

## Functional Differences and Interactions among the Putative RecA Homologs Rad51, Rad55, and Rad57

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**The genes of the *Saccharomyces cerevisiae* RAD52 epistasis group are required for the repair of ionizing radiation-induced DNA damage. Three of these genes, RAD51, RAD55, and RAD57, have been identified as putative RecA homologs. An important feature of RecA is its ability to bind and hydrolyze ATP. RAD55 and RAD57 contain putative nucleotide binding motifs, and the importance of these motifs was determined by constructing site-directed mutations of the conserved lysine residue within the Walker A-box. Changing the lysine residue to arginine or alanine resulted in a mutant phenotype in DNA repair and sporulation for Rad55 but not for Rad57. Protein-protein interactions among Rad51, Rad55, and Rad57 were tested for by the two-hybrid system. Rad55 was shown to interact with Rad51 and Rad57 but not with itself. Additionally, no interaction between Rad57 and Rad51 or between Rad57 and itself was detected. Consistent with the hypothesis that Rad55 and Rad57 may function within, or stabilize, a protein complex, we found that RAD51 expressed from a high-copy-number plasmid suppresses the DNA repair defect of strains carrying *rad55* and *rad57* mutations. These data, in conjunction with other reports, demonstrate the importance of protein-protein interactions in the process of DNA repair.**

In the yeast *Saccharomyces cerevisiae*, three DNA repair pathways have been defined on the basis of genetic analysis of radiation-sensitive mutants. Genes in the RAD3 group are required for nucleotide excision repair, the RAD6 group of genes is required for error-prone repair, and genes in the RAD52 group are implicated in the recombinational repair of double-stranded breaks (12). The RAD52 group mutants (*rad50-57*, *xrs2*, and *mre11*) share the property of extreme sensitivity to ionizing radiation but exhibit considerable heterogeneity in other phenotypes related to recombinational repair. The RAD55 and RAD57 genes form a subgroup with similar properties. Strains carrying *rad55* and *rad57* mutations show reduced spontaneous mitotic recombination, but recombination is still induced by UV light or ionizing radiation (30, 39, 43). In contrast, *rad51* and *rad52* mutations confer defects in both spontaneous and induced mitotic recombination (1, 36, 38, 43). Most of the genes of the RAD52 epistasis group are required for meiosis. There is some evidence to suggest that RAD57 functions late in meiotic recombination. First, when *rad57* diploids are returned to vegetative growth medium after induction of meiosis, quite high levels of intragenic recombination are observed (14), and second, physically recombined DNA molecules are detected at wild-type levels and with normal kinetics during meiosis (6). However, *rad57* strains show decreased viability during meiosis, and spore products are inviable (14). Spore inviability is a common feature of mutants defective in meiotic recombination, because recombination is required for the correct segregation of homologs at the meiosis I division.

Both point and disruption mutations of RAD55 confer cold sensitivity for the repair of ionizing radiation-induced DNA damage (30). Spontaneous mitotic recombination is reduced in

a similar temperature-dependent manner in strains disrupted for either RAD55 or RAD57 or for both genes (39). This cold sensitivity has been interpreted to mean that Rad55 and Rad57 function within or stabilize a protein complex. On the basis of the similarity of mutant phenotypes, it is likely that they function in the same complex. In addition, an extragenic mutation that suppresses the X-ray sensitivity of both the *rad55* and *rad57* mutations (cited in reference 30) was isolated. Further evidence for a complex that functions in recombinational repair of double-stranded breaks derives from studies on the interaction between the Rad51 and Rad52 proteins (32, 47). However, both proteins clearly have roles other than that of the Rad51-Rad52 complex, as single and double mutants have distinct phenotypes in spontaneous mitotic recombination, HO-induced recombination, and integration of linear DNA during transformation (38, 45, 50).

The RAD55 and RAD57 genes were cloned by complementation of the X-ray-sensitive phenotype (8). Sequence analysis indicates that both predicted peptide sequences share homology with prokaryotic RecA and with two other yeast proteins, Rad51 and Dmc1 (22, 29). Rad51 is likely to be a functional homolog of RecA (1, 2, 47). The Rad51 protein is capable of forming nucleoprotein filaments on duplex DNA remarkably similar to those formed by RecA (34) and exhibits strand exchange activity in vitro (51). On the basis of sequence alignment, Dmc1 may also be a RecA homolog (5, 49). However, the *DMC1* gene is expressed only in meiotic cells, and *dmc1* mutants have no known phenotype in mitotic recombination or DNA repair (5). Dmc1 and Rad51 have been shown to colocalize in meiotic nuclei at sites suggested to represent recombination nodules (4). Of the putative RecA homologs, Rad55 and Rad57 have the least amount of homology to RecA (21% identity) and are lacking some of the conserved regions thought to be important in RecA function. Clearly RAD51, RAD55, and RAD57 are not redundant since mutation of any of these genes confers X-ray sensitivity. A lack of functional redundancy was also observed for spontaneous mitotic intrachromosomal recombination, as a *rad51 rad55 rad57* triple

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TABLE 1. Description of yeast strains

