Cell Cycle and Genetic Requirements of Two Pathways of Nonhomologous End-Joining Repair of Double-Strand Breaks in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, an HO endonuclease-induced double-strand break can be repaired by at least two pathways of nonhomologous end joining (NHEJ) that closely resemble events in mammalian cells. In one pathway the chromosome ends are degraded to yield deletions with different sizes whose endpoints have 1 to 6 bp of homology. Alternatively, the 4-bp overhanging 3′ ends of HO-cut DNA (5′-AACA-3′) are not degraded but can be base paired in misalignment to produce +CA and +ACA insertions. When HO was expressed throughout the cell cycle, the efficiency of NHEJ repair was 30 times higher than when HO was expressed only in G_1. The types of repair events were also very different when HO was expressed throughout the cell cycle; 78% of survivors had small insertions, while almost none had large deletions. When HO expression was confined to the G_1 phase, only 21% were insertions and 38% had large deletions. These results suggest that there are distinct mechanisms of NHEJ repair producing either insertions or deletions and that these two pathways are differently affected by the time in the cell cycle when HO is expressed. The frequency of NHEJ is unaltered in strains from which *RAD1*, *RAD2*, *RAD51*, *RAD52*, *RAD54*, or *RAD57* is deleted; however, deletions of *RAD50*, *XRS2*, or *MRE11* reduced NHEJ by more than 70-fold when HO was not cell cycle regulated. Moreover, mutations in these three genes markedly reduced +CA insertions, while significantly increasing the proportion of both small (−ACA) and larger deletion events. In contrast, the *rad50* mutation had little effect on the viability of G_1-induced cells but significantly reduced the frequency of both +CA insertions and −ACA deletions in favor of larger deletions. Thus, *RAD50* (and by extension *XRS2* and *MRE11*) exerts a much more important role in the insertion-producing pathway of NHEJ repair found in S and/or G_2 than in the less frequent deletion events that predominate when HO is expressed only in G_1.

DNA end joining is a common mechanism of double-strand-break (DSB) DNA repair. This repair process is called nonhomologous, or illegitimate, recombination because it requires little or no DNA homology to join the ends of DNA. End-joining reactions also differ from classical homologous recombination in that they are typically nonconservative, such that the joining process results in a loss or gain in nucleotide base number (41, 46). End joining in a wide array of organisms has been studied, from higher eucaryotes (14, 30, 41, 47) or simple eucaryotes (13, 29, 33, 34, 53, 54, 60) to bacteria (7, 27). Even though the systems and the methods used to study end joining vary, the joint molecules formed are strikingly similar. In most cases, the ends have rejoined by apparently using base pairings between opposite single strands, yielding junctions whose end points have 1 to 5 bp of homology. The end-joining product may have either nucleotide insertions or deletions, depending on how the overlapping single-stranded ends are processed (14). Because the postulated overlap intermediates would often have only a few base pairings, it has been suggested that alignment proteins may be used to stabilize these intermediates (41).

The annealing of overlapping microhomologies has been suggested (29, 39) to occur similarly to the repair of DSBs by the homologous recombination mechanism known as single-strand annealing (SSA) (11, 24, 31, 32). In SSA, homologous regions flanking the DSB are joined together, after the ends of

the DSB are degraded by a 5′-to-3′-exonuclease activity, creating long 3′-ended single strands containing complementary sequences of the homologous regions. In *Saccharomyces cerevisiae*, efficient DNA repair by SSA requires a minimum of 60 to 90 bp of perfect homology in the regions flanking the DSB (57). A similar intermediate might occur in nonhomologous end joining (NHEJ), although only 1 to 5 bp of precise homology is sufficient for end joining in *S. cerevisiae* (29, 33, 34). This might explain why these nonhomologous events occur 1 to 2 orders of magnitude less frequently than SSA. Efficient SSA repair of a chromosomal DSB requires *RAD52* (57), although it does not need other genes of the *RAD52* epistasis group (*RAD50*, *XRS2*, *RAD51*, *RAD54*, *RAD55*, or *RAD57*) (20). A key step in SSA is the removal of the long, nonhomologous tails that are 3′ to the annealed complementary sequences. In *S. cerevisiae*, this requires the nucleotide excision endonuclease formed by Rad1 and Rad10 proteins (4, 10, 20). Whether similar processing occurs for the ends of broken DNA in NHEJ reactions is not known.

When HO endonuclease creates a DSB at the mating-type (*MAT*) locus, the break is normally repaired by homologous recombination with one of two silent donors of mating-type information, *HML* or *HMR* (for a review, see reference 15). If homologous recombination is prevented, by deletion of the *RAD52* gene, cells will repair the DSB by nonhomologous pathways (29, 61, 62). We have investigated the genetic requirements for the two types of end joining that we identified previously (29). We find that most of the genes in the *RAD52* epistasis group (*RAD51*, *RAD52*, *RAD54*, and *RAD57*) are not needed for end joining, nor is the *RAD1* gene. However, three genes that play a less important role in homologous DSB

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TABLE 1. Yeast strains

Strain	Genotype	Source
NR238-7c	*HO HMLα MAT**a**/MATα HMR**a** ade1 leu2-3,112 lys5 ura3-52*	N. Rudin
XW119	*HO HMLα MAT**a**/MATα Δhmr::LEU2 leu2-3,112 lys5 ura3-52*	X. Wu
XW133	*HO HMLα MAT**a**/MATα Δ hmr::URA3 leu2-3,112 lys5 ura3-52*	X. Wu
XW157	*HO Δhml::ADE1 MAT**a**/MATα Δhmr::URA3/HMR**a** ade1 leu2-3,112 lys5 ura3-52*	X. Wu
JKM60	*GAL::G1::HO HMLα MATα Δhmr::LEU2 leu2-3,112 lys5 ura3-52*	This study
JKM64	*HO HMLα MATα/MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	This study
JKM65	*GAL::G1::HO HMLα MATα Δhmr::URA3 leu2-3,112 lys5 ura3-52*	XW133 × JKM60
JKM73	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::URA3 leu2-3,112 lys5 ura3-52*	XW157 × JKM60
JKM74	*GAL::G1::HO Δhml::ADE1 MAT**a** Δhmr::LEU2 ade1 leu2-3,112 lys5 ura3-52*	XW157 × JKM60
JKM75	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::LEU2 ade1 leu2-3,112 lys5 ura3-52*	JKM73 × JKM74
JKM81	*Δho HMLα MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	This study
JKM82	*Δho HMLα MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	This study
JKM84	*GAL::G1::HO HMLα MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	JKM81 × JKM65
JKM95	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	JKM75 × JKM84
JKM95-H	*JKM95 transformed with pGAL-HO*	This study
JKM95-C	*JKM95 transformed with pJKM10*	This study
JKM96	*GAL::G1::HO Δhml::ADE1 MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	JKM75 × JKM84
JKM97	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad1::LEU2*	This study
JKM99	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad52::URA3*	This study
JKM101	*Δho HMLα MAT**a** HMR**a** ade1 leu2-3,112 lys5 ura3-52*	JKM82 × JKM91
JKM108	*Δho Δhml::ADE1 MATα HMR**a** ade1 leu2-3,112 lys5 ura3-52*	JKM75 × JKM101
JKM110	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	This study
JKM111	*Δho Δhml::ADE1 MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52*	This study
JKM112	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad1::LEU2*	This study
JKM114	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad51::LEU2*	This study
JKM115	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52*	This study
JKM116	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad50::hisG*	This study
JKM118	*Δho Δhml::ADE1 MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52*	This study
JKM121	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad52::TRP1*	This study
JKM125	*Δho Δhml::ADE1 MAT**a** Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad50::hisG*	JKM116 × JKM118
JKM126	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad57::LEU2*	This study
JKM127	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad2::TRP1*	This study
JKM128	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad54::LEU2*	This study
JKM129	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 xrs2::LEU2*	This study
JKM136	*Δho HMLα MAT**a** HMR**a** ade1 leu2-3,112 lys5 trp1::hisG ura3-52 rad52::TRP1*	JKM121 × JKM120
JKM138	*Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 mre11::hisG*	This study
JKM143	*GAL::G1::HO Δhml::ADE1 MATα Δhmr::ADE1 ade1 leu2-3,112 lys5 ura3-52 rad50::hisG*	This study

