The Yeast Recombinational Repair Protein Rad59 Interacts With Rad52 and Stimulates Single-Strand Annealing

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ABSTRACT

The yeast *RAD52* gene is essential for homology-dependent repair of DNA double-strand breaks. *In vitro*, Rad52 binds to single- and double-stranded DNA and promotes annealing of complementary single-stranded DNA. Genetic studies indicate that the Rad52 and Rad59 proteins act in the same recombination pathway either as a complex or through overlapping functions. Here we demonstrate physical interaction between Rad52 and Rad59 using the yeast two-hybrid system and co-immunoprecipitation from yeast extracts. Purified Rad59 efficiently anneals complementary oligonucleotides and is able to overcome the inhibition to annealing imposed by replication protein A (RPA). Although Rad59 has strand-annealing activity by itself *in vitro*, this activity is insufficient to promote strand annealing *in vivo* in the absence of Rad52. The *rfa1-D288Y* allele partially suppresses the *in vivo* strand-annealing defect of *rad52* mutants, but this is independent of *RAD59*. These results suggest that *in vivo* Rad59 is unable to compete with RPA for single-stranded DNA and therefore is unable to promote single-strand annealing. Instead, Rad59 appears to augment the activity of Rad52 in strand annealing.

DNA double-strand breaks (DSBs) are potentially lethal lesions that are repaired by either homology-dependent recombinational repair or homology-independent mechanisms. The homology-dependent repair of DSBs requires genes of the *RAD52* epistasis group (reviewed in Paques and Haber 1999). Most of these genes were identified by their requirement for the repair of ionizing radiation-induced DNA damage (Game and Mortimer 1974). *RFA1*, which encodes the largest subunit of the heterotrimeric DNA-binding complex, replication protein A (RPA), is also considered to be a member of the *RAD52* group as certain non-null alleles show X-ray sensitivity, recombination deficiency, and genetic interaction with *rad52* alleles (Firmenich *et al.* 1995; Smith and Rothstein 1995; Umezu *et al.* 1998). Although mutation of any of the genes in the *RAD52* group results in sensitivity to ionizing radiation, there is considerable heterogeneity within the group when tested for recombination defects in various assays. In general, the genes within the *RAD52* group fall into four subgroups based on the similarity of mutant phenotypes: *MRE11*, *RAD50*, and *XRS2*; *RAD51* and *RAD54* (and *RDH54*); *RAD52* (and *RAD59*); and *RAD55* and *RAD57*. The phenotype of *rad59* mutants is most like *rad52*, but much less severe—hence the grouping with *RAD52* (Bai and Symington 1996; Bai *et al.* 1999). Protein-protein interactions have been detected between members of most subgroups, as well as between some members of different groups. For example, Mre11, Rad50, and Xrs2 form a stable complex (Usui *et al.* 1998), Rad55 and Rad57 form a stable heterodimer (Hays *et al.* 1995; Johnson and Symington 1995; Sung 1997b), and Rad54 interacts with Rad51 (Jiang *et al.* 1996; Clever *et al.* 1997). In addition, Rad51 interacts with Rad52 and Rad55 (Shinohara *et al.* 1992; Milne and Weaver 1993; Hays *et al.* 1995; Johnson and Symington 1995).

Rad51, Rad52, Rad54, Rdh54, Rad55, Rad57, and RPA are required for efficient homologous pairing and strand exchange *in vitro* (Sung 1994; Sung 1997a,b; New *et al.* 1998; Petukhova *et al.* 1998, 2000; Shinohara and Ogawa 1998; Mazin *et al.* 2000; Van Komen *et al.* 2000). Rad51 has significant homology to bacterial RecA proteins and is the key factor for homologous pairing and heteroduplex DNA formation *in vitro*. However, genetic assays have provided evidence for DSB repair that is independent of the *RAD51*, *RAD54*, *RAD55*, and *RAD57* genes. These *RAD51*-independent events are generally detected using direct or inverted repeats and are thought to occur by a nonconservative mechanism that involves single-strand annealing (SSA; Ivanov *et al.* 1996; Kang and Symington 2000). Repair of a chromosomal DSB by strand invasion followed by replication to the end of the chromosome (*b*reak *i*nduced *r*eplication, or BIR) has also been observed in *rad51, rad54, rad55*, and *rad57* mutants (Malkova *et al.* 1996; Signon *et al.* 2001). Even though BIR and gene conversion both require a common strand invasion step,

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BIR can occur in the absence of *RAD51* and is considered to be nonconservative. These *RAD51*-independent events all require *RAD52* and the annealing function of Rad52 is likely to play a central role in these events (Mortensen *et al.* 1996).