Strain <sup>a</sup>	Relevant genotype or description	Source or reference
W303-1A	<i>MATα leu2-3,112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,15</i>	53
W303-1B	<i>MATα leu2-3,112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,15</i>	53
U672	<i>MATα rad55::LEU2</i>	J. McDonald and R. Rothstein
W826-6C	<i>MATα rad57::LEU2</i>	J. McDonald and R. Rothstein
YRJ20	<i>MATα rad57::URA3</i>	This study
LSY411	<i>MATα rad51::URA3</i>	38
YRJ46	Diploid of W303-1A and W303-1B	This study
YRJ47	Diploid of U672 and W303-1B	This study
YRJ48	Diploid of W303-1A and W826-6C	This study
YRJ50	<i>MATα rad55::LEU2</i>	This study
YRJ51	<i>MATα rad57::LEU2</i>	This study
YRJ52	Diploid of U672 and YRJ50	This study
YRJ53	Diploid of W826-6C and YRJ51	This study
YRJ54	<i>MATα rad55::LEU2 rad57::LEU2</i>	This study
CTY10-5D	<i>MATα leu2-3,112 trp1-901 ade2 his3-200 gal4 gal80 URA3::lexA op-lacZ</i>	S. Fields

<sup>a</sup> All strains used in this study are derivatives of W303-1A or W303-1B, except for CTY10-5D. Only the differences in genotype are noted.

mutant showed the same rate of recombination as a *rad51* single mutant (39). In addition, mutation of any of the four putative RecA homologs confers a meiosis-defective phenotype (5, 35). These observations suggest that each of these yeast proteins has a unique function in the pathway that catalyzes homologous DNA pairing and strand exchange.

In this study, we present genetic evidence for interactions among Rad51, Rad55, and Rad57 based on two-hybrid technology and high copy suppression. In addition, we have characterized the phenotype of disruption and point mutations of *RAD55* and *RAD57* for DNA repair, mating-type switching, and sporulation.

## MATERIALS AND METHODS

**Media, growth conditions, and genetic methods.** Rich medium (YEED), synthetic complete (SC) medium lacking the appropriate amino acid or nucleic acid base, and sporulation media were prepared as described previously (46). Cells were grown at 23, 30, or 37°C as stated for each experiment. Transformations were performed by the lithium acetate method (19). Sporulation was carried out as described previously (46). Briefly, diploid yeast strains containing plasmids were grown overnight on synthetic minimal medium plates which selected for the maintenance of the plasmid. Each strain was then replica plated onto sporulation medium (46) and grown at room temperature (~25°C) for 3 to 5 days. The percent sporulation was determined by microscopic analysis of cells scraped from the sporulation plates and resuspended in water. Percent sporulation values are the averages of two independent cultures of each strain, and no fewer than 200 cells were counted per sample. Cells with three to four visible spores were considered sporulated. Cells with fewer than three visible spores were considered unsporulated. Tetrad dissection was carried out as described previously (46). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium supplemented with 100 µg of ampicillin per ml to maintain plasmids.

**Strains.** A list of the yeast strains used in this study is shown in Table 1. All strains are isogenic to W303 (53), except as noted. U672, which contains the *rad55::LEU2* allele, was constructed by J. McDonald and R. Rothstein with pSTL11 (30). *LEU2* is inserted at codon 166 of the 406-codon open reading frame (ORF). W826-6C was constructed by J. McDonald and R. Rothstein with plasmid pSM51 (a gift from D. Schild, University of California, Berkeley). This strain contains a *LEU2* insertion which replaces codons 193 to 374 of the 460-codon *RAD57* ORF. YRJ20 contains a *URA3* insertion which replaces codons 2 to 374 of the *RAD57* ORF. Plasmids were maintained and amplified in *E. coli* XL1-blue (Stratagene). *E. coli* CJ236 (27) was used as a host for M13 infection.

***RAD55*-containing plasmids.** pSTL4 (30) served as the original parent molecule for all *RAD55*-containing plasmids. pRS316:*RAD55* was constructed by ligating the 1.8-kb *HindIII* fragment from pSTL4 to pRS316 (48) cut with *HindIII*. To create site-directed mutations in *RAD55*, the 1.8-kb *HindIII* fragment from pSTL4 was cloned into the *HindIII* site of M13mp18 to create M13mp18:*RAD55*. Site-directed mutagenesis was performed according to the method of Kunkel et al. (26, 27). Briefly, oRJ18 (5'-TGCCAAAATTAGTCGC GCCAATGCCCGGAG-3') or oRJ19 (5'-CCAAAATTAGTCCTGCCAATGCCCGG-3') was used as the mutagenesis primer. Single-stranded M13mp18:*RAD55* was produced in *E. coli* CJ236 and was used as the template for the

mutagenesis reactions. oRJ18 or oRJ19 was annealed to single-stranded M13mp18:*RAD55* and elongated with T4 DNA polymerase (New England Biolabs) and ligated with T4 DNA ligase (New England Biolabs). DNA from the ligation reaction was transformed into *E. coli* XL1-blue (Stratagene) and then plated to form plaques on a lawn of XL1-blue cells. M13 phage containing the correct mutations was determined by sequence analysis and was given the names M13mp18:*rad55KA49* and M13mp18:*rad55KR49*. M13mp18:*rad55KA49* had two nucleotide changes, an A-to-G change at position 145 of the *RAD55* ORF and an A-to-C change at position 146. These alterations would encode a change of amino acid 49 from lysine to alanine. M13mp18:*rad55KR49* had one nucleotide change, an A-to-G change at position 146 of the *RAD55* ORF which would encode a change of amino acid 49 from lysine to arginine. The 1.8-kb *HindIII* fragments from M13mp18:*rad55KA49* and M13mp18:*rad55KR49* were ligated to pRS316 digested with *HindIII* to generate pRS316:*rad55KA49* and pRS316:*rad55KR49*, respectively. pRS423 derivatives of pRS316:*RAD55*, pRS316:*rad55KA49*, and pRS316:*rad55KR49* were constructed by ligating the 1.8-kb *XhoI-SacI* fragments from each of these plasmids to pRS423 (48) digested with *XhoI* and *SacI*.