repair, *RAD50*, *XRS2*, and *MRE11*, are essential for one type of end-joining event. In addition, we find that there is a cell cycle dependence on the different types of end joining.

MATERIALS AND METHODS

Strains. All strains used in this study were constructed from a DBY745 background (49). The stable chromosome allele *G1::GAL10::HO* was constructed by a two-step gene replacement method (51) utilizing pJKM4, a YIP5 vector carrying the *G1::GAL10::HO* construct from Nasmyth (37) to make the strain JKM60. The *HO* replacement was done in diploid strain XW119 (64), and the plasmid pJKM4 was targeted to *HO* by digesting the plasmid with *Sac*I and selecting Ura⁺ transformants. The *HO* gene duplication was then excised by selection with 5-fluoroorotic acid (FOA) (6); some of the plasmid popouts removed the wild-type sequence, leaving the *G1::GAL10::HO* construct in the chromosome. After tetrad dissection, strains with the *G1::GAL10::HO* construct were detected by their galactose-inducible switching. The strains from which *HO* was deleted, JKM81 and JKM82, were constructed by a similar two-step gene replacement methodology with plasmid pJKM7. However, the *Sac*II site of this plasmid was used to target it to the *HO* gene. Southern blot analysis was done with the nonisotopic Genius system (Boehringer Mannheim) to confirm construction.

In strain JKM64, the *HMR* cassette including an adjacent silencer sequence was deleted and replaced by an *ADE1* marker contained in a *Mlu*I-to-*Pst*I fragment of pJKM5, transforming NR238-7c to Ade⁺. The Ade⁺ isolates were dissected to yield the segregant JKM64, and the *HMR* deletion was subsequently confirmed by Southern analysis. Strains which had both donor cassettes deleted and which had both marked by *ADE1* were generated by a series of crosses ultimately deriving Δhml::ADE1 from XW157 (64) and Δhmr::ADE1 from JKM64 (Table 1). Tetrad dissection and selection of the appropriate segregants yielded isogenic *MAT*α and *MAT**a** cosegregants in the *G1::GAL10::HO* background (JKM95 and JKM96) and the Δho background (JKM110 and JKM111),

respectively. In all cases, cassette deletions remove all sequences homologous to *MAT* and extend beyond to include donor silencing sequences.

Isogenic mutant strains were constructed by the one-step gene disruption method (48) with the DNA substrates described below and by the lithium acetate method of transformation described by Schiestl and Geitz (52). Most mutant strains were constructed in JKM115, which is a *trp1::hisG'* derivative of JKM110 made by methods similar to those described by Alani et al. (2). The *TRP1* knockout was done by transforming JKM110 to Ura⁺ with the 5.3-kb *Eco*RI fragment (*trp1::hisG'::URA3::hisG'*) of pNYK1009 (Eric Alani). The *trp1::hisG'* derivative was obtained by selecting for Ura⁻ deletion recombinants on media containing FOA. Other isogenic mutant strains were made from the following: *rad52::TRP1*, *Bam*HI fragment of pSM21 (55); *rad1::LEU2*, *Sal*I fragment of pL962 (provided by R. L. Keil); *rad50::hisG'*, *Bgl*II and *Eco*RI fragment of pNKY83 (3) followed by Ura⁻ selection described above; *rad51::LEU2*, *Pst*I and *Xba*I fragment of pAM50 (5); *rad54::LEU2*, *Bgl*II and *Pst*I fragment of pXRAD:LEU (43); *rad57::LEU2*, *Sac*I fragment of pSM51 (55); *rad2::TRP1*, *Sal*I fragment of pWS521 (provided by W. Siede); *xrs2::LEU2*, *Bam*HI and *Hin*dIII fragment of pEI40 (22); and *mre11::hisG'*, *Bam*HI fragment of pKJ1112-5 (25). Congenic *MAT**a*** derivatives of the mutant strains were made by crossing the mutant strain with JKM118, which itself is a segregant of a cross between JKM115 and JKM111. A *rad50* mutant with *G1::GAL::HO* (strain JKM143) was made by crossing JKM125 with JKM95 and selecting for the appropriate marked segregant after tetrad dissection. Mutants were screened for their UV sensitivity or sensitivity to methyl methanesulfonate when appropriate.

Plasmids. The centromeric (*CEN4*) plasmid pGAL-HO, which has a *GAL10::HO* transcriptional fusion and is marked by *URA3* (23) was used to transform strains for cell cycle-unregulated *HO* expression. As a control for growth on uracil dropout minimal medium containing galactose (SC-URA,GAL) without pGAL-HO, strains were also transformed with the plasmid pJKM10, which was derived from pGAL-HO by deleting a 2.5-kb *Hin*dIII fragment containing the *HO* gene. Plasmid pJKM4 was constructed by cloning a 4.8-kb *Eco*RI-to-*Sal*I fragment from pHO-M419 (provided by Kim Nasmyth) into a YIP5 vector digested with *Eco*RI and *Sal*I. Plasmid pJKM7 was constructed by

deleting a 2.2-kb fragment from pJKM4. The deletion was made by digesting pJKM4 with SacI and BamHI, which was followed by a T4 polymerase reaction and ligation of the blunt ends. The deletion extends from a region upstream of the promoter sequence to the middle of the *HO* structural gene, leaving up to 500 bp of flanking sequences distal and proximal, which is sufficient for targeting the plasmid into the chromosome. Plasmid pJKM5 was constructed by cloning the *XhoI ADE1* fragment from pUC19-ADE1 (36) into *XhoI*-digested pWAC4U (64). This replaces a *URA3* marker with an *ADE1* marker in an *HMR* deletion plasmid. The plasmid pJH277 contains a 4.0-kb *HindIII* fragment of *MATα* cloned into pGEM3 (constructed by Charles White). Standard molecular genetic techniques for plasmid construction and propagation in *Escherichia coli* MC1061 were performed as described by Sambrook et al. (50).