The *RAD59* gene was identified in a screen for mutants that reduce the rate of *RAD51*-independent recombination between inverted repeats (Bai and Symington 1996). Synergism between *rad51* and *rad59* mutations is also observed for BIR (Signon *et al.* 2001) and for telomere maintenance in the absence of telomerase (Chen *et al.* 2001). *RAD59* encodes a 238-amino-acid protein with significant homology to the N-terminal region of Rad52, the region most highly conserved among Rad52 family members (Bai and Symington 1996). The C-terminal region of Rad52, which is absent from Rad59, is required for interaction with Rad51 (Milne and Weaver 1993). The observation that more than one copy of *RAD52* suppresses the repair defect of *rad59* mutants, and that certain non-null alleles of *RAD52* have phenotypes similar to *rad59* mutants, suggests that the two proteins have overlapping activities and/or form a complex (Bai and Symington 1996; Bai *et al.* 1999).

RAD52, *RFA1*, and *RAD59* are the only members of the *RAD52* epistasis group required for HO-induced deletion formation between direct repeats (Sugawara and Haber 1992; Umezu *et al.* 1998; Bai *et al.* 1999; Sugawara *et al.* 2000). The requirement for *RAD52* in this reaction is consistent with the observation that Rad52 promotes annealing of single-stranded DNA *in vitro* (Mortensen *et al.* 1996; Sugiyama *et al.* 1998). Purified Rad59 binds to single- and double-stranded DNA and also promotes annealing of complementary single-stranded DNA *in vitro* (Petukhova *et al.* 1999). However, the annealing activity of Rad59 is not stimulated by RPA and Rad59 is unable to stimulate Rad51-promoted strand exchange *in vitro* (Petukhova *et al.* 1999). This contrasts with Rad52, which is able to overcome the inhibition to strand exchange imposed by RPA, and shows RPA-stimulated strand annealing (Sung 1997a; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998; Shinohara *et al.* 1998; Sugiyama *et al.* 1998). Rad52 interacts directly with RPA (Park *et al.* 1996) and is thought to displace RPA from single-stranded DNA to promote annealing or load Rad51 in preparation for strand exchange. Thus Rad59 shares several *in vitro* activities with Rad52, except the ability to displace RPA to facilitate loading of Rad51. In this study we present evidence for interaction between Rad52 and Rad59 and suggest that the role of Rad59 in strand annealing is in the context of the Rad52-Rad59 complex.

MATERIALS AND METHODS

Media, growth conditions, and genetic methods: Standard genetic methods were followed. Rich medium (YPD) and synthetic complete (SC) medium lacking the appropriate amino acid or nucleic acid base were prepared as described previously (Sherman *et al.* 1986). Selection for Ura− cells was performed on SC medium containing 5-fluoroorotic acid (5-FOA) at 1 mg/ml (Boeke *et al.* 1987). To induce expression of Rad59, cultures were grown in synthetic medium containing 2% glucose prior to addition of galactose to 2%. To induce expression of *HO* endonuclease, cultures were grown in synthetic medium minus tryptophan containing 2% raffinose prior to addition of galactose to a final concentration of 2%. Yeast mating, sporulation, and tetrad dissection were performed as previously described (Sherman *et al.* 1986). Yeast cells were grown at 30°. Transformations were performed by the lithium acetate method (Ito *et al.* 1983).

