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Suppression of the Double-Strand-Break-Repair Defect of the *Saccharomyces cerevisiae rad57* Mutant

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ABSTRACT

The Rad51 paralogs Rad55 and Rad57 form a heterodimer required to mediate the formation and/or stabilization of the Rad51 filament. To further characterize the function of Rad55-Rad57, we used a combination of *rad57* partial suppressors to determine whether the DNA repair and recombination defects of the *rad57* mutant could be completely suppressed. The combination of all suppressors, elevated temperature, *srs2*, *rad51-I345T*, and mating-type (*MAT*) heterozygosity resulted in almost complete suppression of the *rad57* mutant defect in the recruitment of Rad51 to DNA-damaged sites, as well as survival in response to ionizing radiation and camptothecin. In a physical assay to monitor the kinetics of double-strand-break (DSB)-induced gene conversion, the *rad57* mutant defect was effectively suppressed by *srs2* and *MAT* heterozygosity, but these same suppressors failed to suppress the spontaneous recombination defect. Thus the Rad55-Rad57 heterodimer appears to have a unique function in spontaneous recombination that is not essential for DSB repair. Furthermore, we investigated the currently unknown mechanism of *rad57* suppression by *MAT* heterozygosity and found that it is independent of *DNL4*.

HOMOLOGOUS recombination is required for the faithful repair of DNA double-strand breaks (DSBs) that arise during normal cellular processes or from exposure of cells to DNA-damaging agents. Central to the process of homologous recombination is the Rad51 protein, which facilitates synapsis and strand invasion into homologous duplex DNA (San Filippo *et al.* 2008). Rad51 belongs to the RecA family of homologous pairing proteins (Aboussekhra *et al.* 1992; Basile *et al.* 1992; Shinohara *et al.* 1992). Yeast and humans have two RecA homologs: Rad51 and the meiosis-specific Dmc1 (Bishop *et al.* 1992; San Filippo *et al.* 2008). In addition, the *Saccharomyces cerevisiae RAD55* and *RAD57* genes encode proteins with sequence similarity to RecA and Rad51 and are considered to be Rad51 paralogs (Kans and Mortimer 1991; Lovett 1994). Mutation of *RAD51*, *RAD55*, or *RAD57* confers sensitivity of ionizing radiation (IR) and defects in mitotic and meiotic recombination, indicating that their functions are not redundant (Symington 2002). *rad51* mutants generally exhibit more severe defects than *rad55* or *rad57* mutants in DSB-induced recombination assays; however, *rad55* and *rad57* mutants are more defective than *rad51* in some assays that measure spontaneous recombination between repeated sequences (Rattray and Symington 1995; Mozlin *et al.* 2008).

The molecular details of homologous recombination are largely based on genetic, physical, and cytological studies of DSB repair (DSBR) and on biochemical characterization of purified proteins (Paques and Haber 1999; Sugawara *et al.* 2003; Lisby *et al.* 2004; San Filippo *et al.* 2008). The single-stranded DNA (ssDNA)-binding protein, replication protein A (RPA), initially binds ssDNA that forms by nucleolytic processing of DNA ends at DSBs. *In vitro*, RPA has been shown to be inhibitory to Rad51 binding to ssDNA, but this inhibition can be overcome by addition of Rad52 or the Rad55-Rad57 heterodimer to the reaction (Sung 1997a,b; New *et al.* 1998; Shinohara and Ogawa 1998). Rad52, via its interaction with both RPA and Rad51, recruits Rad51 to the DNA and stimulates the removal of RPA (Sugiyama *et al.* 1998; Song and Sung 2000; Seong *et al.* 2008). Once Rad51 has been recruited to the ssDNA, a nucleoprotein filament forms in an ATP-dependent manner. A competent Rad51 filament is then able to interact with another DNA molecule to search for homologous sequences and initiate strand exchange (Krogh and Symington 2004). Consistent with the *in vitro* assays, *rad52* mutants are completely defective in the recruitment of Rad51 to meiotic and mitotic DSBs *in vivo* (Gasior *et al.* 1998; Sugawara *et al.* 2003; Lisby *et al.* 2004; Miyazaki *et al.* 2004).

Cytological and chromatin immunoprecipitation studies suggest that Rad55 and Rad57 mediate filament formation by facilitating nucleation of Rad51 onto ssDNA

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or by stabilizing the filament once it is assembled. In *rad55* mutants, Rad51 is recruited to DSBs with slower kinetics and forms dimmer IR-induced foci compared to wild-type cells (Sugawara *et al.* 2003; Lisby *et al.* 2004; Fung *et al.* 2006). The IR sensitivity of *rad55* and *rad57* mutants is partially bypassed by overexpression of Rad51, which increases the availability of the protein for filament formation, or by *RAD51* gain-of-function alleles, such as *rad51-I345T*, that encode proteins with a higher affinity for DNA than wild-type Rad51 (Hays *et al.* 1995; Johnson and Symington 1995; Fortin and Symington 2002; Malik and Symington 2008). Deletion of *SRS2*, which encodes a helicase that disrupts Rad51-ssDNA complexes *in vitro*, also suppresses the IR sensitivity of *rad55* and *rad57* mutants (Krejci *et al.* 2003; Veaute *et al.* 2003; Fung *et al.* 2006). The expression of both mating-type alleles in haploid or diploid cells suppresses the IR sensitivity and interhomolog recombination defects of *rad55* and *rad57* mutants through an unknown mechanism (Lovett and Mortimer 1987; Mozlin *et al.* 2008). Its target of action is presumed to be the Rad51 nucleoprotein filament since mating-type heterozygosity suppresses other mutations that result in Rad51 filament defects such as *rad51-K191R* and *rad52-20*, and these mutations are also suppressed by deletion of *SRS2* or by overexpression of Rad51 (Schild 1995; Morgan *et al.* 2002; Fung *et al.* 2006). The DSBR defect of *rad55* and *rad57* mutants is cold sensitive (Lovett and Mortimer 1987; Symington 2002). Cold sensitivity is a property often associated with proteins composed of multiple subunits or large multi-protein complexes (Scheraga *et al.* 1962), consistent with a role for the Rad51 paralogs in stabilizing Rad51 nucleoprotein filaments. Together, these data support the proposed role for Rad55 and Rad57 as accessory factors for Rad51 during the initiation of recombination.

Vertebrates encode five Rad51 paralogs: Rad51B, Rad51C, Rad51D, Xrcc2, and Xrcc3. Mutations in genes encoding the Rad51 paralogs in chicken DT40 cells confer defects in DSB-induced homologous recombination and result in spontaneous chromosomal aberrations, high sensitivity to DNA crosslinking agents, and decreased Rad51 focus formation upon exposure to IR (Takata *et al.* 2001). Overexpression of human Rad51 suppresses the sensitivity of the DT40 Rad51 paralog-defective cell lines to DNA crosslinking agents, consistent with their function as accessory proteins for Rad51 (Takata *et al.* 2001). However, studies in mammalian cells suggest that there could be a later function for the Rad51 paralogs, possibly involving Holliday junction resolution. The human Rad51B-Rad51C-Rad51D-Xrcc2 complex preferentially binds to branched DNA substrates, including a synthetic Holliday junction substrate, over other DNA substrates (Yokoyama *et al.* 2004). *XRCC3*- and *RAD51C*-defective cell lines exhibit longer-than-normal gene conversion tract lengths, which could result from defects in the resolution of recombination intermediates or be due to a different mode of recombination by the Rad51-independent pathway (Brenneman *et al.* 2002; Nagaraju *et al.* 2006; Pohl and Nickoloff 2008). Extracts made from *XRCC3*- or *RAD51C*-defective Chinese hamster ovary cells show reduced levels of Holliday junction resolvase activity (Liu *et al.* 2004). Furthermore, Rad51C colocalizes with the mismatch repair protein Mlh1, which serves as a marker for the later pachytene/diplotene stages during meiosis, and both Rad51C and Xrcc3 associate with the pseudoautosomal region, a crossover hotspot during meiosis (Liu *et al.* 2007).