Generation of DSB. For Δ*ho* strains, overnight cultures of individual isolates were grown in uracil dropout minimal medium containing glucose (SC-URA) to maintain the *URA3*-marked pGAL-HO plasmid. Cultures were washed with sterile water, diluted, and then spread onto plates of SC-URA,GAL or SC-URA. The *G1::GAL10::HO* strains were treated similarly, except that cultures were first grown on complex medium (YEPD), washed with sterile water, diluted, and then spread onto plates of complex medium containing either galactose (YEPGAL) or glucose (YEPD). For *G1::GAL::HO* strains that had been transformed with either pGAL-HO or pJKM10, cultures were grown in SC-URA to maintain these *URA3*-marked centromeric plasmids. The frequency of survival after HO-induced DSBs was determined by dividing the number of colonies growing on SC-URA,GAL or YEPGAL by the number of colonies growing on SC-URA or YEPD.

Independent events were generated by picking different colonies from appropriate glucose-containing media, resuspending cells directly into sterile water, diluting, and spreading directly onto galactose-containing medium. At the same time, the cells were patched onto glucose-containing medium to check their mating type prior to their galactose exposure.

Analysis of repair events. Initial analysis of repair events at *MAT* were determined by the complementation mating phenotype test (56). For healing events at *MATα*, simple matings with single auxotrophs were used in complementation mating tests to determine an α-mating, sterile, or **a**-like phenotype. DNA for sequence analysis of the repair events was obtained by generating a *MAT* PCR fragment and sequencing the fragment by an end-labeled PCR method (fmole; Promega). The *MAT* Z1 primer JKMp2 (TCGAAAGATAAACAACCTCC) and *MATα2* primer JKMp3 (TCTTGCCCACTTCTAAGCTG) generate an 800-bp PCR fragment from *MATα*. The PCR was performed directly on yeast cells by a method described by Huxley et al. (19), with the following modifications. The reaction volume was increased to 50 μl, and 0.03 U of *Taq* polymerase per μl was used in each reaction mixture. Additionally, the MgCl₂ concentration was reduced to 1.5 mM for optimal *Taq* polymerase (Promega) activity. The reaction mixtures were treated in a PTC 100 Peltier-effect thermal cycler (M. J. Research, Inc.) with 34 cycles of 95°C for 1.5 min, 48°C for 2 min, and 72°C for 3 min. The final cycle was followed by an extension step at 72°C for 7 min. The PCRs were assessed by 1.5% agarose gel analysis (SeaKem; FMC) with a 100-bp DNA marker ladder (GIBCO-BRL) to determine approximate deletion sizes. PCR fragments were purified with the Prep-a-gene DNA purification matrix (Bio-Rad) prior to the sequencing reaction. JKMp2 was used as the sequencing primer.

Specific mutations were screened directly by using discriminating PCR primers. Primers JKMp3 (*MATα* specific) and JKMp5 (AAAATTATACTGTGTTG) were used to amplify HO cut site healing events resulting in CA insertions (+CA) in *MATα* strains by using PCRs described above, except that the annealing temperatures were set at 50°C. Primers JKMp3 (*MATα* specific) and JKMp6 (TATAAAATTATACTGCG) were used to amplify HO cut site healing events resulting in ACA deletions (−ACA) in *MATα* strains by using the PCRs described, also with the annealing temperatures set at 50°C. Deletions at *MAT*, which could not be amplified with the primers described above, were amplified with primers in *MAT*-W/X (primer no. 649 [GTCGTCTTGTTCAAGAAGGT]) and *MAT*-Z2 distal (primer no. 9 [AAGTCATGTGAACCGCATGG]) with the annealing temperature set at 48°C. These primers would amplify deletion fragments within the 2.5 kb between these primers. As a positive control for each discriminating PCR, strains containing a predetermined mutation at *MAT* were used as templates to verify the specificity of the amplification. Plasmid pJH277 or strain JKM110, both of which contain wild-type *MATα* sequence, was used as a negative control in the discriminating PCRs.

Media and growth conditions. Complex (YEPD) and minimal synthetic complete (SC) media used for the growth and sporulation of *S. cerevisiae* are described by Sherman et al. (56). Complex medium used for galactose induction of the *G1::GAL::HO* fusion gene, YEPGAL, contained 2% (wt/vol) galactose to replace glucose as the carbon source. SC medium without uracil containing 2% (wt/vol) galactose to replace glucose as the carbon source (SC-URA,GAL) was used to maintain and induce the plasmid-borne *GAL::HO* fusion gene. Medium containing FOA was prepared as described elsewhere (6). The Rad⁻ phenotype was determined by using plates containing 0.015% (vol/vol) methyl methanesulfonate (Aldrich). UV sensitivity was determined by 5- to 15-s exposures to UV light (254 nm; dose rate, 1 J/m²/s).

TABLE 2. Survival frequency in strains with HO-induced DSBs

Source of HO and strain	Relevant genotype	Frequency of survival[a]
pGAL-HO		
JKM110	*RAD⁺*	$(2.2 \pm 0.5) \times 10^{-3}$
JKM116	*rad50Δ*	$(3.0 \pm 2.9) \times 10^{-5}$
JKM129	*xrs2Δ*	$(2.2 \pm 1.2) \times 10^{-5}$
JKM138	*mre11Δ*	$(3.0 \pm 1.8) \times 10^{-5}$
JKM121	*rad52Δ*	$(2.0 \pm 0.4) \times 10^{-3}$
JKM114	*rad51Δ*	2.2×10^{-3}
JKM128	*rad54Δ*	2.1×10^{-3}
JKM126	*rad57Δ*	5.3×10^{-3}
JKM112	*rad1Δ*	$(1.9 \pm 0.5) \times 10^{-3}$
JKM127	*rad2Δ*	2.0×10^{-3}
G1::GAL::HO		
JKM95	*RAD⁺*	$(3.3 \pm 0.8) \times 10^{-5}$
JKM95-C	*RAD⁺*	$(6.8 \pm 2.5) \times 10^{-5}$
JKM95-H	*RAD⁺*	$(1.6 \pm 0.8) \times 10^{-3}$
JKM143	*rad50Δ*	$(1.2 \pm 0.6) \times 10^{-5}$
JKM99	*rad52Δ*	$(1.2 \pm 0.3) \times 10^{-4}$
JKM95[b]	*RAD⁺*	$(2.2 \pm 2.4) \times 10^{-4}$
JKM143[b]	*rad50Δ*	$(1.3 \pm 0.4) \times 10^{-4}$
JKM99[b]	*rad52Δ*	$(2.3 \pm 1.4) \times 10^{-4}$
JKM97[b]	*rad1Δ*	$(4.0 \pm 1.7) \times 10^{-4}$

[a] Frequency of survival for a culture of cells was determined by dividing the number of colonies that grew on minimal medium containing galactose (SC-URA,GAL or SC,GAL) by the number of colonies that grew on minimal medium containing glucose (SC-URA or SC). In all cases for which a standard deviation is given, the calculation is based on a minimum of nine independent measurements.

[b] Frequency of survival was determined as described in footnote *a*, except with rich media (YEPGAL and YEPD).