Yeast strains and plasmids: *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Strains T334 and PJ69-4A have been described previously (James *et al.* 1996; Lewis *et al.* 1998). Briefly, PJ69-4A is a strain designed for detecting protein-protein interactions by the two-hybrid assay and contains *HIS3*, *ADE2*, and *lacZ* reporters driven by the *GAL1*, *GAL2*, and *GAL7* promoters, respectively. T334 contains the *reg1-501* mutation, which allows galactose induction in the presence of glucose (Hovland *et al.* 1989; Lewis *et al.* 1998). All other strains are derivatives of strains W303-1A or W303-1B (Thomas and Rothstein 1989). To construct strain LSY959, W1479-80B (Smith and Rothstein 1999) was first transformed with plasmid pRS414:*MAT*α to allow mating to LSY836. Haploid progeny derived from this cross were grown nonselectively and then screened on SC-Trp to identify plasmid-free segregants. Segregation of *rad52::HIS5* was scored by streaking progeny onto solid YPD medium and irradiating with 50 krad in a Gammacell 220 ^{60}Co irradiator. Progeny that failed to grow were scored as *rad52::HIS5*. Segregation of *rfa1-D228Y* was scored by restriction length polymorphism analysis, as described previously (Smith and Rothstein 1995). To construct LSY997 and LSY999, LSY959-15D and LSY959-23C, respectively, were plated on SC medium containing 5-FOA to select for cells that have undergone a pop-out event, resulting in loss of the *URA3* marker.

To construct pGAD10-*RAD59* and pGBD-*RAD59*, a 951-bp fragment containing the *RAD59* open reading frame (ORF) plus 275 bp of 3′ noncoding sequence and a *Bgl*II site at the 5′ end was generated by PCR using genomic DNA as the template and the following primers: 5′-GGGGAAGATCT TAATGACGTACAAGCGAAGCC and 5′-TTCGTTACCTTG GAATGGTATGT. To construct pGAD10-*RAD59*, the PCR fragment was digested with *Bgl*II and cloned into the *Bgl*II site of pGAD10 (gift from S. Fields). To construct pGBD-*RAD59*, the PCR fragment was digested with *Bgl*II and subcloned into the *Bgl*II site of pNotA/T7 (5 Prime) to generate pNotA/T7:*RAD59*. pNotA/T7:*RAD59* was digested with *Bgl*II and the 918-bp fragment was cloned into the *Bam*HI site of pGBD-C2 (James *et al.* 1996) to generate pGBD-*RAD59*. To construct p*RAD59*-CAD and p*RAD59*-CDBD, a 736-bp fragment containing *Bam*HI sites on the 5′ and 3′ ends and the *RAD59* ORF minus the stop codon was generated by PCR using genomic DNA as the template and the following primers: 5′-GGATCCAAGTCTTATGACGATACAAGCGAAGCCC and 5′-GGATCCGCGGTTTGATATGCGTGCCTTTAGC. The PCR fragment was subcloned into the pGEM-T Easy (Promega, Madison, WI) vector system to generate pGEM-T:*RAD59*. pGEM-T:*RAD59* was digested with *Bam*HI and the 736-bp fragment was cloned into the *Bam*HI site of pCAD2 (Printen and Sprague 1994) to generate p*RAD59*-CAD and into the *Bam*HI site of pCDBD-1e (gift from R. Brazas) to generate p*RAD59*-CDBD. Prior to use, the structures of pGAD10-*RAD59*, pGBD-*RAD59*, p*RAD59*-CAD, and p*RAD59*-CDBD were confirmed by DNA sequencing and in a functional assay by complementation of the gamma