To determine whether the function of Rad55 and Rad57 is limited to the initiation of recombination, we used a combination of *rad55* and *rad57* suppressors that are thought to function by promoting Rad51 filament function to see if these suppressors can make a competent filament that can fully suppress *rad55* and *rad57* defects. We show almost complete suppression of the Rad51 recruitment and DSBR defects of the *rad57* mutant by combining the suppressors, which suggests that the primary function of Rad55-Rad57 in DSBR is recruitment and/or stabilization of Rad51. However, the same combination of suppressors that suppresses the DSB-induced gene conversion defect of *rad57* does not suppress the spontaneous recombination defect, suggesting that Rad55-Rad57 has a role in spontaneous recombination that is distinct from its role in DSBR.

MATERIALS AND METHODS

Media, growth conditions, and genetic methods: Rich medium [yeast extract–peptone–dextrose (YPD)], synthetic complete medium (SC) lacking the appropriate amino acids or nucleic acid bases, sporulation medium, and genetic methods were as described previously (Sherman *et al.* 1986). Synthetic deficient medium containing 2% raffinose and supplemented with adenine, uracil, histidine, and leucine was used for the galactose induction of *I-SCEI* in the direct-repeat recombination assays.

Yeast strains and plasmids: *S. cerevisiae* strains used in this study are listed in Table 1. All strains are in the W303 background (*his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100*) except those listed as BY4742 and LSY1786 (Zou and Rothstein 1997). The yellow fluorescent protein (YFP) fusion strains were made by crossing the appropriate haploid parents, sporulating the resulting diploids, and screening the haploid progeny for the correct phenotype; the expression of YFP was confirmed by epifluorescence microscopy. To construct LSY1957-1 and LSY2004-1, pRS406-*rad51-I345T* was cut with *Bsu*36I and transformed into the *YFP-RAD51* strain W5857-2C or into the *YFP-RAD51 rad57* strain LSY1956-8B, creating a repeat. The resulting Ura^+ transformants were screened for the presence of the *rad51-I345T* allele fused to *YFP* by PCR and for restriction digestion to detect the novel *Hpa*II restriction site introduced by the allele. The second *RAD51* allele in the repeat formed by integration lacks a promoter and is not expressed (Fung *et al.* 2006). Haploid strains expressing both mating types were made by transforming haploids with the opposite mating-type allele on the pRS414 vector. *SIR4*, *DNL4*, and *RAD55* deletion strains were

TABLE 1

Yeast strains

Strain	Genotype	Source or reference
W1588-4C	*MAT***a**	Zou and Rothstein (1997)
W1588-4A	*MAT*α	Zou and Rothstein (1997)
W5857-2C	*MAT***a** *ADE2 YFP-RAD51*	Lisby *et al.* (2004)
YHK597-2B	*MAT***a** *rad55::LEU2*	H. Klein
YHK598-8B	*MAT*α *rad57::LEU2*	H. Klein
YHK1186-5C	*MAT*α *dnl4::URA3*	H. Klein
LSY1421-2A	*MAT*α *srs2::HIS3 ade2-n::URA3::ade2-a*	This study
LSY1422-2A	*MAT*α *rad57::LEU2 ade2-n::URA3::ade2-a rad5-535*	This study
LSY1422-6B	*MAT*α *rad57::LEU2 srs2::HIS3 ade2-n::URA3::ade2-a*	This study
LSY1519-1D	*MAT*α *ade2-n::TRP1::ade2-I*	Mozlin *et al.* (2008)
LSY1682-1B	*MAT*α *rad55::LEU2 dnl4::URA3*	This study
LSY1786	*MAT*α *dnl4::KanMX4 rad55::LEU2 his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	This study
LSY1827-1D	*MAT*α *ADE2 rad51-I345T srs2::HIS3*	This study
LSY1867	*MAT***a** *ADE2 srs2::HIS3*	This study
LSY1892	*MAT*α *ade2-n::URA3::ade2-a*	This study
LSY1894-3B	*MAT*α *rad57::LEU2 ade2-n::URA3::ade2-a*	This study
LSY1895	*MAT***a** *rad57::LEU2 rad51-I345T ade2-n::URA3::ade2-a*	This study
LSY1896-1A	*MAT*α *rad57::LEU2 rad51-I345T srs2::HIS3 ade2-n::URA3::ade2-a*	This study
LSY1898	*MAT*α *srs2::HIS3 sir4::KanMX4 ade2-n::URA3::ade2-a*	This study
LSY1900	*MAT*α *rad57::LEU2 srs2::HIS3 sir4::KanMX4 ade2-n::URA3::ade2-a*	This study
LSY1956-8B	*MAT***a** *ADE2 YFP-RAD51 rad57::LEU2*	This study
LSY1957-1	*MAT***a** *ADE2 YFP-rad51-I345T-URA3-RAD51*	This study
LSY2004-1	*MAT*α *ADE2 YFP-rad51-I345T-URA3-RAD51 rad57::LEU2*	This study
LSY2005-8A	*MAT*α *ADE2 YFP-rad51-I345T-URA3-RAD51 rad57::LEU2 srs2::HIS3*	This study
LSY2029	*MAT*α *rad57::URA3 ade2-I dnl4::KanMX4*	This study
LSY2032-1C	*MAT***a** *ade2-n::TRP1::ade2-I lys2::GAL-I-SCEI rad57::LEU2 srs2::HphMX4 sir4::KanMX4*	This study
LSY2032-10C	*MAT***a** *ade2-n::TRP1::ade2-I lys2::GAL-I-SCEI*	This study
LSY2032-12A	*MAT***a** *ade2-n::TRP1::ade2-I lys2::GAL-I-SCEI rad57::LEU2*	This study
LSY2086-2B	*MAT*α *ADE2 YFP-rad51-I345T-URA3-RAD51 srs2::HIS3*	This study
LSY2113-4	*MAT*α *sir4::KanMX4 srs2::HIS3 ade2::TRP1::ade2-I lys2::GAL-I-SCEI*	This study
BY4742 *dnl4::KanMX4*	*MAT*α *dnl4::KanMX4 his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Winzeler *et al.* (1999)
BY4742 *sir4::KanMX4*	*MAT*α *sir4::KanMX4 his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Winzeler *et al.* (1999)
BY4742 *rad55::KanMX4*	*MAT*α *rad55::KanMX4 his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Winzeler *et al.* (1999)

made by a one-step gene replacement of the relevant locus with a linear PCR fragment containing homologous 5′ and 3′ flanking sequences and the *KanMX4* selectable marker from the appropriate BY4742 deletion strain. LSY1786 was constructed by one-step gene replacement of *RAD55* in BY4742 *dnl4::KanMX4* with a linear PCR fragment containing homologous 5′ and 3′ flanking sequences and *LEU2* from YHK597-2B. Most other haploid strains were made by mating appropriate haploid strains, sporulating the resulting diploids, and screening the haploid segregants for the desired genotype. Unless otherwise indicated, null alleles of all genes were used in these studies.