RESULTS

Nonhomologous DNA repair in Rad⁺ cells. In our previous studies of NHEJ, chromosomal repair of the DSB at *MAT* was prevented by a *rad52* mutation (29, 61, 62). In the following experiments, gene conversion repair of the DSB at *MAT* was prevented by deletion of both of the homologous donors, *HML* and *HMR*. Klar et al. (28) first showed that when HO was expressed in cells lacking the two donor sequences, nearly all cells died because they could not repair the DSB. We constructed *MATα* strain JKM110, which lacks *HML* and *HMR* and carries the *URA3*-marked plasmid pGAL-HO, in which the *HO* gene is under the control of a galactose-inducible promoter (23). Galactose induction results in a high level of *HO* expression throughout the cell cycle, and previous studies showed that HO cleavage and the normal switching of *MAT* have the same kinetics throughout the cell cycle (8, 23). When cells were plated on both glucose and galactose plates lacking uracil (to retain the pGAL-HO plasmid), the frequency of survivors on galactose medium was 2.2×10^{-3} relative to growth on glucose plates (Table 2). This galactose-induced lethality was dependent on the *GAL::HO* gene, since the same strain carrying a *URA3*-marked plasmid (pJKM10) that lacks the *GAL::HO* gene had a plating efficiency of 81% on galactose relative to glucose.

The frequency of survivors does not depend on the way in which homologous repair is prevented. Strain JKM136, containing both *HML* and *HMR* but from which the *RAD52* gene was deleted, was constructed in the JKM110 strain background. When transformed with the plasmid pGAL-HO and induced for *HO* expression, the frequency of survivors was 1.1×10^{-3}. Similarly, JKM121, a donor-deleted strain with the *rad52Δ* mutation, had a frequency of survivors of 2.0×10^{-3}.

In addition to *RAD52*, we have examined the roles of a number of other genes implicated in the repair of DSBs. A major group of important recombination genes belong to the *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *XRS2*, and *MRE11*). Despite their common X-ray-sensitive phenotypes, deletions of these genes show very different phenotypes in the repair of HO-induced DSBs. *RAD52* is absolutely required for DSB repair, while *RAD51*, -*54*, -*55*, and -*57* are required when the chromatin structures of the donor sequences are not sufficiently accessible (58). In contrast, *RAD50*, *XRS2*, and *MRE11* are not required for HO-induced events, although their absence significantly slows down the kinetics of recombination (20, 22, 57). We analyzed the effect of these mutations on the frequency of survival after HO-induced DSBs. Deletions of *RAD51*, *RAD52*, *RAD54*, and *RAD57* showed no effect on the frequency of healing DSBs compared with the wild type (Table 2). Surprisingly, deletions of *RAD50*, *XRS2*, and *MRE11* had profound effects, reducing the number of survivors by approximately 70-fold.

We also examined *RAD1* and *RAD2*, two genes that are essential for nucleotide excision repair, for their role in NHEJ. The *RAD1* and *RAD10* genes are necessary for efficient SSA repair of DSBs, when there are nonhomologous ends at the DSB site (10, 20). It has been proposed that the Rad1/Rad10 endonuclease removes the nonhomologous ends formed in the SSA intermediate (10). Despite the fact that nonhomologous deletions could be imagined to require a similar removal of nonhomologous unpaired ends (29, 39), the *rad1* deletion had no effect on the frequency of NHEJ (Table 2). Recently, the Rad2 gene product has been suggested to have a 5′-to-3′ exonuclease activity in DNA repair (16). A *rad2* deletion, which has no effect on SSA (20), also has no effect on NHEJ (Table 2).

Types of end-joining repair in Rad+ cells. We then characterized the types of repair events that were obtained after HO induction in JKM110 and its derivatives. The survivors were first analyzed by determining their mating types, which can be used as a rough determinant of the kind of healing event that had occurred. In *MAT*α strains, survivors can be either **a**-like, sterile, or still α mating (18, 29) (Fig. 1). An **a**-like phenotype results from a large deletion that removes the HO cut site and at least part of the adjacent *MAT*α*1* and *MAT*α*2* genes (Fig. 1C). Sterile derivatives have mutations in *MAT*α*1* that alter the HO cut site without extending into the *MAT*α*2* gene (Fig. 1B). In addition to frameshift mutations and large deletions, an in-frame insertion or deletion of a single amino acid in the coding region covered by the HO cut site is sufficient to abolish *MAT*α*1* activity (results presented here). PCR analysis can be used to classify sterile mutants into those that have deletions of >50 bp and those that have smaller alterations around the HO cut site (see Materials and Methods).

Of 127 survivors of strain JKM110, none were either **a**-like or sterile because of a deletion of more than 50 bp. Five were α mating; however, subsequent DNA sequencing showed that they all had a wild-type *MAT*α sequence. These derivatives may have become mutant in the HO gene or may have become unable to induce HO. The remaining 122 were all sterile. Forty-five of these independent sterile survivors were sequenced after PCR amplification. Although mutations that alter the HO endonuclease recognition site have been shown to extend over a 22-bp region (38), most of the sequence changes in these sterile derivatives occurred within or immediately adjacent to the actual site of cleavage at *MAT* (Table 3). The most common mutation found was a 2-bp (+CA) insertion in the 4-bp overhang of the HO cut site (Fig. 2A). The healing event for this mutation can be imagined as a 1-bp overlap
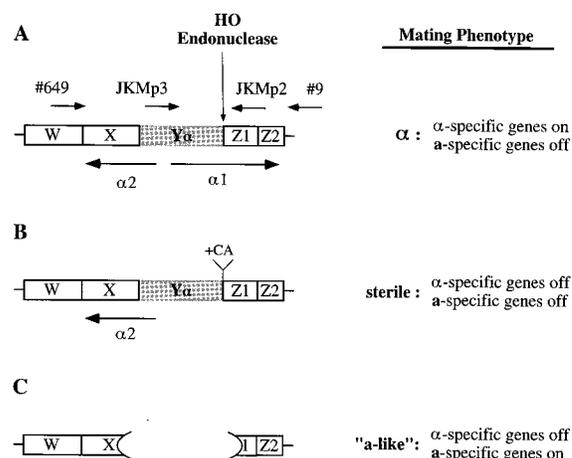

FIG. 1. Alternative mating types of NHEJ healing events of a HO endonuclease DSB at the *MAT*α locus. (A) The structure of the wild-type *MAT*α locus and its mating phenotype. The relative positions and directions of the *MAT*α*1* and *MAT*α*2* transcripts are indicated below the wild-type *MAT*α locus. The site of the HO endonuclease DSB is at the Yα and Z1 border. The positions of the PCR primers and their 5′-to-3′ orientations relative to the *MAT*α sequence are drawn above the *MAT*α locus. (B) The structure of a CA insertional end-joining event at the *MAT*α DSB site which mutates *MAT*α*1* and results in a sterile mating phenotype. (C) The structure of a large deletion end-joining event which results in the mutation of *MAT*α*1* and *MAT*α*2* and causes an **a**-like mating phenotype.

which provides a 3′ base-paired end on the top strand as a template for DNA polymerase. Synthesis on the bottom strand would require the removal of the terminal 3′ nucleotide prior to DNA synthesis. The resulting +CA insertion causes a frameshift mutation in *MAT*α*1* and is sterile. Another insertional mutant that was much less frequently found (1 in 45 cases) is a 3-bp (+ACA) insertion, which is also sterile despite being an in-frame mutation that inserts a glutamine residue into the Matα1 protein. It is curious that this +ACA insertion mutation is so rare compared with the +CA insertion, because it is easier to imagine a single base-pairing overlap in this healing event between the terminal 3′ A of the top strand and the terminal T of the bottom strand that does not necessitate