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype	Source
PJ69-4A	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	James *et al.* (1996)
T334	*MAT*α *pep4-3 prb1-1122 ura3-52 leu2-3 reg1-501 gal1*	Lewis *et al.* (1998)
W303-1A	*MAT***a**	Thomas and Rothstein (1989)
W303-1B	*MAT*α	Thomas and Rothstein (1989)
LSY836[a]	*MAT***a** *RAD5 rad59::LEU2 met17::ADE2*	Bartsch *et al.* (2000)
W1479-11C[a]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII	Smith and Rothstein (1999)
W1479-80B[a]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad52::HIS5 rfa1-D228Y lys2ΔSpe*I	Smith and Rothstein (1999)
LSY959-15D[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad59::LEU2*	This study
LSY959-18D[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad52::HIS5*	This study
LSY959-23C[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad59::LEU2 rad52::HIS5 lys2ΔSpe*I *met17::ADE2*	This study
LSY959-28C[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rfa1-D228Y met17::ADE2*	This study
LSY959-43C[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad59::LEU2 rfa1-D228Y met17::ADE2 lys2ΔSpe*I	This study
LSY959-9B[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad52::HIS5 rfa1-D228Y met17::ADE2*	This study
LSY959-8A[a,b]	*MAT::HIS3 RAD5 leu2ΔEco*RI::*URA3-HOcs::leu2ΔBst*EII *rad59::LEU2 rad52::HIS5 rfa1-D228Y met17::ADE2*	This study
LSY997[a]	*MAT::HIS3 RAD5 rad59::LEU2*	This study
LSY999[a]	*MAT::HIS3 RAD5 rad59::LEU2 rad52::HIS5 lys2ΔSpe*I *met17::ADE2*	This study

[a] Strains are in the W303 background (*his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100*); only differences from this genotype are noted.

[b] Several strains with the same genotype were used, but only one is noted.

ray sensitivity of a *rad59* null strain (data not shown). The *RAD52* two-hybrid plasmids were generous gifts from D. Weaver and P. Berg (Milne and Weaver 1993; Hays *et al.* 1998). Plasmid YDL059 contains the *RAD59* gene tagged at the C terminus with the V5 epitope and six histidine residues and is regulated by the *GAL1* promoter (Invitrogen, San Diego). Rad52 was overexpressed using plasmid p52.1 (Sung 1997a). pFH800 contains the *HO* gene regulated by the *GAL1* promoter on a *CEN4 TRP1* vector (Nickoloff *et al.* 1989). To construct pADW6, which was used to express Rad59 tagged at its carboxy terminus with the V5 epitope (Rad59-V5) from its native promoter, a 1800-bp fragment containing *RAD59* tagged at its C terminus with the V5 epitope tag was amplified using the following primers: 5′-AAGCTTGATGATCCACT AGTACGG and 5′-GGATCCGATTCATTAATGCAGGG with plasmid YDL059 as a DNA template. The PCR fragment was subcloned into the pGEM-T Easy (Promega) vector system to generate pGEM-T:*GAL-RAD59*-V5. pGEM-T:*GAL-RAD59*-V5 was digested with *Spe*I and the resulting 757-bp fragment containing the 3′ end of *RAD59* tagged with the V5 epitope was cloned into the *SpeI* sites of pRS416:*RAD59*, replacing a 1200-bp fragment containing the 3′ end of *RAD59*. PET14b:*RAD59* was made by cloning a *RAD59*-containing fragment, digested with *Nde*I and *Bgl*II, between the *Nde*I and *Bam*HI sites of pET14b. The *RAD59* fragment was made by PCR using primers BP59c: 5′-GGAT AAACAGACAAACATATGACGATACAAGCGAAGCCCAG and BP59d: 5′-TTCGTTACCTTGGAATGGTATGT with the plasmid pRS416:*RAD59* as the DNA template.

Two-hybrid analysis: β-Galactosidase activity was assayed quantitatively in permeabilized cells as described previously (Adams *et al.* 1998). At least four individual transformants of each category were assayed.

Co-immunoprecipitation: For immunoprecipitations from cells overexpressing Rad52 and Rad59-V5, T334 cells overexpressing Rad52, a V5 epitope-tagged Rad59 (Rad59-V5), or both Rad52 and Rad59-V5 were grown to mid-log phase in selective synthetic medium. Galactose was added to a final concentration of 2% and incubation was continued for 16 hr. For immunoprecipitations from cells expressing Rad59-V5 in single copy, LSY999 cells (*rad52 rad59*) and LSY997 cells (*rad59*) carrying pRS416 or pRS416:*RAD59-V5* were grown to mid-log phase in selective synthetic medium. For both sets of immunoprecipitations, cells (50 ml) were harvested, washed twice with 20 mm Tris at pH 7.4, 200 mm NaCl and stored at −70°. Extracts were prepared and immunoprecipitation performed as described previously (Strahl-Bolsinger *et al.* 1997). α-V5 monoclonal antibody (Invitrogen) was used to immunoprecipitate Rad59 and α-Rad52 crude serum (kindly provided by R. Rothstein) was used to precipitate Rad52. The V5 immunoprecipitation was probed for Rad52 using affinity-purified α-Rad52 antibody (kindly provided by R. Rothstein) and affinity-purified α-Rad51 antibody (kindly provided by P. Sung). The Rad52 immunoprecipitation was probed for Rad59-V5 using α-V5-HRP monoclonal antibody (Invitrogen).