To construct pRS406-*rad51-I345T*, a PCR fragment containing the *rad51-I345T* complete open reading frame was made using pRS413-*rad51-I345T* (Fortin and Symington 2002) as the PCR template. The PCR fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and subsequently cloned into the pRS406 vector using the *Sac*I and *Not*I restriction sites. pRS414-*MAT***a** and pRS414-*MAT*α were gifts from R. Rothstein.

Microscopy: Cells were grown in SC or SC–TRP liquid medium until an optical density at 600 nm (OD_{600}) of 0.2, at which time the liquid cultures were exposed to the defined doses of radiation in a Gammacell-220 irradiator containing ^{60}Co, or treated with camptothecin (CPT) at a concentration of 7 μg/ml for 3 hr. Aliquots of the cultures were processed immediately for imaging as described by Lisby *et al.* (2001). Live cell images were captured as described (Lisby *et al.* 2004). YFP fluorescence was acquired using Openlab software (Improvision) and quantified using Volocity software (Improvision). For each set of strains shown in Figures 3 and 6, samples were processed at the same time because of day-to-day variation in focus brightness.

Clastogen sensitivity tests: For IR sensitivity tests, cells were grown in liquid medium to mid-log phase at 23° or 30°. The cultures were serially diluted, and aliquots of each 10-fold dilution were spotted onto YPD or SC–TRP plates. The plates were left unirradiated or irradiated in a Gammacell-220 irradiator containing ^{60}Co for the designated dose and then incubated for 3 or 5 days at 30° or 23°, respectively. For CPT sensitivity tests, cells were grown in SC–TRP overnight at 30° or 23°. Strains were diluted to a concentration of 7×10^6 cells/ml and then 10-fold serially diluted and spotted onto SC–TRP plates or onto SC–TRP plates containing the specified concentration of CPT buffered with 0.25% dimethyl sulfoxide (DMSO). A stock solution was made by dissolving CPT in

DMSO at 1 mg/ml. Control plates contained 0.25% DMSO. The plates were incubated for 3 or 5 days at 30° or 23°, respectively.

DSB-induced gene conversion assay: SC–TRP glucose cultures (5 ml) were grown overnight at 30°. Cells were diluted to a concentration of 1×10^5 cells/ml in 300 ml SC–TRP with raffinose replacing glucose. Cultures were grown overnight to a concentration of 3×10^6 cells/ml, and galactose was added to the cultures for a final concentration of 2%. Fifty milliliters of cells were harvested at each indicated time point after galactose induction. DNA was isolated from each time point and used as template for PCR with the following primers amplifying the 2.5-kb region encompassing the *ade2-I* allele: 5GCA 5′-GTTGTGTGGAATTGTGAGCG-3′ and 3GCA 5′-CGCCATACTGGAGGCAATAA-3′. PCR in the linear range was performed using 25 cycles and 5 ng of genomic DNA. Control primers amplifying 1.8 kb of chromosome *IV* (*TRP1* locus) were used in a PCR reaction with the *ade2* reporter primers to normalize the amount of PCR product. Control primers used were 5QTrp1 5′-CACGGCAGAGACCAATCAGTA-3′ and 3QTrp1 5′-GCACTCCTGATTCCGCTAATA-3′. PCR products were analyzed on a 1.5% agarose gel. To amplify repaired products, PCR was performed for 35 cycles and with 500 ng template DNA. PCR products were digested with *Aat*II and analyzed on a 1.8% agarose gel.

Determination of spontaneous mitotic recombination rates: Mitotic recombination rates between *ade2* direct repeats were determined as described previously by Mozlin *et al.* (2008).

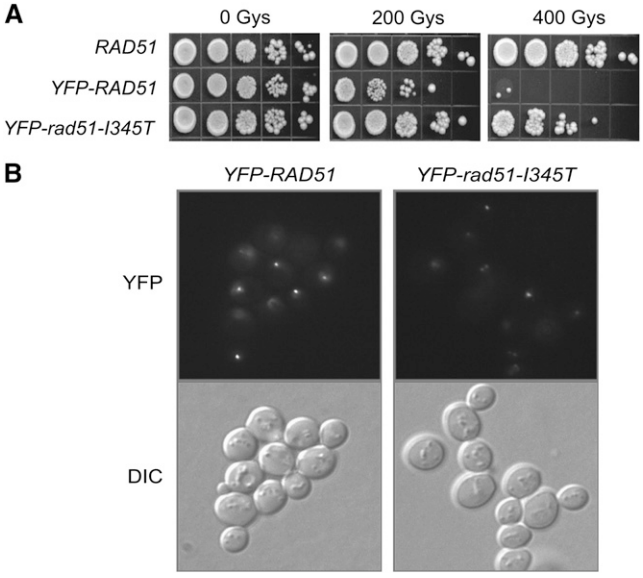

Figure 1.—YFP-rad51-I345T is more functional *in vivo* than YFP-Rad51. (A) Serial dilutions of log-phase cultures of LSY1519-1D (*RAD*), W5857-2C (*YFP-RAD51*), and LSY1957-1 (*YFP-rad51-I345T*) were spotted onto YPD plates and left unirradiated or irradiated at 200 and 400 Gy. Survival was assessed following growth for 3 days at 30°. (B) Log-phase cultures of strains were exposed to 200 Gy of γ-irradiation, followed by microscopy to monitor focus formation.

RESULTS

***YFP-rad51-I345T* is functional:** Previous studies have shown a defect in the formation and/or brightness of DSB-induced Rad51 foci in *rad55* or *rad57* mutants (Gasior *et al.* 1998, 2001; Mozlin *et al.* 2008). To determine whether the defect in Rad51 recruitment to DSBs observed in the *rad57* mutant could be rescued by factors thought to act at the level of the Rad51 filament—namely elevated temperature, deletion of *SRS2, rad51-I345T,* and expression of both mating-type alleles—we monitored Rad51 foci formation by epifluorescence microscopy. To study the suppression conferred by the *rad51-I345T* allele, the endogenous Rad51-I345T protein was tagged with YFP. First, a strain expressing this fusion protein in a *RAD57* background was tested for IR sensitivity and foci formation. Several groups have noted previously that terminally tagged versions of Rad51 are not fully functional (Lisby *et al.* 2004; Kojic *et al.* 2005). Surprisingly, the *YFP-rad51-I345T* strain was almost as IR resistant as the untagged *RAD51* strain, in contrast to the *YFP-RAD51* strain (Figure 1A). Although the number of cells with IR-induced Rad51 foci was comparable for the *YFP-RAD51* and *YFP-rad51-I345T* strains, the foci formed by the YFP-Rad51 fusion protein were on average 1.5 times brighter than the IR-induced foci formed by the more functional YFP-Rad51-I345T protein (Figure 1B). The brighter YFP-Rad51 foci could be a result of inappropriate recruitment, retention, aggregation, or turnover of YFP-Rad51 fusion protein.