A DNA fragment which contained *BamHI* restriction sites at the 5' and 3' ends of the *RAD55* ORF was made by a PCR, with oRJ10 (5'-GGGGATCCAT ATGTCGCTTGGTATACCAC-3') and oRJ11 (5'-GGGGATCCTTAACCTTC ACTATCATAAATTATGTC-3') being used as the primers and pSTL4 as the template. The 1.2-kb *RAD55*-containing PCR fragment was digested with *BamHI* and ligated to pBTM116 (which contains the complete LexA coding sequence, amino acids 1 to 202, under the control of the yeast *ADHI* promoter) cut with *BamHI* to generate plexA:*RAD55*. pGAD10:*RAD55* was constructed by ligating the 1.2-kb *BamHI* fragment to pGAD10 (which contains amino acids 768 to 881 from the *trans*-activating domain of the yeast Gal4 protein under the control of the yeast *ADHI* promoter [9]) digested with *BglII*. Both plexA:*RAD55* and pGAD10:*RAD55* were sequenced to confirm that the *RAD55* ORF was in frame with the *lexA* ORF or the *GAL4* activating domain ORF. plexA:*RAD55* was tested and shown to complement the  $\gamma$ -ray sensitivity phenotype of yeast strain U672.

***RAD57*-containing plasmids.** YEp13:*RAD57* (a gift from D. Schild, University of California, Berkeley) served as the original parent molecule for all *RAD57*-containing plasmids. pRS313:*RAD57* was constructed by ligating the 2.0-kb *DraI-NruI* fragment from YEp13:*RAD57* with pRS313 digested with *SmaI*. To create site-directed mutations in *RAD57*, the 1.1-kb *SaII* fragment from pRS313:*RAD57* was cloned into the *SaII* site of M13mp18 to create M13mp18:*RAD57*. Site-directed mutagenesis was performed as described previously for *RAD55*. oRJ3 (5'-CATAAGTAATTGAGATGCACCAGTAGAGCTCT-3') or oRJ9 (5'-AA TTGAGATCTACCAGTAG-3') was used as the mutagenesis primer, and single-stranded M13mp18:*RAD57* was used as the template. Mutagenesis resulted in the generation of phage M13mp18:*rad57KA131* and M13mp18:*rad57KR131*. The correct mutations were confirmed by sequence analysis. M13mp18:*rad57KA131* had two nucleotide changes, an A-to-G change at position 391 of the *RAD57* ORF and an A-to-C change at position 392. These alterations would encode a change at amino acid 131 from lysine to alanine. M13mp18:*rad57KR131* had one nucleotide change, an A-to-G change at position 392 of the *RAD57* ORF which would encode a change at amino acid 131 from lysine to arginine. The 1.1-kb *SaII* fragments from M13mp18:*rad57KA131* and M13mp18:*rad57KR131* were ligated to pRS313:*RAD57* digested with *SaII* to generate pRS313:*rad57KA131* and pRS313:*rad57KR131*, respectively. pRS423 derivatives of pRS313:*RAD57*, pRS313:*rad57KA131*, and pRS313:*rad57KR131* were constructed by ligating the 2.0-kb *XhoI-SpeI* fragments from each of these plasmids to pRS423 digested with *XhoI* and *SpeI*.

A DNA fragment which contained *Bam*HI restriction sites at the 5' and 3' ends of the *RAD57* ORF was made by a PCR, with oRJ2 (5'-GGGGATCCTC ATGCTAGGGCCCTTATCA-3') and oLS24 (5'-GGGGATCCTCAGGCTGT TTCTATTCC-3') being used as the primers and YEp13-*RAD57* as the template. The 1.4-kb *RAD57*-containing PCR fragment was digested with *Bam*HI and ligated to pBTM116 cut with *Bam*HI to generate plexA:*RAD57*. pGAD10:*RAD57* was constructed by ligating the 1.4-kb *Bam*HI fragment to pGAD10 digested with *Bgl*II. Both plexA:*RAD57* and pGAD10:*RAD57* were sequenced to confirm that the *RAD57* ORF was fused in frame with the *lexA* ORF or the *GAL4* activating domain ORF. plexA:*RAD57* was tested and shown to complement the  $\gamma$ -ray sensitivity phenotype of yeast strain W826-6C.

**Other plasmids.** pRS423:*RAD51* was constructed by ligating a 3.7-kb *Bam*HI fragment from YEp13:*RAD51* (8) to pRS423 digested with *Bam*HI. pGAD10:*RAD51* was a gift from Christian Bendixen and contains the complete *RAD51* ORF fused in frame with the *GAL4* activating domain encoded on pGAD10. YCp50:*HO* and pGAL:*HO* were generously provided by I. Herskowitz (17, 42).

**$\gamma$ -Irradiation survival assays.** Cells were grown in liquid medium at various temperatures to mid-log phase. The cultures were serially diluted (five 10-fold serial dilutions), and then aliquots of each dilution were plated on solid medium. The plates were irradiated in a Gammacell-220 irradiator containing  $^{60}\text{Co}$  (Atomic Energy of Canada) at 0, 5, 10, 15, 20, 25, or 30 kilorads. Cells grown at 23°C were incubated for 4 to 5 days before survivors were counted, and cells grown at 37°C were incubated for 2 days before survivors were counted. Each strain was assayed three separate times, and the mean survival for each dose is presented.