Physical analysis of *HO*-induced single-strand annealing: Strains containing pFH800 (Nickoloff *et al.* 1989) were grown for 24 hr in 20 ml SC (raffinose) −Trp −Ura medium, which selects for retention of pFH800 and the *leu2* duplication,

respectively. Cells were then used to inoculate 350 ml SC (raffinose) −Trp. Cultures were grown to an OD_{600} of 0.3–0.5. A 50-ml sample was removed for the 0-hr time point and then galactose was added to a final concentration of 2%. Incubation was continued and 50-ml samples were removed at the given times. For the overnight time point samples, the final aliquot was removed, centrifuged, resuspended in sterile water, and incubated overnight at 30°. For all samples, cells were harvested by centrifugation and washed with water. Cell pellets were then frozen in liquid nitrogen. DNA was extracted from the thawed cell pellets and digested with *Spe*I. DNA fragments were separated by electrophoresis through 0.8% agarose gels, transferred to nylon membranes, and hybridized with a 400-bp PCR fragment generated by amplification of sequences from the YCL017 ORF, which is adjacent to *LEU2*. Quantitation of the HO-cut and product bands was done using a Molecular Dynamics (Sunnyvale, CA) Storm 445 SI phosphorimager and IMAGE-QUANT software.

Determination of rates of spontaneous deletion formation: At least two independent isolates of each strain were used to determine the rates of spontaneous deletion formation in three to seven trials. Mean rates from at least three trials of each strain are presented. Single colonies of each isolate were grown on YPD for 2–4 days. Nine individual colonies of each strain were suspended in water and appropriate dilutions were plated on SC to determine total cell number and on 5-FOA plates to determine the number of Ura− cells. Median recombination frequencies (5-FOA-resistant cells/total cells) were determined and rates (events/cell/generation) were calculated according to the following formula: rate = (0.4343 × median frequency)/(Log N − Log N_0), where N is the total number of cells in the colony and N_0 (number of initial cells) = 1 (Drake 1970).

Proteins: Rad59 was purified as the fusion protein with the N-terminal His_6-affinity tag from the *Escherichia coli* strain BL21/DE3 carrying plasmids pET14b-*RAD59* and pLysS. Cells were grown in Luria broth medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37° to OD_{600} = 0.3–0.4. The cultures were cooled to 18° and isopropyl-1-thio-β-d-galactopyranoside was added to 0.4 mm. Cells were cultured at 18° for an additional 16 hr. Cells were harvested and resuspended in 40 ml buffer A [20 mm Tris, pH 8.0, 300 mm NaCl, 10% glycerol, 7 mm mercaptoethanol, 0.5 mm phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton, 1 mg/liter pepstatin A, and 0.5 mg/liter leupeptin]/liter culture volume. Cells were lysed by three freeze/thaw cycles and then briefly sonicated. The lysate was centrifuged at 15 K for 45 min and the supernatant removed to fresh tubes containing 1 ml Talon resin (CLONTECH, Palo Alto, CA)/liter culture volume equilibrated with buffer A. After mixing at 4° for 20 min the solution was transferred to an empty column and washed extensively with buffer A and then with 20 ml buffer A + 10 mm imidazole. Rad59 was eluted with buffer A containing 100 mm imidazole. Fractions containing Rad59 were dialyzed against 20 mm Tris, pH 8.0, 10% glycerol, 10 mm mercaptoethanol, 1 mm EDTA, and 0.1 mm PMSF until the conductivity was equivalent to 60 mm NaCl and then applied to a 1-ml Q-sepharose column (Pharmacia, Piscataway, NJ). Fifty percent of Rad59 was in the unbound fraction and directly applied to a 1-ml heparin agarose column (Pharmacia). Rad59 was eluted with a gradient of 100–800 mm NaCl in 20 mm Tris, pH 8.0, 10% glycerol, 10 mm mercaptoethanol, 1 mm EDTA, and 0.1 mm PMSF. Fractions containing Rad59 were stored at −80°. Purified Rad52 and RPA were generous gifts from S. Kowlaczykowski.