TABLE 3. Sequenced repair events of sterile mating survivors

Derivative	Sequence	No. of repair events	
		G1::GAL::HO	pGAL-HO
Wild type	CGC<u>AACA</u>GTATA[a]		
+CA	CGCAACAC**CA**GTATA	12/42	35/45
+ACA	CGCAACA**ACA**GTATA[b]	—[c]	1/45
+AA	CGC**AA**AACAGTATA[b]	—	1/45
−ACA	CGCAGTATA	10/42	5/45
−A	CGCACAGTATA	6/42	1/45
−GC	CAACAGTATA	2/42	—
−C	CGCAACAGTATA	2/42	—
−G	CCAACAGTATA	—	1/45
−AGT	CGCAACATA	1/42	—
−GCA	CACAGTATA	—	1/45
C→T	CGCAATAGTATA	1/42	—
T→A	CGCAACAGTAAA	1/42	—
Δ<700 bp		7/42	—

[a] Sequence starts at the proximal boundary of the Z1 region in *MAT*. The 3′ 4-bp overhang created after HO endonuclease activity is underlined.
[b] Base insertions are indicated by boldface type.
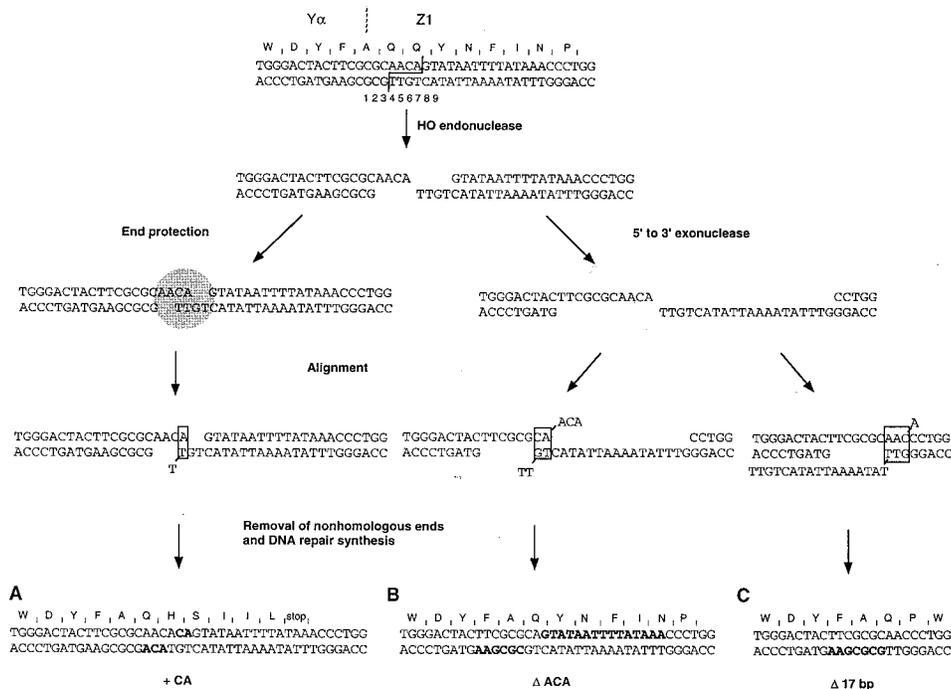[c] —, not found.

FIG. 2. Specific healing events after a HO endonuclease DSB at *MATα* and proposed mechanisms of NHEJ. The sequence for the HO endonuclease cut site is shown for *MATα*, and the relevant amino acid sequence corresponding to a portion of *MATα1* is given above it. The nucleotides in the Z region are also shown. The 4-bp 3′ overlapping sequence cleaved by HO endonuclease is indicated. Possible end processing and subsequent alignment of complementary base pairs that could lead to insertions or deletions are shown. New DNA synthesis is indicated in boldface type. Actual DNA sequences and amino acid changes of *MATα1* are shown for three representative end-joining events.

removal of a nucleotide prior to fill-in DNA synthesis. A third rare insertional mutation (1 in 45 cases) was a 2-bp (+AA) insertion at position Z4, at one end of the region cleaved by HO.

The second most common mutation seen among the sterile mutants of JKM110 was a 3-bp −ACA deletion of the nucleotides at the 3′ end of the top strand in the 4-bp overhang of the HO cut site (Table 3). This mutation could also have occurred by alternative base pairings forming within the cut site sequences which, after 3′ end processing and DNA synthesis, would heal the DSB (Fig. 2B). This in-frame deletion removes a glutamine residue from the Matα1 protein and is sufficient to abolish Matα1 function. Another deletion mutation within the HO cut site (1 in 45 cases) is the removal of a single A residue at position Z4. This mutation, therefore, also occurred within the 4 bp cleaved by HO (Table 3).

The two remaining mutations that were sequenced did not have changes directly in the 4-bp overhang produced by HO endonuclease but were deletions directly proximal to the cut site. One mutation was a 1-bp −G deletion at Z2, and the other was a deletion of −GCA at positions Z2 to -4. These deletions could be imagined as products of recision and overlap of the DSB. However, since they do involve nucleotides directly in the cut site, they could have occurred by some other mechanisms prior to HO induction.

We conclude that the vast majority of mutations that we recovered were not preexisting but resulted from repair of HO-cleaved DNA. This is first evident from the frequency of these mutations, which is much higher than the normal mutation rate in *S. cerevisiae*. Second, mutations in at least a 22-bp region of the HO endonuclease recognition site will reduce or abolish HO cutting (38), yet nearly all of the mutations that we

recovered appear to involve the ends of the DNA created by DNA cleavage. This argues that few of the mutations existed prior to HO cleavage. Moreover, the types of mutations were highly nonrandom in character, suggesting that they arose by nonhomologous DNA repair of the DSB rather than by spontaneous replicational errors. The differences in the types of NHEJ events that we recovered in different genetic backgrounds or when HO was induced at different times of the cell cycle (see below) also argue that nearly all of the events that we recovered were induced by HO cleavage. Most of the healing events can be imagined to proceed through an intermediate which allows opposite strands to reanneal in some fashion. None of the sterile survivors in more than 100 different isolates that we sequenced were either 4-bp insertions or 4-bp deletions that could have occurred by the filling in or removal of the entire 4-bp 3′ overhang to create blunt ends prior to joining, although the −AA and −GCA deletion events could be imagined as a resection of bases to the left of the cut site and a filling in of three overhanging bases in the cut end on the right.

One class of NHEJ repair is dependent on *RAD50*, *XRS2*, and *MRE11*. We then analyzed the spectrum of NHEJ events in different *rad* mutant backgrounds. Mating phenotype was used to roughly determine the kind of healing event (e.g., **a**-like, sterile, or α mating), and then PCR amplification with different primers was used to identify the two most prominent healing events in wild-type cells: +CA insertions and −ACA deletions. Specific discriminating primers were designed such that PCR amplification could occur only if the survivor had one of these specific mutations (see Materials and Methods).