**Two-hybrid interaction assay.** The LexA fusion constructs and the GAD10 fusion constructs were cotransformed into CTY10-5d. Initially,  $\beta$ -galactosidase production was determined by a filter assay as described by Breeden and Namyth (7). Quantitation of  $\beta$ -galactosidase production was determined as described previously (33), with the following alteration. Because of differences in growth rates, the strains were grown on solid medium. Cells were scraped from the plates and then resuspended in Z buffer. Each strain was assayed three times, and the mean value for each strain is reported.

## RESULTS

***rad55*, *rad57*, and *rad55 rad57* mutants are cold sensitive for the repair of ionizing radiation-induced DNA damage.** The original studies indicating cold sensitivity for DNA repair of *rad57* mutants were performed with a single allele, *rad57-1* (cited in reference 13). To determine whether other alleles conferred the same phenotype, haploid strains that contained either a *LEU2* insertion which replaced the middle one-third of the *RAD57* ORF or a *URA3* insertion which replaced the first two-thirds of the *RAD57* ORF were constructed. Haploid strains containing these two disruption alleles were equally sensitive to  $\gamma$ -irradiation, and those grown at 37°C showed a 10-fold greater resistance to radiation than those grown at 23°C at the highest dose examined (Fig. 1; only data from the *rad57::LEU2* strain is shown). An isogenic strain containing a disruption of the *RAD55* gene showed resistance to ionizing radiation similar to that observed for the *rad57* mutant at both temperatures (Fig. 1). Although the alleles of *RAD55* and *RAD57* used in this study do not remove the entire ORF, they are likely to be representative of the null phenotype, as a deletion of the entire *RAD55* ORF has the same phenotype as the *LEU2* insertion (30). A *rad55 rad57* double mutant was found to have sensitivity to  $\gamma$ -irradiation at 23°C equal to that of either of the single mutants, but at 37°C, it was slightly more sensitive than either of the two single-mutant strains. For purposes of comparison, the survival curves of a *rad51* mutant at both temperatures are shown. The *rad51* mutant is equally sensitive to radiation at both temperatures; thus, temperature suppression is not a general feature of *rad* mutants.

It has been reported that homozygous *rad55* and *rad57* diploid strains exhibit greater resistance to ionizing radiation than the corresponding haploid strains (30, 43). This phenomenon, referred to as diploid-specific repair, is also observed in *rad50* and *xrs2* mutants (20, 43) and was confirmed with diploids containing *rad55* or *rad57* disruption mutations (Fig. 2). The suppression of the radiation sensitivity caused by temperature and mating-type heterozygosity was less than that reported

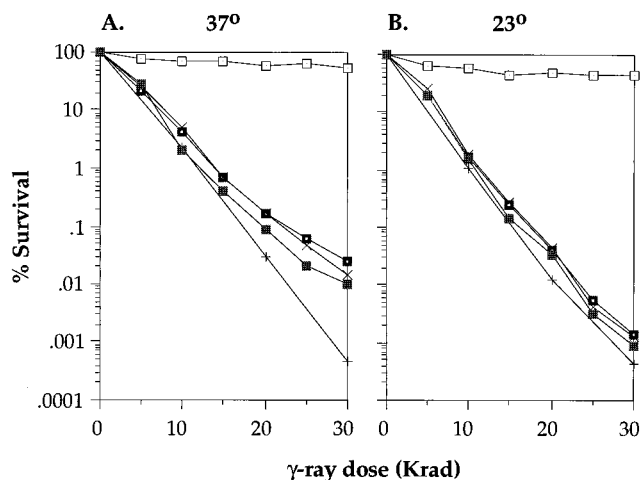


FIG. 1. *rad55* and *rad57* single mutants and *rad55 rad57* double mutants are cold sensitive for ionizing radiation repair. The strains used for these experiments were W303-1A (*RAD*) (□), LSY411 (*rad51*) (+), U672 (*rad55*) (■), W826-6C (*rad57*) (×), and YRJ54 (*rad55 rad57*) (⊞). Experiments were carried out at 37°C (A) and 23°C (B).  $\gamma$ -Ray sensitivity was determined as described in Materials and Methods. Krad, kilorads.

previously for *rad55* strains (30). Since the same allele of *RAD55* was used in both studies, this difference is likely to be due to genetic background differences. These results confirm previous observations of suppression of the *rad55* and *rad57* phenotypes by temperature and mating-type heterozygosity. Furthermore, the similar phenotypes of the *rad55*, *rad57*, and *rad55 rad57* strains suggest that they function at the same step in DNA repair.