The Rad51 foci formation defect of the *rad57* mutant strain can be suppressed: Rad51-I345T focus formation was monitored following treatment of cells with CPT (7 μg/ml) for 3 hr. CPT stabilizes the covalent DNA-Top1 intermediate that forms during the catalytic DNA nicking–closing cycle of Top1, and these stable nicks can then be converted into recombinogenic DSBs during replication (Hsiang *et al.* 1989). CPT was used instead of IR to maintain cells at a constant temperature throughout the experiment. For this analysis, we measured two parameters: the number of CPT-induced foci and the foci brightness. At 30° in the wild-type *RAD57* background, YFP-Rad51-I345T formed at least one focus in 91% of the cells, whereas, in the *rad57* mutant strain, only 26% of the cells had at least one YFP-Rad51-I345T focus (Figure 2B). In addition, the brightness of the YFP-Rad51-I345T foci in the *rad57* mutant strain was only 31% of the brightness of the foci in the *YFP-rad51-I345T RAD57* strain (Figure 2C). When the suppressors *srs2* and *MAT* heterozygosity were combined with the *rad51-I345T* allele in the *rad57* strain, 90% of cells had at least one focus at 30°, which is comparable to wild type. Although the brightness of the foci in the *rad57*-suppressed strain was increased to 90% of the *RAD57 YFP-rad51-I345T* strain, suppression was not complete ($P = 0.0012$). The degree of suppression of foci brightness was temperature dependent; at 23°, the brightness of the YFP-Rad51-I345T foci in the *rad57* suppressor strain was only 73% of the brightness of the foci in the *YFP-rad51-I345T* strain, in contrast to the 90% brightness of YFP-Rad51-I345T foci in the *rad57* suppressor strain at 30° ($P = 0.0001$) (Figure 2C). In the wild-type *RAD57*

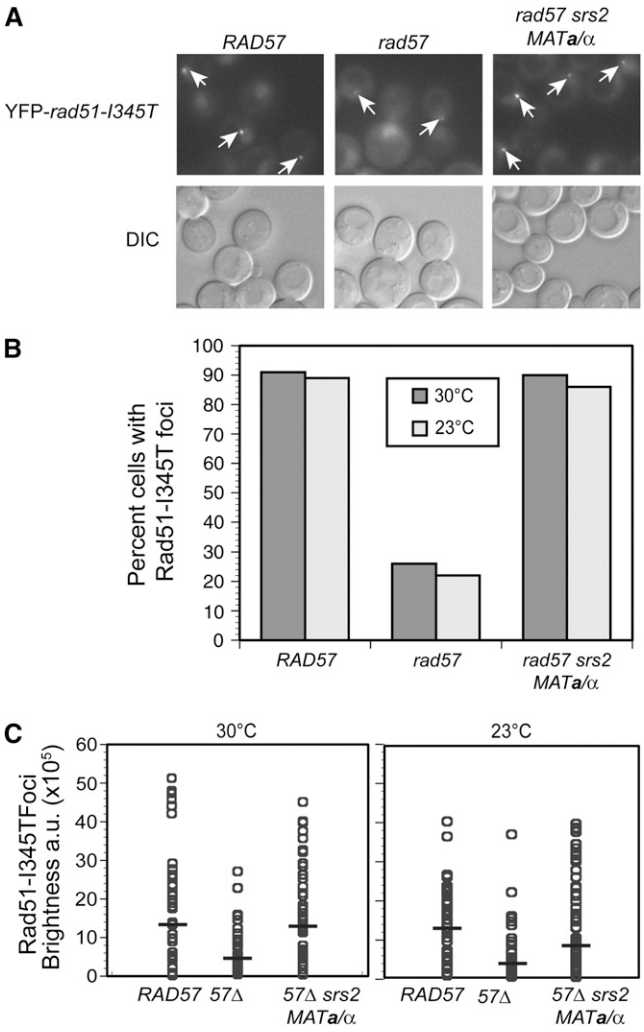

FIGURE 2.—The combination of the suppressors *srs2* and mating-type heterozygosity almost fully suppresses the Rad51 foci formation defect of *rad57* in response to treatment with camptothecin. (A) To express both mating types, LSY2005-8A (*MAT*α *YFP-rad51-I345T rad57::LEU2 srs2::HIS3*) was transformed with pRS414-*MAT***a** whereas LSY1957-1 (*RAD57*) and LSY2004-1 (*rad57::LEU2*) were transformed with the pRS414 empty vector. Log-phase SC–TRP cultures of strains were treated with camptothecin at a concentration of 7 μg/ml for 3 hr at 30° or 23° followed by microscopy to monitor focus formation. (B) The percentage of cells with at least one focus was calculated; at least 100 cells were analyzed for each strain. (C) The mean focus brightness was normalized for each strain relative to LSY1957-1 (*RAD*57 *YFP-rad51-I345T*). The brightness of each focus was quantified and plotted; at least 80 foci were analyzed for each strain. A solid bar represents the mean focus brightness for each strain; a.u. represents arbitrary units.

background, YFP-Rad51-I345T formed at least one focus in 89% of the cells at 23°, whereas the *rad57*-suppressed strain had no defect with 86% of the cells having at least one YFP-Rad51-I345T focus ($P = 0.08$), suggesting that suppression of the number of foci formed is not temperature dependent (Figure 2B).

The IR and CPT sensitivity of the *rad57* null strain can be fully rescued by combining partial suppressors of the *rad57* mutant: A prediction from the above finding is that, if Rad55 and Rad57 function solely in recruitment and/or stabilization of Rad51 at damaged sites, then combinations of partial suppressors of *rad55* and *rad57* that suppress the Rad51 foci defect should additively suppress the sensitivity of *rad55* and *rad57* mutants to genotoxic agents. If, however, Rad55-Rad57 has a function independent of Rad51 filament formation, then suppressing the Rad51 recruitment defect of a *rad57* strain would not be expected to suppress cell survival after DNA damage. The DNA repair defect of *rad57* strains with all combinations of suppressors was assessed by the plating efficiency at 30° and 23° after exposure to IR or on medium containing CPT. DSBs are the toxic lesion generated by IR or CPT exposure.

Of the single suppressors, *MAT* heterozygosity showed the greatest suppression of the IR sensitivity of the *rad57* mutant, and, when combined with *srs2* or *rad51-I345T*, there was additive suppression (Figure 3). We found that there was almost full suppression of the IR and CPT sensitivity of the *rad57* mutant when at least three of the four suppressors were combined. Because the *srs2* mutation also confers sensitivity to IR and CPT, it appears that the suppression of the *rad57* strain with all of the suppressors is complete. At both 23° and 30°, the *rad57 srs2 rad51-I345T MAT***a**/α strain showed equal or higher resistance than the *srs2* single-mutant strain. Fewer than three suppressors resulted in incomplete suppression, especially at 23° (Figure 3).