There was no significant change in the types of events recovered in strains from which *RAD52* was deleted (Table 4). In contrast, there was a profound change in strains from which

TABLE 4. Specific repair events

Relevant genotype	% a-like phenotype (deletion, >700 bp)[a]	% Sterile phenotype[b]			
		Deletion, <700 bp	ΔACA	+CA	Other[c]
Rad⁺ (pGAL::HO)	0 (0/122)	0 (0/45)	11 (5/45)	78 (35/45)	11 (5/45)
rad52Δ (pGAL::HO)	0 (0/39)	0 (0/39)	10 (4/39)	54 (21/39)	36 (14/39)
rad50Δ (pGAL::HO)	6 (4/64)	0 (0/64)	69 (44/64)	2 (1/64)	23 (15/64)
xrs2Δ (pGAL::HO)	8 (2/26)	0 (0/26)	65 (17/26)	15 (4/26)	12 (3/26)
mre11Δ (pGAL::HO)	3 (2/74)	1 (1/74)	78 (58/74)	1 (1/74)	16 (12/74)
G1::GAL::HO Rad⁺	26 (22/86)	12 (7/42)	18 (10/42)	21 (12/42)	23 (13/42)
G1::GAL::HO Rad⁺ (pGAL::HO)	0 (0/40)	0 (0/40)	7.5 (3/40)	72.5 (29/40)	20 (8/40)
G1::GAL::HO rad50Δ	86 (37/43)	5 (2/43)	0 (0/43)	0 (0/43)	9 (4/43)

[a] a-like phenotypes determined by mating complementation testing are given as percentages of total sample sizes.

[b] For Rad⁺ (pGAL-HO) and G1::GAL::HO Rad⁺ only a fraction of the total number of the sterile class was analyzed by sequencing, and these are shown in Table 3. The percentages shown here for these strains were calculated by using the data from Table 3 to determine the relative fractions of a specific mutation in each sterile class. For the remaining strains, the mutations were determined by PCR analysis with the primers described in Materials and Methods.

[c] For Rad⁺ (pGAL-HO) and G1::GAL::HO Rad⁺, the other mutations are shown in Table 3. For the remaining strains, the exact mutations for the other class of sterile mutants were not determined. For these strains, the other class represents isolates which did not produce PCR products with JKMp3 and either the discriminating primer JKMp5 (+CA) or JKMp6 (ΔACA) but which did produce a product with JKMp3 and JKMp2 that had a size similar to that of the wild-type control. Deletions of less than 50 bp could not be detected by this method.

RAD50, XRS2, or MRE11 was deleted (Table 4). Approximately 3 to 8% of the events were large deletions, identified by their a-like phenotype; these types of large deletions were not found in the Rad⁺ parental strain. Conversely, the proportion of +CA insertions was greatly reduced, from 78% to 2 to 15%. There was also a very dramatic increase in −ACA deletions, from 11% to between 65 and 78%. It seems that these three genes are much more important in the formation of +CA insertion events than in the formation of deletions.

Cell cycle dependence of NHEJ. In a previous study of RAD52-independent nonhomologous repair events (29), no +CA insertion events were found when the HO gene was expressed only in the G₁ phase of the cell cycle. All of the events were deletions of various sizes, whereas cell cycle-unregulated HO expression led to in a majority of repair events resulting in +CA insertion. This raised the possibility that repair events in G₁ differed from repair events generated at other times in the cell cycle. To compare the events created by pGAL-HO at all times in the cycle, we took advantage of a modified HO promoter developed by Nasmyth (37), in which the upstream regulatory sequences that normally allow HO expression only in mother cells were replaced by the GAL10 upstream activating sequence. In this G1::GAL::HO construct, nearly normal levels of G₁-expressed HO could be induced with galactose, in both mother and daughter cells (37). We constructed a strain, JKM95, in which the G1::GAL::HO allele was created in chromosome IV to replace the resident HO allele. Strain JKM95 was transformed with a URA3-containing plasmid (pJKM10) to make strain JKM95-C, which could then be compared directly with JKM110 for growth on SC-URA, GAL. Ten independent colonies of cells from strain JKM95-C (with the chromosomal G1::GAL::HO allele) were resuspended in water and plated on glucose and on galactose medium lacking uracil. Galactose induction of the HO endonuclease in these donor-deleted strains was generally a lethal event (Table 2). The frequency of survivors for strain JKM95-C on uracil dropout medium was 6.8×10^{-5}. The survival of cells was somewhat better (2.2×10^{-4} versus 6.8×10^{-5}) when strain JKM95 was plated on rich medium containing galactose (YEP-GAL). This is an effect of growth on rich medium and not plasmid loss, since JKM95 grown on SC,GAL medium had a frequency of survivors that was 3.3×10^{-5}. Thus, the rate of survival when HO was expressed only in the

G₁ phase of the cell cycle was approximately 1/30 to 1/60 of the rate when HO was expressed from pGAL-HO.

To emphasize that the change in survival is a function of when HO is expressed during the cell cycle, we transformed strain JKM95 with plasmid pGAL-HO to make JKM95-H, a strain carrying the chromosomal G1::GAL::HO allele and the cell cycle-unregulated plasmid GAL::HO allele. The frequency of survivors for strain JKM95-H grown on SC-URA,GAL was 1.6×10^{-3}, about 20-fold higher than when this same strain without pGAL-HO was grown under the same conditions, and equivalent to the survival rate of JKM110 carrying only pGAL-HO (Table 2). This strongly suggests that DSBs are not as efficiently repaired by end-joining reactions in G₁ as they are in other phases of the cell cycle.

We note that the rate of survival for the G1::GAL::HO strains is most likely an overestimate. When we analyzed the types of healing events, in terms of whether they were a-like, sterile, or still α mating, we found that the distributions within many independent cultures were not uniform. For example, the proportion of sterile derivatives ranged from 40 to 88%, with a mean of 65% and a large standard deviation (± 16%). Similar fluctuations were found with the a-like (mean, 21% ± 12%) and α-mating (mean, 14% ± 11%) classes. This suggests that the G1::GAL::HO gene is somewhat leaky and that some healing events may have been generated in colonies prior to galactose induction; hence, the survival rate is overestimated by the presence of preexisting (HO-induced) mutations prior to the addition of galactose. In no culture were all of the events of only one type; most probably, then, many of the events were generated after HO induction. This conclusion is also supported by the lack of a wide fluctuation in overall survival after galactose induction, among 10 independent colonies (Table 2). In any case, because the G1::GAL::HO gene is still G₁ regulated, we assume that the mutations that we recovered were generated in G₁. However, to ensure the independence of healing events in all subsequent analyses for all strains, cells from each of a large number of independent colonies were individually spread on galactose plates, and only one survivor from each independent sample was randomly selected for DNA sequence and PCR analysis.

DSBs are repaired differently when expression of HO is confined to G₁. When the repair events resulting from DSBs made in G₁ were compared with those made in all stages of the

cell cycle, there was a marked difference (Table 3). One major change is in the frequency of +CA insertions, which account for 78% of the healing events when HO makes cuts in all phases of the cell cycle, but only 21% of the healing events in G_1 cells. Another big difference is the appearance of deletions that are greater than 50 bp in size when the $G1::GAL::HO$ construct is used to generate breaks. These larger deletion events were not detected (<1%) when DSBs were made throughout the cell cycle but account for 38% of the healing events when DSBs were made with $G1::GAL::HO$.