***rad55* and *rad57* mutants are unable to switch mating type.** Various DNA lesions, including strand breaks and modified bases, are generated by exposure of cells to ionizing radiation. To determine the response of *rad55* and *rad57* mutants to a single well-defined double-stranded break, we have utilized the mating-type switching system. The *HO* endonuclease initiates mating-type switching by the introduction of a site-specific double-stranded break at the *MAT* locus (24). Failure to repair the break by recombination results in lethality. By dissection of

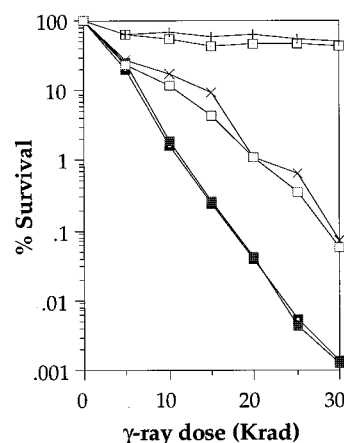


FIG. 2.  $\gamma$ -Ray sensitivity of haploid and diploid *rad* strains. Yeast strains W303-1A (*RAD*) (□), YRJ46 (*RAD/RAD*) (+), U672 (*rad55*) (■), YRJ52 (*rad55/rad55*) (×), W826-6C (*rad57*) (⊞), and YRJ53 (*rad57/rad57*) (□) were analyzed for  $\gamma$ -ray sensitivity at 23°C as described in Materials and Methods. Krad, kilorads.

tetrads from diploids heterozygous for *HO* and for *RAD51* or *RAD52*, the pattern of spore inviability demonstrated that expression of the *HO* endonuclease is incompatible with either *rad* mutation (1, 31). We used a similar approach to determine whether *HO* expression is lethal in *rad55* and *rad57* mutants. Initially, diploids heterozygous for either gene were transformed with a low-copy-number (*CEN*) plasmid containing the *HO* gene regulated by its native promoter (*YCp50:HO*). Tetrads from these strains were dissected, and the viability and mating phenotype of the resulting spores were determined. For both strains, the percent spore viability was the same as that observed for the wild-type diploid. Furthermore, 75% of the spores that contained the *rad* mutation and the *HO* gene were nonmaters, indicating that mating-type switching and diploidization had occurred. This result suggested either that there is sufficient *RAD55* and *RAD57* gene product inherited by the mutant spores to allow them to go through the first switch or that *RAD55* and *RAD57* are not required for mating-type switching. This was addressed in two ways. First, we showed that haploid *rad55* and *rad57* strains could not be transformed with *YCp50:HO*, whereas they were readily transformed with *YCp50*. Second, a plasmid in which *HO* expression is regulated by the *GAL10* promoter was introduced to the haploid *rad* mutants. The plating efficiency of the *rad55* and *rad57* strains was reduced 100-fold on galactose-containing medium compared with that of the wild-type strain. Thus, *RAD55* and *RAD57* are required for mating-type switching, consistent with the results of Sugarawa et al. (50).

**The nucleotide binding site of Rad55, but not that of Rad57, is important for function.** The region of the Rad55 and Rad57 proteins most highly conserved with RecA encompasses the nucleotide binding site (29). This region contains two sequence motifs which are highly conserved among nucleotide binding proteins and which are called the Walker A-type and B-type boxes (55). Mutation of the conserved lysine residue within the A motif (GXXXXGKT/S) has been shown to confer a mutant phenotype in a number of proteins (28, 47). By site-directed mutagenesis, the conserved lysine residue within the A-type box of Rad55 (lysine 49) and of Rad57 (lysine 131) was changed to either an arginine or alanine residue. To test the phenotype of these mutations, each was cloned into a centromere-containing plasmid and then was introduced into a strain containing the appropriate *rad* disruption allele. Comparisons of their abilities to complement the null mutant phenotype were made among the strains containing the vector alone, the vector with a wild-type copy of the gene, and either of the point mutations. Strains containing the *rad55KR49* and *rad55KA49* alleles were deficient in DNA repair after growth at 23°C (Fig. 3). However, strains containing these alleles were more resistant to radiation than strains with the vector alone. To determine whether these alleles were also defective for sporulation, the four plasmids were used to transform a *rad55* homozygous diploid and the resulting strains were sporulated at 23 or 30°C. Because most yeast strains fail to sporulate at temperatures greater than 34°C (15), we were unable to test for the phenotype at 37°C. At both of the temperatures examined, less than 1% sporulation was observed in diploids containing the vector or the *rad55KA49* and *rad55KR49* mutations (Table 2).

The *RAD57* alleles were tested in a similar fashion by transforming each plasmid into haploid or diploid strains containing a disruption of the *RAD57* gene. However, in this case, both the *rad57KA131* and the *rad57KR131* plasmids were able to complement the DNA repair defect for strains grown at 23°C (Fig. 3). These alleles were tested for their ability to complement the sporulation defect of a diploid strain homozygous for the *rad57::LEU2* disruption mutation. Both plasmids comple-

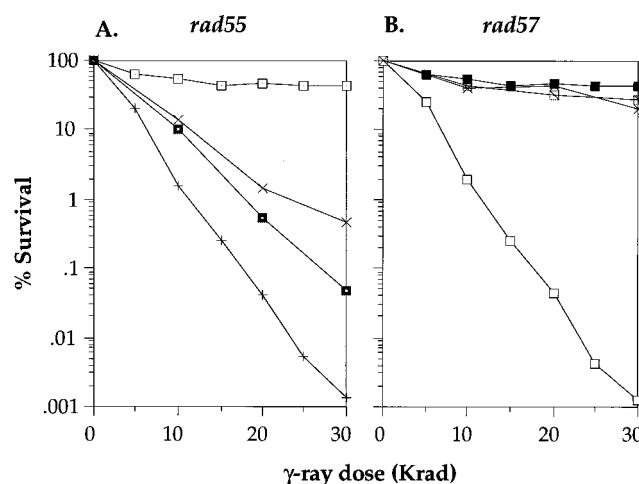


FIG. 3. The nucleotide binding site of Rad55, but not Rad57, is essential for function. (A) The yeast strain U672 was transformed with the following plasmids: pRS316 (+), pRS316:*RAD55* (■), pRS316:*rad55-KA* (●), and pRS316:*rad55-KR* (×). (B) The yeast strain W826-6C was transformed with the following plasmids: pRS313 (□), pRS313:*RAD57* (■), pRS313:*rad57-KA* (\*), and pRS313:*rad57-KR* (○).  $\gamma$ -Ray sensitivity was determined as described in Materials and Methods. All experiments were carried out at 23°C. Krad, kilorads.

mented the sporulation defect of this strain whether it was grown at 23 or 30°C (Table 2). Twenty tetrads from each of these strains were dissected and shown to have greater than 95% spore viability, the same as that observed for the wild-type strain.