Suppression of the DSB-induced gene conversion defect of the *rad57* mutant: Although we observed suppression of the IR and CPT sensitivity of the *rad57* mutant, the survival assays offer insight only into the end result of repair: the ability to complete repair and form a colony or cell death. Because the survival assays are uninformative in regards to the timing of recombination, we used a DSB-induced gene conversion assay to monitor the kinetics of repair. The substrate used contains a direct repeat of alleles of the *ade2* gene separated by vector sequences and a copy of the *TRP1* gene integrated at the endogenous *ADE2* locus (MOZLIN *et al.* 2008) (Figure 4A). One allele contains a 2-bp fill-in mutation of the *Nde*I site (HUANG and SYMINGTON 1994); the other allele contains an insertion of the I-*Sce*I nuclease recognition site disrupting the *Aat*II site. The strains also contain a fusion of the *I-SCEI* gene to the *GAL1* promoter to provide regulated expression of the nuclease. After inducing I-*Sce*I expression at 30°, collecting cells at appointed time points, and isolating genomic DNA, gene conversion events can be detected by PCR with primers that anneal to the vector sequence upstream of the *ade2-I* allele and to the *ade2* sequence downstream of the I-*Sce*I cut site. Uncut DNA or gene conversion using the *ade2-n* as the donor will produce a PCR product, whereas events repaired by single-strand annealing or unrepaired DNA will not (Figure 4, A and B). By quantitative PCR, the suppressed *rad57* strain displayed

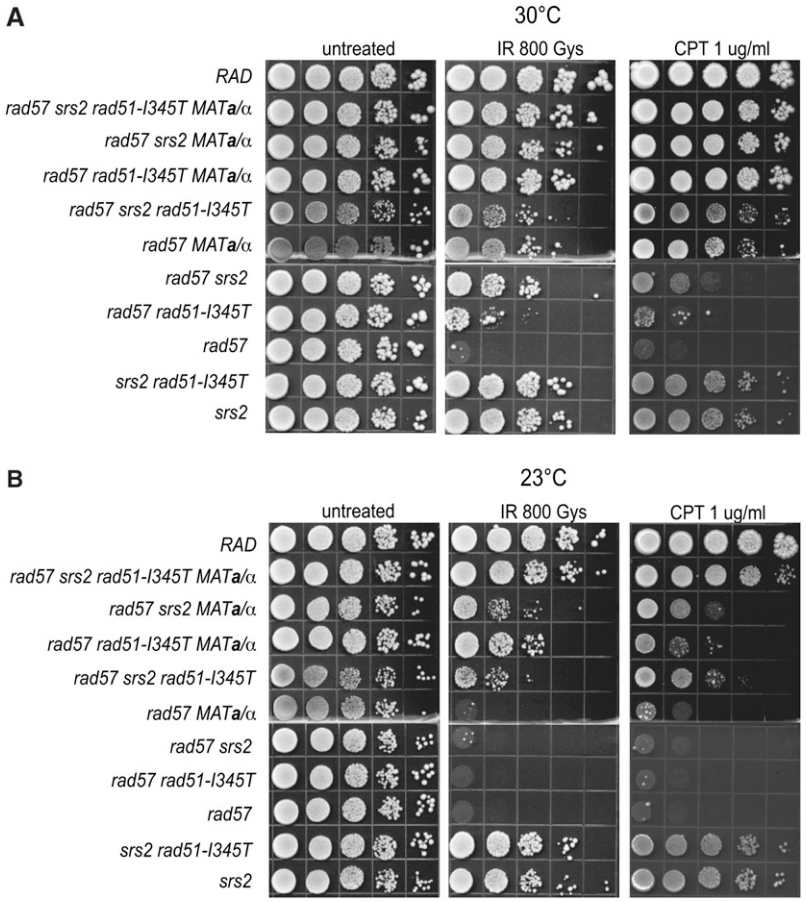

FIGURE 3.—Combining known suppressors of *rad57* fully suppresses the sensitivity of *rad57* to DSB-inducing genotoxic agents. (A) LSY1519-1D (*RAD*), LSY1896-1A (*rad57::LEU2 srs2::HIS3 rad51-I345T*), LSY1422-6B (*rad57::LEU2 srs2::HIS3*), LSY1895 (*rad57::LEU2 rad51-I345T*), LSY 1894-3B (*rad57::LEU2*), LSY1827-1D (*srs2::HIS3 rad51-I345T*), and LSY1867 (*srs2::HIS3*) were transformed with either pRS414-*MAT*α or pRS414-*MAT***a** to express both mating-type alleles or pRS414 empty vector as a control. Ten-fold serial dilutions of log-phase SC–TRP cultures grown at 30° were spotted onto SC–TRP plates and left unirradiated or exposed to 800 Gy of γ-irradiation. Dilutions were also spotted onto SC–TRP plates containing 1 μg/ml camptothecin buffered with 0.25% DMSO. Survival was assessed following 3 days of growth at 30°. (B) The same experiment as in A done at 23°.

increased PCR product at the later time points, comparable to wild type, but the *rad57* single mutant did not (Figure 4B). If the I-*Sce*I-induced DSB is repaired to the wild-type *Aat*II sequence, the PCR product should be digested with *Aat*II and the percentage of gene conversion calculated from the ratio of *Aat*II$^-$ to *Aat*II$^+$ DNA (Figure 4, D and E). Nonlinear PCR was performed to obtain sufficient product for restriction digestion (see MATERIALS AND METHODS). The percentage of gene conversion was then normalized to the amount of PCR product from the quantitative PCR. In a wild-type strain, 45% of the amplified DNA was *Aat*II$^+$ by 6 hr post-induction, and gene conversion plateaued after 8 hr at 80% (Figure 4, D and E). Compared to wild type, the *rad57* single mutant was strongly defective in gene conversion with only 0.8% repair after 24 hr (Figure 4, D and E). The *rad57* mutant combined with *srs2* and *sir4* [to eliminate silencing of the *HMR***a** and *HML*α loci (RINE and HERSKOWITZ 1987)] displayed increased gene conversion and faster kinetics compared to the *rad57* single mutant, but the timing of repair was slower compared to wild type. The *rad57* suppressor strain had only 30% of repair at 6 hr post-induction but approached the wild-type level of repair at the 24-hr time point with 72% gene conversion (Figure 4, D and E). Consistent with previous results (AYLON *et al.* 2003), the *srs2 sir4* mutant showed reduced efficiency of repair compared with wild type and was even slightly lower than the *rad57 srs2 sir4* strain. Thus, the DSB-induced gene conversion defect of the *rad57* mutant can be fully suppressed by *srs2* and *MAT* heterozygosity.

The spontaneous recombination defect of *rad57* is not suppressed by combining *srs2* and mating-type heterozygosity: Because the *rad57* suppressed strain shows close to wild-type levels of DSB-induced gene conversion, we were interested to see if the same was true for spontaneous gene conversion between direct repeats. Previous studies showed a severe defect in spontaneous recombination between *ade2* direct repeats in the *rad57* mutant (MOZLIN *et al.* 2008). Spontaneous recombination rates were determined using a *ade2* repeat construct similar to the one described above, except the second allele contained a fill-in mutation of the *Aat*II site and a *URA3* marker was present between the repeats (HUANG and SYMINGTON 1994). Unequal sister-chromatid or intrachromatid gene conversion between the two *ade2* repeats can generate Ade$^+$ Ura$^+$ recombinants that retain the duplication (Figure 5A). The rate of recombination in the *rad57* mutant was 1000-fold lower than in the wild-type strain (Figure 5B). As shown previously, the *sir4* mutation resulted in a small, but significant, suppression of the *rad57* spontaneous recombination defect ($P < 0.05$) (MOZLIN *et al.*