Some of these **a**-like deletions were confirmed by PCR amplification. Eleven **a**-like survivors did not produce an amplified product with primers JKMp3 ($MAT\alpha2$ specific) and JKMp2 (MAT-Z1 specific) (see Fig. 1A for primer positions). Six of the 11 **a**-like survivors yielded a DNA product when primers 649 (MAT-W specific) and 9 (MAT-Z2 specific) were used in PCRs. The deletions varied in size as determined by agarose gel analysis of the PCR products, ranging in size between 700 bp and 1.3 kb. The remaining 5 **a**-like survivors presumably were deletions in MAT that were greater than 1.3 kb, extending beyond the position of primer no. 649. Deletions that extend beyond primer no. 9 would not have been recovered in our experiments because primer no. 9 lies within the C-terminal end of the essential $TSM1$ gene directly distal to MAT (45). Sequence analysis of five of the six PCR-amplified deletions showed that the junction points exhibited microhomology base pairing (2 to 6 bp).

Forty-two sterile derivatives that had small changes of the HO cutting site were analyzed by DNA sequencing (Table 3). Compared with the results when HO was expressed at all cell cycle stages, the $G1::GAL::HO$ strain JKM95 had a threefold decrease in +CA insertions and almost twice as many −ACA deletions. In addition, nearly half of the other events listed in Table 3 for the $G1::GAL::HO$ case (11% of the total) were the single A deletion at Z4 in the cut site region; this deletion was observed in 2% of the sterile derivatives when HO was expressed throughout the cell cycle. The remainder of the other G_1 events were a scattering of other point mutations and 1- to 2-bp insertion/deletions in the cut site sequence (Table 3). Thus, the proportion of insertion events was markedly higher in cells when HO was expressed at all phases of the cell cycle. In addition, when HO was expressed throughout the cell cycle, there was a general shift toward healing events that were confined to the 4-bp 3′ overhanging ends of the cut site and away from more extensive degradation of the cut site ends seen in G_1.

These conclusions are supported by the analysis of healing events in strain JKM95-H, carrying pGAL-HO as well as the chromosomal $G1::GAL::HO$ allele, which are very similar to the events analyzed for JKM110 (Table 4). Survivors from the JKM95-H strain had no large deletion events, and all had a sterile mating type with +CA insertions as the predominant healing event. Analysis of the mating types of survivors from the control strain JKM95-C grown on the same galactose minimal medium lacking uracil showed that they had a similar array of healing events compared with events from strain JKM95 grown on synthetic complete medium with galactose (data not shown).

Finally, we examined α-mating survivors. In the $G1::GAL::HO$ strains, more than 10% of the survivors yielded α-mating colonies; this is significantly higher than the 2% observed for the pGAL-HO-containing colonies (see above). However, as before, most of these colonies proved to have wild-type $MAT\alpha$ loci and apparently had been selected because the HO endonuclease became mutant or was not well expressed. DNA sequence analysis performed on 19 independent α-mating survi-

vors from both Rad⁺ and *rad* mutant cells (see below) showed that 1 had a point mutation in the HO endonuclease recognition site (Z2, G to T) that had been shown previously to prevent HO cleavage, without changing the amino acid sequence of $MAT\alpha1$ (a $MAT\alpha$-inc mutation) (61). Three other mutations had a G-to-C point mutation at position Z3 that changed a glutamine residue to glutamate (Fig. 2). The remaining 15 had a wild-type $MAT\alpha$ sequence.

As a further way to test α-mating colonies, we crossed four additional α maters with an HO $mata1$-inc strain. If the $MAT\alpha$ locus is mutant ($MAT\alpha$-inc), so that HO cannot cut it, then the resulting diploid should be $mata1$-inc/$MAT\alpha$-inc and have an α-mating phenotype. However, if the $MAT\alpha$ locus is wild type, then the resulting $mata1$-inc/$MAT\alpha$ diploid, carrying a normal HO gene, will switch and eventually the cells will be gene converted to $mata1$-inc/$mata1$-inc (**a** mating). In all four cases, this test showed that the α-mating colonies still carried a wild-type $MAT\alpha$ sequence. Thus, of 23 α maters that we analyzed in JKM95-related strains, only 4 had mutations in the HO cut site. We have not further investigated α maters in these studies.

Effects of deleting *RAD52*, *RAD50*, and *RAD1* on G_1-induced repair events. *rad52*, *rad50*, and *rad1* deletion mutants were also constructed in the $G1::GAL::HO$ strain JKM95. The *rad52* and *rad1* mutations in $G1::GAL::HO$ strains caused only minor alterations in viability, as was also found when HO was expressed throughout the cell cycle (Table 2). Similarly, the *rad50* mutation in a $G1::GAL::HO$ strain caused only a 2- to 3-fold reduction in viability compared with a 70-fold drop when HO was expressed throughout the cell cycle (Table 2). This suggests that *RAD50* (and presumably *XRS2* and *MRE11*) does not affect the types of repair events found in G_1 cells (i.e., mostly deletions) nearly as much as it affects the more frequent (mostly insertion) events when HO is expressed throughout the cell cycle. In keeping with this interpretation, nearly all of the survivors in $G1::GAL::HO$ *rad50*Δ cells were large deletions (86%), and nearly all of the remaining events were smaller deletions (Table 4). Thus, the absence of *RAD50* seems to prevent predominantly the types of end-joining events that are generated in the non-G_1 portion of the cell cycle.

DISCUSSION

Although it is evident that all eucaryotic organisms are capable of repairing DSBs by NHEJ, very little is known about the precise mechanisms of repair. In this study, we make two important observations. First, we show that NHEJ is strongly influenced by the stage of the cell cycle in which DSBs are induced, both in the frequency of survival and in the types of repair events that are obtained. Second, we show that three genes that play a minor role in homologous recombinational repair of a single DSB—*RAD50*, *XRS2*, and *MRE11*—play a central role in some, but not all, types of nonhomologous repair. Deletion of these genes not only decreased the efficiency of NHEJ but also nearly eliminated the end-filling (insertion) events that account for 80% of repair when HO is expressed throughout the cell cycle.

NHEJ G_1 repair proceeds predominantly by deletion. Nonhomologous repair of chromosomal DSB is 30-fold less efficient when DSBs occur in the G_1 stage of the cell cycle than at other times. In addition, a much larger proportion of G_1 repair events are deletions of various sizes. How such deletions might be formed is still a matter of conjecture. A large body of data from *S. cerevisiae* suggests that DSBs are degraded by 5′-to-3′ exonucleases to create long stable single-stranded 3′ ended tails (44, 57, 58, 63). When the DSB is flanked by a substantial

region of homology, the break can be repaired by SSA (11, 31, 57), which in *S. cerevisiae* requires the *RAD1* and *RAD10* genes to excise the nonhomologous tails present after annealing (10, 20). Whether nonhomologous deletion end joining also proceeds with the same 5′-to-3′ degradation of the DSB which is followed by complementary base pairing between stable 3′-ended single strands is unknown. Most of the nonhomologous deletion junctions have 1 to 6 bp of homologous base pairing. It is difficult to imagine these regions of microhomology as a stable SSA intermediate; however, it should also be noted that these deletions arise 10 times less frequently than when a DSB is flanked by 90 bp of homology (57). Possibly, there is some alignment protein that helps to stabilize this weak interaction (41). Alternatively, if DNA polymerase is able to initiate replication from the 3′ end of a transiently base-paired intermediate such as those shown in Fig. 2, new DNA synthesis (perhaps displacing the resident strand) could rapidly produce a long, base-paired duplex that would be stable. In any case, the deletion-forming pathway in NHEJ differs significantly from SSA in that it proceeds independently of Rad52 and of Rad1 (Table 2).