If Rad55 forms nucleoprotein filaments analogous to those formed by RecA, a dominant negative phenotype might be expected from the expression of the *rad55KA49* allele in wild-type strains. This was tested by transferring this allele to a high-copy-number plasmid and determining the radiation resistance of a wild-type strain transformed with the plasmid. No increase in radiation sensitivity was observed at any of the temperatures tested (data not shown). Even though *rad57KA131* does not have a mutant phenotype, we also tested this allele for a dominant negative phenotype. No increase in radiation sensitivity was observed when the plasmid containing this mutation was introduced into a wild-type strain.

**Interactions between the Rad51, Rad55, and Rad57 proteins.** Because of the similar phenotypes of *rad55* and *rad57* mutants, we considered the possibility that these two proteins are part of a protein complex. To test this hypothesis, the two-hybrid interaction system of Chien et al. (9) was used. The *RAD55* ORF or *RAD57* ORF was fused to the sequence encoding the LexA DNA binding domain in plasmid pBTM116

TABLE 2. Sporulation of *rad* mutants

Relevant genotype	Plasmid	% Sporulation <sup>a</sup>
<i>RAD/RAD</i>	None	81.9
<i>rad55/rad55</i>	pRS316	<0.5
	pRS316: <i>RAD55</i>	77.8
	pRS316: <i>rad55-KA</i>	<0.5
	pRS316: <i>rad55-KR</i>	<0.5
	pRS313	<0.5
<i>rad57/rad57</i>	pRS313: <i>RAD57</i>	74.4
	pRS313: <i>rad57-KA</i>	47.8
	pRS313: <i>rad57-KR</i>	51.0
	pRS313	<0.5

<sup>a</sup> Sporulation was carried out on plates at 23°C as described in Materials and Methods.

TABLE 3. Physical interactions among Rad proteins

LexA fusion plasmid	GAD fusion plasmid	Sp act (nmol/min/mg of protein) <sup>a</sup>	Fold increase <sup>b</sup>
pBTM116	pGAD10	0.35	NA <sup>c</sup>
plexA: <i>RAD55</i>	pGAD10	3.4	1
	pGAD: <i>RAD51</i>	801	236
	pGAD: <i>RAD55</i>	5.1	1.5
	pGAD: <i>RAD57</i>	27	7.9
plexA: <i>RAD57</i>	pGAD10	0.48	1
	pGAD: <i>RAD51</i>	0.62	1.3
	pGAD: <i>RAD55</i>	65	135
	pGAD: <i>RAD57</i>	0.33	0.7

<sup>a</sup> Specific activity was determined as described in Materials and Methods.

<sup>b</sup> To determine the fold increase for strains carrying plexA:*RAD55*, the specific activity of each strain was divided by the specific activity of CTY10-5d (plexA:*RAD55* and pGAD10). To determine the fold increase for strains carrying plexA:*RAD57*, the specific activity of each strain was divided by the specific activity of CTY10-5d (plexA:*RAD57* and pGAD10).

<sup>c</sup> NA, not applicable.

and to the sequence encoding the Gal4 transcriptional activation domain in plasmid pGAD10. All of the resulting fusion proteins were shown to complement the DNA repair defect of the corresponding disruption mutant strain. Pairs of plasmids were then used to cotransform CTY10-5d, a strain containing four LexA DNA binding sites upstream of the *E. coli lacZ* gene, to test for transcriptional activation mediated by protein-protein interactions. As can be seen from Table 3, we were unable to detect interactions between Rad55 and itself or between Rad57 and itself; however, we observed interaction between Rad55 and Rad57. On the basis of their homologies to RecA and the potential for forming mixed filaments, Rad51 was also tested for interactions with Rad55 or Rad57. We found a strong interaction between Rad51 and Rad55 but no detectable interaction between Rad57 and Rad51 by this method.

**High-copy suppression of the  $\gamma$ -ray sensitivity of *rad55* and *rad57* strains by *RAD51*.** The data presented above suggest that Rad51, Rad55, and Rad57 are part of a multiprotein complex. The cold sensitivity conferred by *rad55* and *rad57* mutations for repair of ionizing radiation-induced DNA damage suggests that the complex may be unstable at lower temperatures in the

absence of Rad55 or Rad57. We considered the possibility that, as seen with other protein complexes, overexpression of one of the members of the complex would partially suppress the defect caused by a mutation in one of the other members of the complex (44). To test this hypothesis, *rad51*, *rad55*, and *rad57* mutants were transformed with a high copy vector containing either *RAD51*, *RAD55*, or *RAD57*. The plasmid containing *RAD51* suppressed the  $\gamma$ -ray sensitivity of both the *rad55* and *rad57* strains by 100-fold at the highest radiation dose tested (Fig. 4). However, plasmids containing *RAD55* or *RAD57* did not suppress the repair defect of the *rad51* mutation, and *rad51* was not suppressed by *RAD55* and *RAD57* together. Furthermore, the plasmid with *RAD55* did not suppress the DNA repair defect of the *rad57* mutant, and the *RAD57* plasmid did not suppress the DNA repair defect of the *rad55* strain.