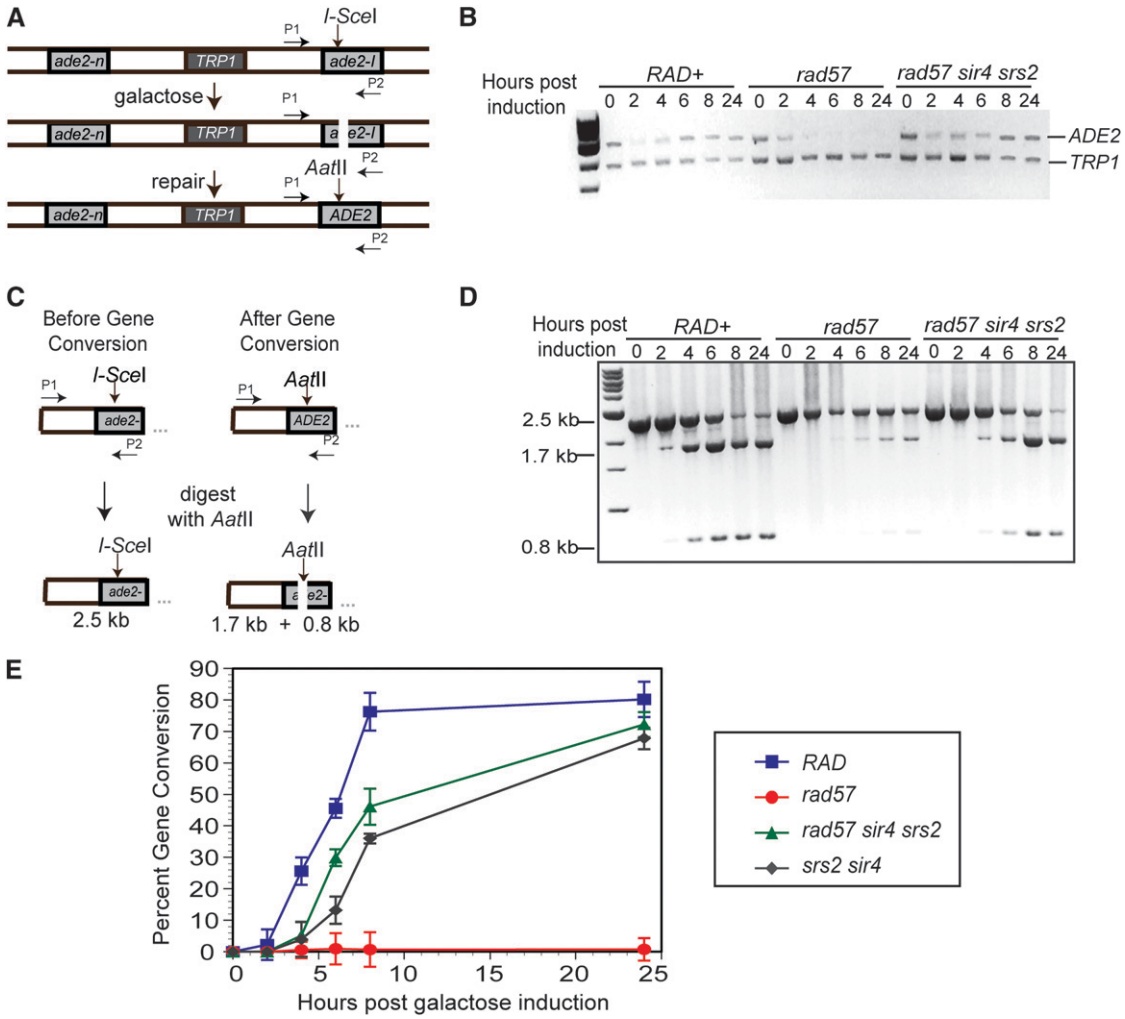

FIGURE 4.—The DSB-induced gene conversion defect of *rad57* is suppressed by *srs2* and *MAT* heterozygosity. (A) The direct-repeat recombination substrate contains 3.6-kb repeats with different *ade2* alleles integrated at the endogenous locus on chromosome *XV* separated by plasmid sequences and the *TRP1* gene. Upon galactose induction, I-*Sce*I is expressed and makes a DSB within the *ade2-I* allele. Primers were designed to anneal upstream of the *ade2-I* allele within vector sequences as well as downstream of the I-*Sce*I cut site. (B) Quantitative PCR. Uncut DNA or gene conversion using *ade2-n* as the donor will result in a PCR product, whereas single-strand annealing or unrepaired DNA will not. The top band is the *ADE2* PCR product and the bottom band is the control *TRP1* PCR product. Strains used were LSY2032-10C (*RAD*), LSY2032-12A (*rad57::LEU2*), LSY2113-4 (*srs2::HIS3 sir4::KanMX*), and LSY2032-1C (*rad57::LEU2 srs2::HphMX sir4::KanMX*). (C) Gene conversion will restore the wild-type *Aat*II site in the *ade2*-I allele. After PCR, products can be digested with *Aat*II to monitor the kinetics of gene conversion within each strain. DNA uncut by I-*Sce*I will result in a 2.5-kb fragment whereas gene conversion will result in 1.7- and 0.8-kb fragments. (D) PCR was performed to saturation (35 cycles), and the *Aat*II-digested PCR products were analyzed by agarose gel electrophoresis. (E) The percentage of gene conversion was calculated as the ratio of *Aat*II cut to uncut DNA and normalized to the amount of PCR product from the quantitative PCR (B).

2008). Although *srs2* did not cause a significant increase in the rate of spontaneous recombination in the *rad57* background, it did result in a small, but significant, suppression of the *rad57 sir4* recombination defect ($P < 0.05$). Despite these small increases, the recombination rate of the *rad57 srs2 sir4* strain was only 6-fold higher than that of *rad57* and 170-fold lower than that of wild type (Figure 5B). This is the same combination of suppressors that resulted in an almost wild-type frequency of DSB-induced gene conversion in the *rad57* background. As expected, the *srs2* mutant showed an increased rate of spontaneous recombination compared with wild type (AGUILERA and KLEIN 1988).

The mechanism of suppression of *rad55* and *rad57* mutants by *MAT* heterozygosity is not through down-regulation of *SRS2* or nonhomologous end joining: The mechanism for suppression of the *rad57* mutant by *srs2*, overexpression of *RAD51*, or by *rad51-I345T* is most likely by increased recruitment or stabilization of Rad51 nucleoprotein filaments (HAYS *et al.* 1995; JOHNSON and SYMINGTON 1995; FORTIN and SYMINGTON 2002; FUNG *et al.* 2006). However, the mechanism behind the