DNA annealing: Annealing of a ^{32}P-labeled 48-mer oligonucleotide (oligo-25) and a complementary unlabeled 48-mer oligonucleotide (oligo-26) was performed as described in Sugiyama *et al.* (1998) except that the reaction buffer contained 30 mm Tris-Cl (pH 7.5), 5 mm $MgCl_2$, and 1 mm dithiothreitol. For the reaction containing RPA, Rad52, and Rad59, Rad52 and Rad59 were incubated together at room temperature for 1 min before addition to the preformed RPA-oligonucleotide complexes. In all reactions, DNA concentrations were 200 nm, Rad59 concentration was 50 nm, Rad52 concentration was 20 nm, and RPA concentration was 30 nm. Annealing was monitored by separation through 10% polyacrylamide gels in TAE buffer and quantified with a Molecular Dynamics Storm 445 SI phosphorimager and IMAGE-QUANT software. Numbers presented are the mean of three to six trials.

TABLE 2

Two-hybrid interaction of Rad59 and Rad52

Activation domain	DNA-binding domain			
	GBD-Rad59[a]	Rad59-GBD	Rad52-GBD	GBD
GAD-Rad59	0.39[b]	0.59	4.24	0.59
Rad59-GAD	0.53	0.59	6.03	1.93
GAD-Rad52	0.20	1.26	3.91	2.05
Rad52-GAD	40.39	24.14	51.89	2.72
GAD	0.12	0.68	3.20	0.82

[a] Gene name written before GAD or GBD indicates a carboxy-terminal fusion to the indicated Gal4 domain and gene name written after GAD or GBD indicates an amino-terminal fusion to the indicated Gal4 domain.

[b] β-Galactosidase activity is presented as Miller Units (Adams *et al.* 1998). Results are the mean values of trials on 4–12 individual transformants.

RESULTS

Rad59 and Rad52 interact in the two-hybrid system: *RAD52* present in more than one copy partially suppresses the gamma ray sensitivity of *rad59* mutants, which suggests a physical interaction between Rad59 and Rad52 or overlapping functions (Bai and Symington 1996). The yeast two-hybrid system was used to determine whether Rad52 and Rad59 interact. Previous studies indicated that Rad52 homomeric interactions could be detected only by the two-hybrid system using fusion proteins in which the carboxy terminus of Rad52 was fused to either the Gal4 DNA-binding domain (GBD) or the Gal4 transactivating domain (GAD), but not by using amino-terminal fusion proteins (Hays *et al.* 1998). We reasoned that interaction between Rad59 and Rad52 may also require the use of Rad52 carboxy-terminal fusions. The two-hybrid strain PJ69-4A (James *et al.* 1996), expressing a carboxy-terminal Rad52-GAD fusion protein and either an amino-terminal or carboxy-terminal GBD-Rad59 fusion protein, activated expression of the *GAL7-lacZ* reporter gene, as determined by β-galactosidase activity (Table 2). However, there was no activation if an amino-terminal GAD-Rad52 fusion protein was used. Activation required the presence of both Rad52 and Rad59 because no activation was detected when fusion proteins were coexpressed with the GBD- or GAD-encoding plasmids (Table 2).