## DISCUSSION

We have characterized mutations of *RAD55* and *RAD57* and have demonstrated that both genes are required for the repair of ionizing radiation-induced DNA damage, mating-type switching, and sporulation. Point mutations within the putative nucleotide binding domain of *RAD55* confer a mutant phenotype in all assays, suggesting that Rad55 protein requires nucleotide binding and/or hydrolysis to carry out its biological function. The same mutations in *RAD57* confer barely detectable defects in DNA repair and sporulation, which argues that Rad57 does not require a nucleotide cofactor for function. Several lines of evidence suggest that Rad55 and Rad57 interact, and we confirmed this interaction by the two-hybrid system. In addition to detecting an interaction between Rad55 and Rad57, we also discovered that Rad55 interacts with Rad51. High copy plasmids containing *RAD51* suppress the DNA repair defect of *rad55* and *rad57* mutants, supporting the idea that these interactions are biologically relevant. These results are discussed in more detail below.

**Relationship of Rad55 and Rad57 to prokaryotic RecAs.** Through sequence analysis and by modeling of the primary amino acid sequence onto the three-dimensional structure of the *E. coli* RecA protein, four proteins from *S. cerevisiae* have been classified as RecA homologs (18). Such comparisons make the prediction that these proteins should have at least

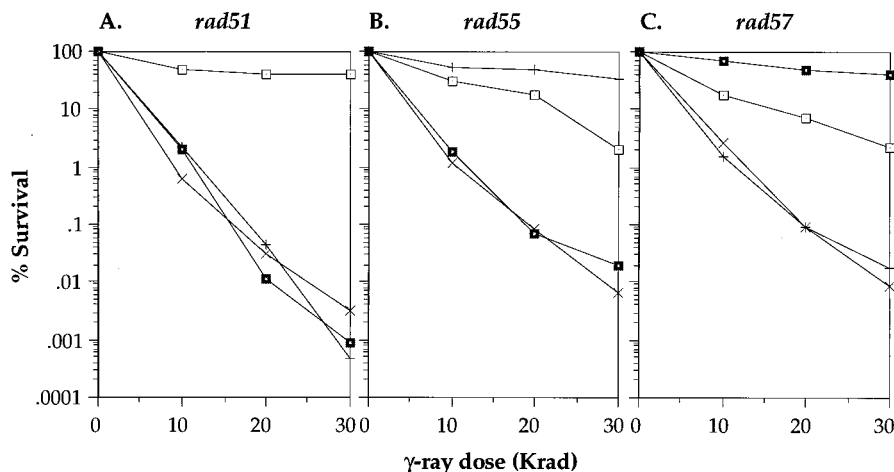


FIG. 4. Overexpression of *RAD51* partially suppresses the ionizing radiation sensitivity phenotype of *rad55* and *rad57* mutants. Strains LSY411 (*rad51*) (A), U672 (*rad55*) (B), and W826-6C (*rad57*) (C) were transformed with the following plasmids: pRS423 ( $\times$ ), pRS423:*RAD51* ( $\square$ ), pRS423:*RAD55* (+), and pRS423:*RAD57* ( $\blacksquare$ ).  $\gamma$ -Ray sensitivity was determined as described in Materials and Methods. All experiments were carried out at 23°C. Krad, kilorads.

some of the biochemical properties of RecA. For Rad51, this comparison seems to be valid. Like RecA, Rad51 has a DNA-dependent ATPase activity and is able to form filaments on double-stranded DNA similar in structure to filaments formed by RecA (34). In addition, Rad51 has recently been shown to catalyze ATP-dependent strand exchange in vitro (51). The human homolog of Rad51 also forms filaments on double-stranded DNA and has the additional RecA properties of forming filaments on single-stranded DNA and causing ATP-dependent untwisting of superhelical DNA (3). To date, no strand exchange activity has been reported for human Rad51. Although these results are consistent with the notion of Rad51 playing a direct role in recombination, quite high levels of recombination occur in *rad51* mutants (38, 45). Furthermore, recent results suggest that the primary role of the Rad51, Rad54, Rad55, and Rad57 proteins may be in facilitating strand invasion into normally inaccessible DNA (50).

In their analysis showing a structural similarity between RecA and Dmc1, Story et al. (49) defined four classes of invariant or conserved amino acid residues, each class being involved in a different aspect of RecA structure and function. When the same residues in Rad55 and Rad57 are considered, Rad55 is 83% conserved and Rad57 is 92% conserved, about twice the degree of similarity expected if the conserved residues were distributed completely at random. Conservation of the structural and functional domains supports the hypothesis that Rad55 and Rad57 are RecA homologs. However, we should point out that Rad55 does not contain glycine 211 and glycine 212, and Rad57 contains only glycine 211. In prokaryotic RecA proteins, these two glycines are invariant and are thought to interact with DNA and/or participate in a conformational change in the RecA protein upon ATP binding. The absence of these residues may reveal a basic difference between RecA and Rad55 and Rad57, indicating that Rad55 and Rad57 have retained only a part of the biological function of RecA.