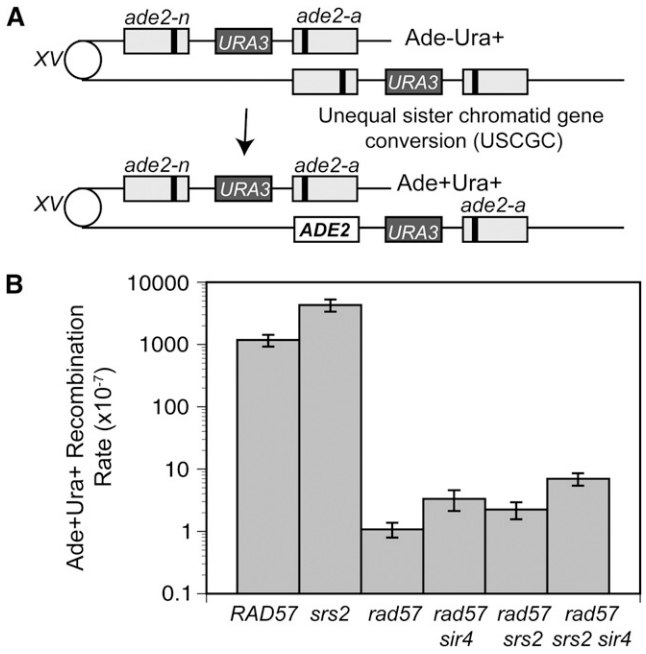

FIGURE 5.—The spontaneous recombination repair defect of *rad57* is weakly suppressed by combining *srs2* and mating-type heterozygosity. (A) The direct-repeat recombination substrate contains 3.6-kb repeats with different *ade2* alleles integrated at the endogenous locus on chromosome *XV* separated by plasmid sequences and the *URA3* gene. Unequal sister-chromatid or intrachromatid gene conversion between the two *ade2* repeats can generate Ade^+Ura^+ recombinants that retain the duplication. Either allele could be converted; only one type of conversion is shown here. (B) Spontaneous sister-chromatid recombination rates at 30°. Strains used were LSY1892 (*RAD*), LSY1894-3B (*rad57::LEU2*), LSY1898 (*rad57:LEU2 sir4::kanMX*), LSY1422-6B (*rad57::LEU2 srs2::HIS3*), LSY1421-2A (*srs2::HIS3*), and LSY1900 (*rad57::LEU2 srs2::HIS3 sir4::KanMX*).

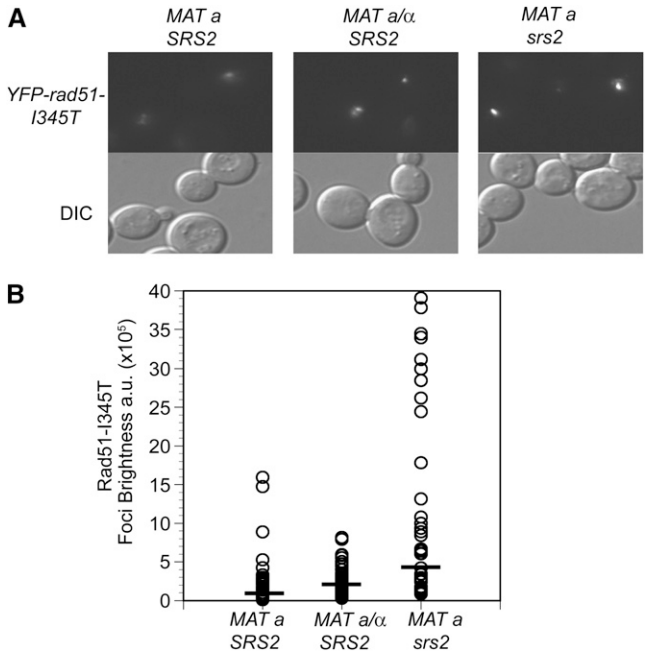

FIGURE 6.—Mating-type heterozygosity and *srs2* have different effects on *rad51-I345T* foci. (A) LSY1957-1 (*MAT***a** *YFP-rad51-I345T*) was transformed with pRS414 empty vector or pRS414-*MAT*α to express both mating types, and LSY2086-2B (*YFP-rad51-I345T srs2::HIS3*) was transformed with pRS414 empty vector. Log-phase cultures of the strains were exposed to 200 Gy of γ-irradiation, followed by microscopy to monitor focus formation. (B) The brightness of each focus was quantified and plotted; at least 50 foci were analyzed for each strain. A solid bar represents the mean focus brightness for each strain.

suppression of *rad55* and *rad57* mutants by *MAT* heterozygosity has yet to be fully elucidated. We have previously shown that steady-state Rad51 protein levels are unchanged by *MAT* heterozygosity, suggesting that transcriptional induction of *RAD51* is not the cause (MORGAN *et al.* 2002). Furthermore, *rad51-I345T* fails to show greater suppression when present in high copy compared with single copy (FORTIN and SYMINGTON 2002), but is additive with *MAT* heterozygosity in suppression of *rad57* (Figure 3). Another possibility is that *MAT* heterozygosity decreases expression and/or functionality of *SRS2* (GALITSKI *et al.* 1999). If *MAT* heterozygosity functioned via Srs2 regulation, then we would not have expected to see additive suppression of the IR sensitivity of the *rad57* mutant by *srs2* and *MAT***a**/α, contrary to our findings (Figure 3). As a further test of whether *srs2* and *MAT* heterozygosity have the same effect on Rad51 recruitment to damaged sites, we examined IR-induced YFP-Rad51-I345T foci in both genetic backgrounds (Figure 6). Both the *srs2* mutant and mating-type heterozygous strain showed an increase in brightness of YFP-Rad51-I345T foci over the *MAT***a** *YFP-rad51-I345T SRS2* strain. However, the foci in the *srs2* strain appeared larger and brighter compared to those in the mating-type heterozygous strain, indicating that the two suppressors have qualitatively different effects on the Rad51 recruitment or filament dynamics.

A prior report suggested that *MAT* heterozygosity suppresses *rad55* and *rad57* mutants solely through downregulation of the competing repair pathway, nonhomologous end joining (NHEJ) (VALENCIA-BURTON *et al.* 2006). The haploid-specific *NEJ1* gene, which is required for the NHEJ pathway of DSBR, is one of the genes repressed by the Mat**a**1-α2 repressor (FRANK-VAILLANT and MARCAND 2001; KEGEL *et al.* 2001; OOI *et al.* 2001; VALENCIA *et al.* 2001). However, we found that abrogation of NHEJ by deletion of *DNL4*, which encodes a DNA ligase essential for NHEJ, did not suppress the IR sensitivity of *rad55* or *rad57* in the W303 strain background (Figure 7). Because the previous study was performed with S288C derivatives, we constructed a *dnl4 rad55* double mutant in the S288C background, but still failed to see suppression of the IR sensitivity conferred by *rad55*. Interestingly, suppression of the CPT sensitivity of the *rad55* mutant by

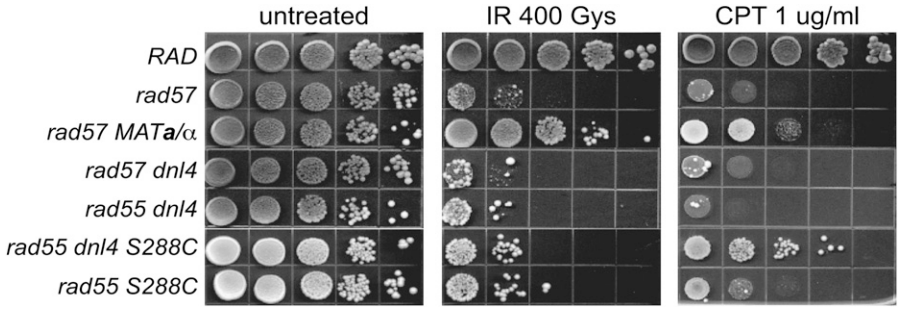

FIGURE 7.—*MAT* heterozygosity does not suppress the IR sensitivity of *rad57* by downregulating NHEJ. Strains used were W1588-4A (*RAD*), YHK598-8B (*rad57::LEU2*), LSY2029 (*rad57::LEU2 dnl4::KanMX*), LSY1682-1B (*rad55::LEU2 dnl4::URA3*), LSY1786 (*rad55::LEU2 dnl4::KanMX*), and BY4742 *rad55::KanMX*. To express both mating types, YHK598-8B (*MATα rad57::LEU2*) was transformed with pRS414-*MAT***a** while the rest of the strains were transformed with the pRS414 empty vector. Ten-fold serial dilutions of log-phase cultures were spotted onto either SC–TRP-only plates or onto SC–TRP plates containing 1 μg/ml camptothecin buffered with 0.25% DMSO. SC–TRP-only plates either were left unirradiated or were exposed to 400 Gy of γ-irradiation.