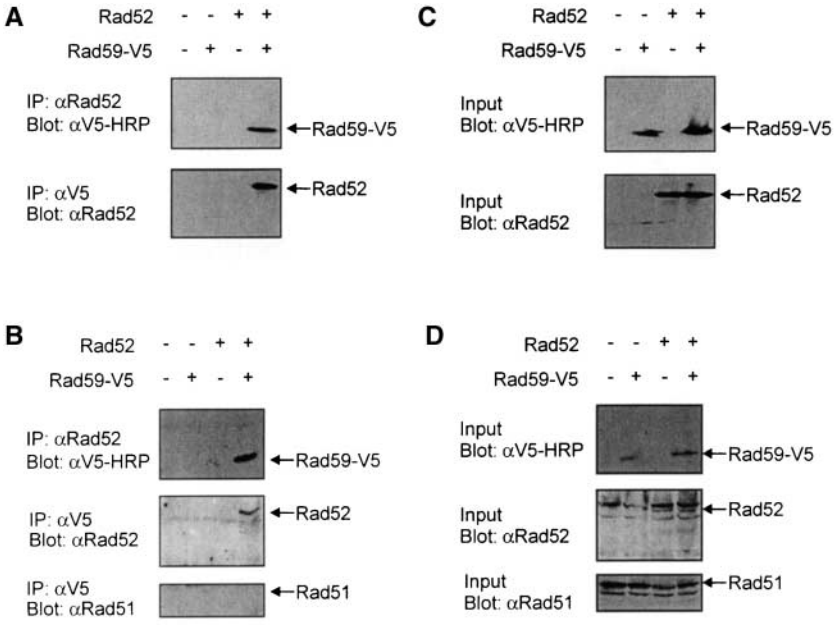

FIGURE 1.—Co-immunoprecipitation of Rad59-V5 and Rad52. (A) Protein extracts (1 mg) were prepared from T334 cells overexpressing the indicated proteins. Rad52 and Rad59-V5 were immunoprecipitated (IP) with α-Rad52 polyclonal antibody and α-V5 monoclonal antibody, respectively. (B) Protein extracts (5–14 mg) were prepared from LSY999 cells (*rad59 rad52*) and LSY997 cells (*rad59*) carrying pRS416 or pRS416:*RAD59*-V5. Rad52 and Rad59-V5 were immunoprecipitated as in A. (A and B) The proteins were separated by SDS-PAGE and immunoblotted with α-V5 monoclonal antibody to detect co-immunoprecipitation of Rad59-V5 with Rad52 and α-Rad52 affinity-purified polyclonal antibody or α-Rad51 affinity-purified polyclonal antibody to detect co-immunoprecipitation of Rad52 or Rad51 with Rad59-V5, respectively. (C) Proteins in the input crude extracts (200 μg) from cells overexpressing Rad52 and Rad59-V5 were immunoblotted with α-Rad52 affinity-purified polyclonal antibody and α-V5 monoclonal antibody. (D) Proteins in the input crude extracts (100–350 μg) from cells expressing Rad52 and Rad59-V5 from the native promoters were immunoblotted with α-Rad52 affinity-purified polyclonal antibody, α-V5 monoclonal antibody, and α-Rad51 affinity-purified polyclonal antibody. Higher- and lower-molecular-weight proteins in the Rad52 immunoblot are cross-reacting species that are present in extracts from strains that lack *RAD52*.

We were unable to detect an interaction between Rad59 and Rad52 using a Rad52-GBD carboxy-terminal fusion protein and either an amino-terminal or carboxy-terminal Rad59 fusion to GAD (Table 2). We were also unable to detect a Rad59 homomeric interaction using any combination of fusion proteins. Both the amino- and carboxy-terminal Rad59 fusions to the Gal4 activation are functional as assayed by complementation of the gamma ray sensitivity of a *rad59* null strain (data not shown), but fusion of GAD to Rad59 may interfere with the ability of GAD to activate transcription.