As was expected, the substitution of alanine or arginine for lysine 49 within the nucleotide binding domain of Rad55 results in reduced resistance to ionizing radiation and complete loss of the ability to sporulate. This result is consistent with the notion of Rad55 requiring nucleotide binding and/or hydrolysis for biological activity. However, the same alteration (lysine 131 to alanine or arginine) in Rad57 resulted in a mutant that was proficient in DNA repair and sporulation. Although mutations of the conserved lysine residue in the Walker A-box frequently result in a mutant phenotype, there are exceptions in which proteins with the same mutations retain biological activity. For example, transformation of a plasmid containing *priAK230*(R or A) into *E. coli* strains with a *priA* null mutation restores the normal growth rate, viability, and morphology of the cell (56). Also, *recA-K72R* is defective in DNA repair and its ability to support plaque formation in red<sup>-</sup> *gam*<sup>-</sup> phage  $\lambda$  (28); however, in vitro RecA-K72R is capable of catalyzing strand exchange (40). The examples of PriA and RecA show that mutation of the lysine in the nucleotide binding domain does not always result in a completely defective protein.

Self-association of RecA monomers is essential for the ability of RecA to form nucleoprotein filaments and carry out strand exchange. Self-association of Rad51 has been demonstrated in vivo by the two-hybrid system (10). In addition, *rad51KA191* confers a dominant negative phenotype for DNA repair, consistent with the formation of a protein complex. It would be predicted that if Rad55 polymerizes to form a filament similar to those of RecA and Rad51, the *rad55KA49* mutation should confer a dominant negative phenotype. Although the failure to observe a dominant negative phenotype

could be interpreted as evidence against Rad55 being a RecA homolog, it is worth noting that the same mutation in the *Ustilago maydis REC2* gene also does not confer a dominant negative phenotype (41). The Rec2 protein has been purified and promotes ATP-dependent homologous pairing and strand exchange in vitro, as was expected for a RecA homolog (23).

**Requirement for protein-protein interactions for DNA repair and recombination.** Numerous processes require the formation of biologically active protein complexes. For example, *E. coli* recombination requires the RecBCD nuclease-helicase protein complex, the RuvAB protein complex is required for branch migration, and the RecA protein self-associated complex is required for strand exchange (25). The requirement for protein complexes to carry out DNA repair in *S. cerevisiae* is becoming more evident. UV excision repair involves the TFIIH complex, which can be purified as a large complex associated with all of the proteins required for the incision step of nucleotide excision repair (52). Mismatch repair in *S. cerevisiae* requires two MutL homologs, Mlh1 and Pms1, which form a heterodimer (37). In conjunction with Msh2, Mlh1 and Pms1 are likely to form a ternary complex during the initiation step of mismatch repair. Recombinational repair is likely to involve interactions between Rad51 and Rad52. In vitro, Rad51 has been shown to interact with itself and Rad52 (47). Experiments with dominant negative alleles of Rad51 and Rad52 illustrate the importance of these types of interactions in vivo and support a model in which a complex of at least Rad51:Rad51 and Rad51:Rad52 is important for DNA repair (10, 32). The yeast single-stranded DNA-binding protein (RPA or RFA) may also participate in this complex. An allele of *rfa1* was recently identified in a screen for mutants deficient in HO-induced recombination (11). The recombination and repair defects of this strain were found to be suppressed by *RAD52* in a dosage-dependent manner.

Several lines of evidence suggest that Rad55 and Rad57 are also part of this complex. First, both *rad55* and *rad57* mutations show cold sensitivity for DNA repair. Second, as was detected by the two-hybrid system, Rad55 interacts with Rad57 and Rad51. And third, high copy plasmids containing *RAD51* suppress the  $\gamma$ -ray sensitivity phenotypes of *rad55* and *rad57* mutants. Hays et al. (16) have also shown interactions among Rad51, Rad55, and Rad57 by two-hybrid technology and high copy suppression. In addition, they have shown that *RAD52*, when present on a high copy number plasmid, suppresses the DNA repair and recombination defects caused by *rad55* and *rad57* mutations (16).

There are at least three models which are consistent with these data. One model predicts that Rad51, Rad52, Rad55, and Rad57, and possibly other factors, form a multiprotein complex involved in recombinational repair. Rad55 and Rad57 could be accessory factors which stabilize Rad51 interactions with single-stranded DNA or enhance its activity. When Rad51 is present at a high concentration, for example, when it is expressed from a high copy number plasmid, recombinational repair would be less dependent on the accessory proteins. Two examples of accessory proteins that enhance the activity of prokaryotic strand exchange proteins have been reported. First, the bacteriophage T4 uvsY protein interacts with a single-stranded DNA-binding protein (gp32) and facilitates binding, by protein-protein interactions, of the uvsX strand exchange protein to DNA (21). Second, the *E. coli* RecF, RecO, and RecR proteins form a complex that stimulates RecA-promoted strand exchange by displacement of SSB from single-stranded DNA (54). Rad55 and Rad57 could potentially play a similar role in stabilizing the Rad51 presynaptic filament. Another model predicts that Rad51, Rad55, and Rad57

act in a recombinational pathway in a sequential manner. Results from experiments on spontaneous mitotic recombination with a chromosomal inverted repeat support this idea. Rattray and Symington (39) proposed that recombinogenic lesions are channeled into a recombination pathway which includes Rad51, Rad54, Rad55, and Rad57. Protein-protein interactions are an appealing method by which these proteins may be localized to the site of DNA repair. Finally, it could be argued that in *S. cerevisiae*, efficient strand exchange is carried out by a mixed filament of the putative RecA homologs.

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