Table 1), we determined the normalized MHMR frequency in the absence and presence of irradiation (Fig. 8A), in which the relative frequency of NHI was multiplied by the fraction of MHMR events (described in Fig. 8). We also determined the normalized frequencies of non-MHMR events (Fig. 8B). In the wild-type cells, radiation caused a 14-fold increase in the normalized frequency of MHMR events and a 1.4-fold increase in that of non-MHMR events (P values shown in Fig. 8). This suggests that radiation specifically induces the MHMR pathway in the wild-type cells. Interestingly, in the *rad51* mutant radiation caused a 27-fold and 5.6-fold increase, respectively, in MHMR and non-MHMR events. In the *rad52* mutant, 10 Gy caused a 70-fold increase in MHMR events and an 8.7-fold increase in non-MHMR events while 50 Gy caused a 247-fold and 48-fold increase, respectively, in MHMR and non-MHMR events. This indicates that radiation enhances the normalized frequencies of both MHMR and non-MHMR events in the *rad51* and *rad52* mutants. It is possible that radiation-induced NHI occurs at radiation-induced DSBs or other damaged sites in these mutants. The normalized frequency of non-MHMR events in the *rad52* mutant irradiated with 10 Gy was about 4.1-fold higher than that of the irradiated wild-type cells (3.17

compared to 0.770), indicating a specific induction of a non-MHMR NHI pathway in the *rad52* mutant cells. It was found previously that the yeast mutant strains deficient in Rad52 and Yku70 are more sensitive to ionizing radiation than the *rad52* single mutant strain (7). The hypersensitivity of the *rad52/yku70* double mutant could be explained by our finding that a non-MHMR pathway is induced by radiation in the *rad52* mutant that might be mediated by Ku and is partly taking over the DSB repair in the absence of Rad52. The normalized frequency of MHMR in the *rad52* mutant irradiated with 10 Gy was similar to that of irradiated wild-type cells (3.17 compared to 2.82), indicating that the *rad52* mutation does not have any effect on the overall efficiency of MHMR but rather on the length of microhomology relative to events in wild-type cells based on sequencing results.

In contrast, in the *rad1* and *rad10* mutants, radiation caused a 5.6- and 8.1-fold increase, respectively, in the normalized frequency of MHMR events and did not have much effect on non-MHMR events, similar to the wild-type cells. The normalized frequencies of MHMR in the irradiated *rad1* and *rad10* mutants were about 2.2- and 2.8-fold lower than that of irradiated wild-type cells; these observations are consistent with the sequencing results indicating that Rad1 and Rad10 are partly required for radiation-induced MHMR. In the *rad2* mutant, radiation caused a 12-fold increase in MHMR and the normalized frequency of non-MHMR events before and after irradiation was not significantly different, similar to wild-type cells.

Increase of Microhomology Length during Radiation-Induced MHMR is Reduced by Mutation of *rad52*

Both yeast and human Rad52 proteins have been shown to promote strand exchange as well as annealing of complementary single-stranded DNA *in vitro* (22,37). Our results suggest that Rad52 facilitates microhomology search and annealing of short homologous sequences in radiation-induced MHMR that is likely *in trans* at non-targeted sites (see model in Fig. 9). Our results are consistent with the observation that fission yeast Rad22, a homolog of the budding yeast Rad52, is required for microhomology-mediated end joining of transformed linearized substrates containing flanking microhomologies (38). A RAD52-dependent pathway of DSB rejoining has been proposed in yeast based on the observations that rejoining of complementary DSBs with overhangs of 8 nucleotides or longer was partially dependent on RAD52 (39). In contrast, our results show that after irradiation, microhomologies of 4 to 7 bp are dependent on Rad52.

It has been shown that Rad52 involves only annealing of 3' overhangs in homologous recombination (1), but our results, in agreement with others (39), imply that Rad52 also involves annealing of short 5' overhangs. In addition, our results suggest the existence of a Rad52-independent MHMR pathway that has been proposed previously (39,40). Rad52 is known to play an important role in homologous recombination to repair DSBs (41). It was suggested that either Rad52 or Ku binds to DSBs in which Rad52 directs into homologous recombination whereas Ku initiates the NHEJ pathway (36). Our findings suggest a novel role for Rad52 in promoting the MHMR pathway of end joining; thus Rad52 seems to be able to direct into both homologous recombination and the end-joining pathway. On the other hand, the observation that Rad51 is not required for radiation-induced MHMR indicates that Rad51 is exclusively required for extensive homology annealing in homologous recombination. In fact, the presence of Rad51 prevents the occurrence of recombination between short homologous sequences (42).