Not all of the repair events in *G1*::*GAL*::*HO* Rad$^+$ cells were deletions; 21% were healed by the formation of +CA insertions. We speculate that many of these +CA insertion events may actually have been the result of the repair of DSBs created at times in the cell cycle other than G_1, either because *G1*::*GAL*::*HO* control is not as precise as normal G_1-regulated HO or because some galactose-induced HO endonuclease activity persists into later stages of the cell cycle. This leakiness need not be very great; for example, given that the efficiency of repair in non-G_1 cells is 30 times higher than in G_1 and strongly favors +CA insertions, the expression of HO in only 1% of non-G_1 cells could produce the 20% +CA insertions that we observe. We favor this idea because of a previous study of nonhomologous repair events in a *rad52* strain in which *HO* was under its normal G_1 transcription regulation but was expressed only infrequently because of a *swi1-1* mutation (62). All of the NHEJ events in that *rad52 swi1-1 HO* strain were deletions.

NHEJ non-G_1 repair proceeds by end preservation. The increase in viability when DSBs are induced in all stages of the cell cycle could be due to several reasons. First, end filling may require one or more DNA replication or repair enzymes whose synthesis or activity is S-phase dependent and which might also be present in G_2 cells but not in G_1 cells. These proteins could include one of the DNA polymerases or an end-binding protein analogous to the Ku protein in mammalian cells (59, 42). A second explanation for improved NHEJ in S or G_2 cells is the presence of a sister chromatid. There is substantial evidence showing close physical cohesion of sister chromatids (35) that might play an important role in stabilizing the ends of the DSB and preventing the often extensive degradation that leads to deletions. The close alignment of sister chromatids has been used to explain the preference for sister chromatids in UV-light-stimulated recombinational repair (26) as well as the so-called G_2 repair phenomenon in X-irradiation experiments (9). Our previous studies have shown that the level of *HO* expression from a plasmid-borne *GAL*::*HO* gene is sufficient to cleave both sister chromatids in at least the vast majority of cells with the same kinetics as those in G_1 cells (8). Perhaps the cell initiates the first pairing steps of homologous recombination, enveloping the ends of both broken sister chromatids in a nonproductive recombinase protein complex which protects ends from degradation. The two identically cut sister chromatids could also interact, possibly directly mutually forming heteroduplex DNA to stabilize the ends which could be processed

in an end-joining event. If there is such synaptic protection of the ends, it must be independent of the recombination genes *RAD51*, *RAD52*, *RAD54*, and *RAD57* (Table 2).

Yeasts, like other eucaryotes, exhibit cell cycle arrest in response to DNA damage (17). One might speculate, therefore, that the difference in viability between cells with G_1-induced breaks and those with DSBs induced later in the cell cycle might reflect the probability that a cell would be arrested at a check point long enough for nonhomologous repair to be effected. This may be so, but the increased survival of non-G_1 cells is not merely a quantitative increase; there is a qualitative difference in the types of repair events that argues in favor of the existence of a mechanism of repair in S and/or G_2 that is lacking in G_1.

Roles of *RAD50*, *XRS2*, and *MRE11* in NHEJ repair. There are at least two distinctive pathways of end joining in *S. cerevisiae*: the formation of nonhomologous deletions ranging in size from a few bp to 2.5 kb or the filling in of gaps created by base pairing between overlapping DNA ends, to produce 2- or 3-bp insertions. *RAD50*, *XRS2*, and *MRE11* appear to be required for the +CA fill-in insertions, but these genes are apparently less critical in forming nonhomologous deletions. The effects of *rad50*, *xrs2*, and *mre11* roughly parallel the effect of restricting DSBs to the G_1 phase of the cell cycle in that +CA insertions are markedly reduced. However, there are distinct differences. When HO was expressed in all phases of the cell cycle in a *rad50* (or *xrs2* or *mre11*) strain, nearly 70% of the events were −ACA deletions and 4 to 8% were larger deletions; in contrast, when DSBs were generated in G_1 cells, the proportion of −ACA deletions was only 18%, while 38% were larger deletions. This suggests that the role of *RAD50*, *MRE11*, and *XRS2* is not exclusively in the end-filling pathway.

RAD50, *XRS2*, and *MRE11* have previously been implicated in a surprisingly wide range of recombination processes, including X-ray sensitivity, spontaneous recombination, and the induction of meiotic DSBs (1, 12, 21, 40). Recently, by the use of two-hybrid interaction analysis, these three gene products have been shown to interact in vivo (25, 40). The absence of either Rad50 or Xrs2 retards, but does not prevent, mitotic gene conversion or SSA of a single HO-induced DSB, because of a significant reduction in 5′-to-3′ exonucleolytic degradation of HO-induced DSBs, as well as an effect on later steps in recombination (22, 57). If the reduced 5′-to-3′ exonucleolytic degradation seen in *rad50* deletion mutants is the primary effect on NHEJ, we would expect that deletions might be reduced in favor of end-filling events that should not need 5′-to-3′ DNA degradation. Surprisingly, the absence of *RAD50* increased the proportion of deletions and nearly eliminated +CA insertions in pGAL-HO-induced cells (Table 3). This suggests that the effect of Rad50 and Xrs2 is not simply on 5′-to-3′-end degradation.

What role could Rad50, Xrs2, and Mre11 play in nonhomologous end filling? We suggest that the Rad50, Xrs2, and Mre11 complex influences pairing or cohesion between the sister chromatids which in turn forms a protective complex and prevents extensive degradation. The absence of a close sister chromatid association in *rad50* or *xrs2* mutants can also explain why they show an increased rate of spontaneous heteroallelic recombination in diploids; lesions that would be repaired by a sister, with no genetic change, are more likely to recombine with a nonsister. Of special relevance to our results is the observation that haploid cells from which *RAD50* is deleted do not show in G_2 cells the normal increase in resistance to radiation attributed to repair of a lesion by a sister chromatid (G_2 repair) (9). However, these *rad50* mutant cells do show nearly normal radiation resistance of diploid cells in G_1, when a lesion

can be repaired by a homologous chromosome (diploid repair). This supports the hypothesis that *RAD50* (and possibly *MRE11* and *XRS2*) may be required for establishing sister chromatid interactions that might help to stabilize the cut ends of a DSB.

Multiple pathways of NHEJ. All of the events illustrated in Fig. 2 can be imagined to be alternative outcomes of a single process of nonhomologous end repair. Our data argue that this is not the case. If the formation of +CA insertions, −ACA deletions, and larger deletions were all derived from one process, all using the same proteins, then a change in physiological state or a mutation should affect all of these processes in the same fashion. Instead, we find that restricting the expression of HO to G_1 cells significantly shifts repair toward the creation of large deletions, while eliminating the *RAD50* family of genes in cells expressing HO throughout the cell cycle leads to the accumulation of −ACA deletions. Both of these changes occur at the expense of +CA end-filling events. Thus, even though all of these events involve the formation of DNA junctions involving a few base-pairs of homology, *RAD50* affects both end filling and the insertion of nonhomologous transforming fragments (54) much more than the formation of nonhomologous deletions. Therefore, not all NHEJ repair events arise by simple alternative base pairings and filling in of a single-stranded intermediate. Further experiments will be designed to understand how each of these outcomes is produced.

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