dnl4 is observed in the S288C but not the W303 background (Figure 7). This difference in suppression is probably due to strain background differences. Regardless of the strain background, it is evident that suppression of the IR sensitivity of *rad55* and *rad57* mutants by *MAT* heterozygosity is not due to downregulation of NHEJ. We tested mutations in other genes regulated by the Mat**a**1-α2 transcriptional repressor (GALGOCZY *et al.* 2004), but none of these was able to suppress the IR sensitivity conferred by *rad57* (data not shown).

DISCUSSION

Suppression of the DSBR defect of the *rad57* mutant: Several lines of evidence support a role for Rad55 and Rad57 as accessory proteins to promote nucleation or stabilization of the Rad51 nucleoprotein filament (HAYS *et al.* 1995; JOHNSON and SYMINGTON 1995; SUNG 1997b; GASIOR *et al.* 1998; FORTIN and SYMINGTON 2002; SUGAWARA *et al.* 2003). One important question in the field is whether the Rad51 paralogs have other roles in recombination in addition to Rad51 presynaptic filament formation. We investigated this possibility by combining partial suppressors of *rad55* and *rad57* that are thought to function by improving the stability or activity of the Rad51 nucleoprotein filament. The defect in the number of CPT-induced Rad51 foci (YFP-Rad51-I345T) observed in the *rad57* strain was suppressed by elevated temperature, *srs2*, and *MAT* heterozygosity. Even though in the most suppressed strain the focus brightness was slightly reduced compared with the *RAD57 YFP-rad51-I345T* strain, the focus brightness was far greater than observed in the *rad57* mutant ($P = 0.0001$) (Figure 2). We found an almost complete rescue of the IR and CPT sensitivity of the *rad57* mutant when at least three of the suppressors were combined and the residual sensitivity reflected the innate DNA damage sensitivity conferred by the *srs2* mutation (Figure 3). Furthermore, by monitoring the kinetics of DSB-induced gene conversion between direct repeats, the *rad57 srs2 sir4* strain showed close to wild-type levels of recombination (72% *vs.* 80%), whereas the efficiency of repair in the *rad57* mutant was <1%. These results show that the DSBR defect of *rad57* mutants can be effectively suppressed by combining the partial suppressors. Because these suppressors are thought to act at the level of the Rad51 filament, we conclude that the main function of Rad57 in DSBR repair is in the assembly or maintenance of the Rad51 filament. However, we cannot rule out the possibility of a late function that is redundant with another protein(s).

Srs2 has been shown to dismantle Rad51-ssDNA filaments *in vitro* (KREJCI *et al.* 2003; VEAUTE *et al.* 2003). This function of Srs2 is thought to be important in the context of replication fork stalling in the presence of DNA damage; removal of Rad51 by Srs2 allows repair to proceed by the error-free template-switching branch of post-replication repair instead of by recombination (PFANDER *et al.* 2005). Consequently, *srs2* suppresses the methyl methanesulfonate (MMS) sensitivity of *rad6* and *rad18* mutants by channeling lesions to the recombination pathway and *srs2* mutants show elevated rates of spontaneous recombination (SCHIESTL *et al.* 1990; RONG *et al.* 1991; ROBERT *et al.* 2006). However, *srs2* mutants show decreased efficiency of DSB-induced recombination and an altered outcome of these events in favor of crossovers (AYLON *et al.* 2003; IRA *et al.* 2003). The efficiency of DSB-induced recombination was lower in the *sir4 srs2* strain than in wild type or the *rad57 srs2 sir4* strain. Thus *srs2* and *rad57* show mutual suppression. Semidominant mutations of *RAD51* are known to suppress the methyl methanesulfonate sensitivity of *srs2* homozygous diploids (CHANET *et al.* 1996). One possible explanation is that Rad51 is recruited to inappropriate DNA sites in *srs2* mutants and less Rad51 is available to bind to induced DNA damage, resulting in the reduced efficiency of DSBR in *srs2* mutants (OSMAN *et al.* 2005). If Rad55-Rad57 mediates recruitment of Rad51 at these inappropriate sites, then in the absence of Rad57 more Rad51 would be available to bind at induced DSBs and would form a stable filament in the absence of Srs2, resulting in the observed suppression.

The spontaneous recombination defect of *rad57* is poorly suppressed by *srs2* and *MAT* heterozygosity: We previously demonstrated a severe defect in the rate of spontaneous gene conversion between *ade2* alleles oriented as direct repeats in *rad55* and *rad57* mutants. Surprisingly, this recombination defect was not suppressed by temperature and was only weakly (3-fold) suppressed by *MAT* heterozygosity or by overexpression of Rad51 (Mozlin *et al.* 2008). Here we show additive suppression of the *rad57* recombination defect by combining *MAT* heterozygosity and *srs2* at 30°, but the rate is still 170-fold less than that observed in wild type (Figure 5); these same suppressors fully suppress the DSB-induced gene conversion defect of the *rad57* mutant.

We assume that most spontaneous recombination events occur during S-phase. Because the phenotype of the suppressed *rad57* mutant in the spontaneous recombination assay is different from that observed for DSB-induced recombination at the same locus, we suggest that spontaneous recombination initiates at ssDNA gaps formed during DNA synthesis. The observation that *srs2* and *MAT***a**/α strongly suppress the sensitivity of the *rad57* mutant to CPT, which makes DSBs in the context of the replication fork, further supports the idea that collapsed replication forks are not the primary initiating lesion for spontaneous recombination. A block to leading-strand synthesis is thought to result in uncoupling of leading and lagging strands and in formation of ssDNA at the replication fork. Srs2 may act to prevent Rad51 binding to these structures, thereby promoting fork reversal and lesion repair or bypass. Single-stranded gaps that form on the lagging strand or following restart of the leading strand downstream of the lesion are bypassed by template switching, homologous recombination, or error-prone translesion DNA synthesis. These pathways appear to compete for the same substrate because *rad57* mutants have an elevated spontaneous mutation rate that is dependent on *REV3* (Rattray *et al.* 2002). Although recombination and template switching had previously been considered as separate mechanisms, recent studies suggest that Rad51 and Rad18 have overlapping functions in the formation of sister-chromatid joint molecules after MMS treatment (Branzei *et al.* 2008). Rad55-Rad57 may have a specific role in formation or maintenance of Rad51 at ssDNA gaps, perhaps by preventing filament extension into adjacent dsDNA. Alternatively, the pairing of the Rad51-bound ssDNA gapped substrate with the intact sister chromatid may be more dependent on Rad55-Rad57 than DSBR in which the ends are not torsionally constrained. Another possibility is that Rad55-Rad57 acts indirectly in spontaneous recombination by antagonizing the post-replication repair pathway, a hypothesis that we are currently testing.

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