Radiation-Induced MHMR Partly Requires RAD1 and RAD10

When more than 4 bp of microhomology are used during NHI, a 3' single-strand DNA is displaced from the integrating DNA after base-pairing between short homologous sequences

of the integrating DNA and the genomic target sequence (Fig. 9). Our results suggest that the Rad1/Rad10 endonuclease is required for removal of the displaced 3' single-strand DNA end from the integrating DNA during NHI, which is consistent with the observation that the Rad1 protein is involved in 3' flap removal during microhomology-mediated end joining in the repair of HO-induced DSBs (40). This biochemical activity of Rad1/Rad10 is required to specifically remove 3' single-strand DNA displaced from the duplex DNA molecules (26), which arises in single-strand annealing (27) and in nucleotide excision repair (28). On the other hand, Rad2, a 5' flap endonuclease required for 3' incision of UV-radiation-induced lesions in nucleotide excision repair (30), is not required for radiation-induced MHMR in our experiments. This indicates that the *RAD1* and *RAD10* genes, and not general nucleotide excision repair genes, are specific for radiation-induced MHMR.

Spontaneous and Radiation-Induced NHI are Dependent on DNL4

Our results showed that the *dnl4* mutation decreased the frequency of spontaneous and radiation-induced NHI events to the same extent, suggesting that *DNL4* is required for both non-MHMR and MHMR pathways. It is likely that the NHI events in the *dnl4* mutant are mediated by *CDC9*, which encodes the mammalian homologue of ligase I required for DNA replication (42). Two junctions from one irradiated *dnl4* mutant were sequenced. These junctions did not involve any microhomology, exhibited deletions from the ends of the integrating plasmid, involved a large deletion at the integration target site, implying that NHI events occur inefficiently in the *dnl4* mutant. Since the 4-bp overhangs of the transforming plasmid that are lost in the *dnl4* mutant are used for MHMR, it is likely that NHI events in the *dnl4* mutant do not involve microhomology. Based on these results, it is likely that radiation-induced MHMR events are dependent on *DNL4*, which is supported by previous results showing that the *dnl4* mutant exhibited a reduced frequency of microhomology-mediated end joining in the repair of HO-induced DSBs in yeast (40).

The Radiation-Induced MHMR Pathway Resembles Single-Strand Annealing

We report here that radiation-induced MHMR partly requires the *RAD52*, *RAD1* and *RAD10* genes, which are also involved in single-strand annealing, suggesting that MHMR resembles single-strand annealing in its genetic requirements, and that it may be mediated by part of the single-strand annealing apparatus. Our results are in agreement with a recent study that the fission yeast protein Rad22 (homologue of the budding yeast protein Rad52) and Exo1 are required for microhomology-mediated end joining in fission yeast, and the authors proposed that microhomology-mediated end joining is related to single-strand annealing (38). Our result that radiation-induced MHMR does not require Rad51 further supports this similarity since it also does not play any role in the single-strand annealing pathway. Both MHMR and the single-strand annealing pathway are accompanied by deletions of the intervening sequences between the homologous sequences, and only one of the repeats remains in the recombined products. However, the minimum homology requirement in single-strand annealing is 29 bp (43) whereas MHMR uses 2–7 bp during nonhomologous integration (20). Several models of MHMR that mimic the mechanism of single-strand annealing have been proposed that involve 5' to 3' resection of the DSB and annealing of microhomology sequences, followed by gap-filling and ligation (5,40).

MHMR is used preferentially in NHEJ-deficient cells and has been proposed as a backup pathway of NHEJ (44). Nonetheless, MHMR is a mutagenic pathway that generates large deletions and other genomic rearrangements, leading to human cancers and genetic diseases (13–17). Based on our data, it is plausible that part of the single-strand annealing apparatus interacts with other genetic factors that are involved in modulating the homology requirement in MHMR and allowing illegitimate recombination between a few base pairs of

homology. Investigation of these genetic factors will be important in understanding the molecular mechanism of the MHMR pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

The distribution of microhomology use in spontaneous and radiation-induced NHI events in (panel A) wild-type strain RSY12 [data from ref. (20)] and is shown here for comparison. Panel B: *rad51* and (panel C) *rad52* mutants, in which 10 Gy to the *rad52* mutant is an equitoxic dose to 50 Gy in wild-type cells.

FIG. 3. Target sequences of NHI events in *rad52* mutant. Panel A: Spontaneous events; panel B: after exposure to 50 Gy of γ radiation; panel C: after exposure to 10 Gy of γ radiation. The genomic location, locus and gene of the target sites are shown in Table S2.

FIG. 4. The distribution of microhomology use in spontaneous and radiation-induced NHI events in (panel A) *rad1*, (panel B) *rad10* and (panel C) *rad2* mutants.

FIG. 5. Target sequences of NHI events in *rad1* mutant. Panel A: Spontaneous events; panel B: after exposure to γ radiation. The genomic location, locus and gene of the target sites are shown in Table S3.

FIG. 6.
Target sequences of NHI events in *rad10* mutant. Panel A: Spontaneous events; panel B: after exposure to γ radiation. The genomic location, locus and gene of the target sites are shown in Table S4.

FIG. 8.

The actual effect of each mutation on MHMR and non-MHMR events after normalization with the relative frequency of NHI. Panel A: The normalized frequency of MHMR events. Panel B: The normalized frequency of non-MHMR events. The relative frequency of NHI resulted from each transformation (the mean shown in Table 1) was multiplied by the fraction of MHMR or non-MHMR events among the nonhomologous integrants isolated from each transformation. The average normalized frequencies of MHMR or non-MHMR were shown in the graph. The normalized frequencies of MHMR or non-MHMR events in different mutants were compared by Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

FIG. 9.

Schematic diagram of the mechanism of spontaneous and radiation-induced MHMR. In unirradiated cells, the majority of spontaneous nonhomologous integration events are mediated by illegitimate recombination that does not use microhomology. In irradiated cells, the MHMR pathway is induced to facilitate illegitimate recombination that is probably *in trans*. Rad52 mediates microhomology search and annealing between short homologous sequences of one end of the integrating DNA and the genomic sequences (shown by base-pairing). The Rad1/Rad10 complex is required for cleavage of the 3' displaced single strand from the integrating DNA when more than 4 bp of microhomology is involved during integration. The bold arrows indicate the dominant mechanism.

TABLE 1

Relative Frequency of NHI Events

Genotype	No. of transformants/ μg^d										Relative induction ^e		
	YEplac195 ^b			NHI, Bg/III linearized pM15 ^c									
	0 Gy	10 Gy ^f	50 Gy	0 Gy	10 Gy	50 Gy	0 Gy	10 Gy	50 Gy	0 Gy	10 Gy	50 Gy	10 Gy
RSY12	12,500		1,300	0.940		0.451	0.75 $\pm$ 0.09		3.47 $\pm$ 1.17			4.70	***
<i>rad51</i> Δ	11,300		1,250	0.430		0.688	0.38 $\pm$ 0.05		5.33 $\pm$ 1.22			14.0	**
<i>rad52</i> Δ	399	210	51.3	0.0164	0.127	0.148	0.41 $\pm$ 1.01	6.05 $\pm$ 2.46	28.8 $\pm$ 10.5	14.7	***	70.1	***
<i>rad1</i> Δ	9,350		3,500	0.670		0.630	0.71 $\pm$ 0.12		1.81 $\pm$ 0.17			2.55	*
<i>rad10</i> Δ	6,500		2,500	0.470		0.470	0.72 $\pm$ 0.36		1.88 $\pm$ 0.46			2.61	***
<i>rad2</i> Δ	8,950		900	0.650		0.430	0.73 $\pm$ 0.13		4.80 $\pm$ 1.7			6.60	***
<i>dhf4</i> Δ	40,200		6,500	0.350		0.250	0.09 $\pm$ 0.04		0.390 $\pm$ 0.085			4.50	***

Note. Data on the wild-type strain RSY12 (20) are included for comparison.

^aThe average from three or more experiments with 10 μg of DNA for each transformation.

^bPlasmid YEplac195 was used to measure the transformation efficiency.

^cBg/III-linearized plasmid pM151 was used to assay nonhomologous integration.

^dThe relative NHI frequency is the number of illegitimate transformants per μg of DNA per 10^4 YEplac195 transformants.

^eThe relative increase of NHI events; the NHI frequency at 50 Gy was compared to 0 Gy.

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.005$.

^fThe *rad52* mutant was irradiated with 10 Gy, an equitoxic dose to 50 Gy in the wild-type cells.