Rad59 and Rad52 co-immunoprecipitate from yeast extracts: To obtain biochemical evidence for physical interaction between Rad59 and Rad52, we introduced plasmids overexpressing Rad52 and Rad59 tagged at the carboxy terminus with the V5 epitope (Rad59-V5) into strain T334, a *trp1* derivative of strain 334 (HOVLAND *et al.* 1989; LEWIS *et al.* 1998). Rad52 was found to co-immunoprecipitate with Rad59-V5, and Rad59-V5 co-immunoprecipitated with Rad52 (Figure 1A). In control experiments, Rad52 did not co-immunoprecipitate when Rad59-V5 was not expressed and Rad59-V5 did not co-immunoprecipitate well when Rad52 was not overexpressed (Figure 1A). The interaction was unaffected by DNaseI or ethidium bromide, indicating that it was not due to independent association of the proteins with DNA present in the extract (data not shown). These results indicate that the interaction between Rad59 and Rad52 is specific.

To verify that interaction occurs when the proteins are expressed at normal levels, these experiments were repeated using *rad59* or *rad52 rad59* strains containing a *CEN* plasmid with *RAD59* tagged at the carboxy terminus with the V5 epitope (Rad59-V5) and regulated by the native promoter. Rad52 was found to co-immunoprecipitate with Rad59-V5, and Rad59-V5 co-immunoprecipitated with Rad52 (Figure 1B), indicating that overexpression of the proteins is not required to detect their interaction. In control experiments, Rad52 did not co-immunoprecipitate when Rad59-V5 was not expressed and Rad59-V5 did not co-immunoprecipitate when Rad52 was not expressed (Figure 1B).

Previous studies have shown that Rad51 and Rad52 co-immunoprecipitate from yeast extracts (SUNG 1997a). We were unable to detect Rad51 with Rad59 in immunoprecipitations from the *RAD52* strain using the α-V5 antibody, even though it was present in crude extracts (Figure 1, B and D). This result suggests that the Rad52/Rad59 complex is distinct from the Rad51/Rad52 complex.

In Figure 1, C and D, equal amounts of total protein were loaded into the lanes containing crude protein extracts. When both *RAD59*-V5 and *RAD52* were overexpressed, a greater amount of Rad59-V5 was detected than when *RAD59*-V5 was overexpressed alone (Figure 1C). Likewise, when both *RAD59*-V5 and *RAD52* were expressed from their native promoters, a greater amount of Rad59-V5 was detected than when *RAD59*-V5 was expressed alone (Figure 1D). These results suggest that Rad52 may play a role in stabilizing the Rad59 protein.

Rad59 promotes the annealing of complementary oligonucleotides and RPA-oligonucleotide complexes: Several groups have demonstrated *in vitro* annealing activity

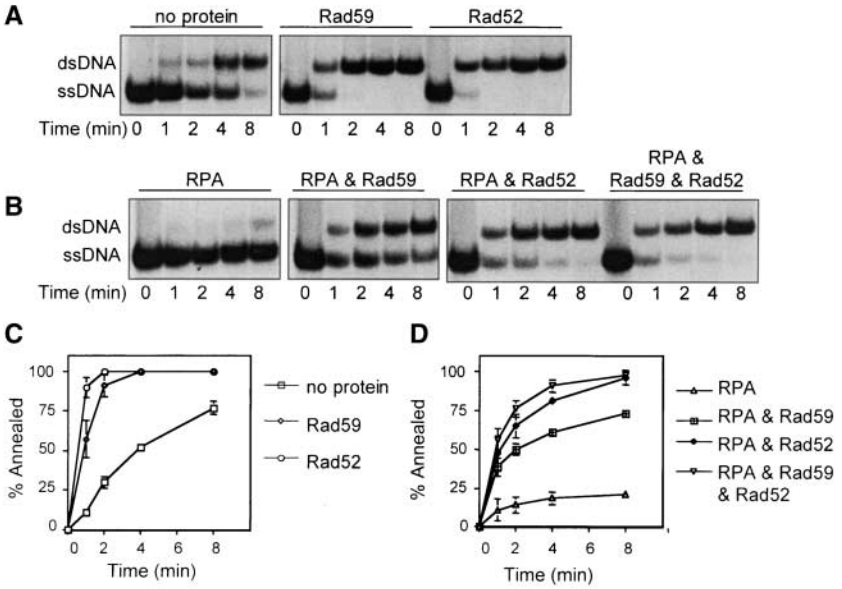

FIGURE 2.—Rad59 stimulation of oligonucleotide annealing. A ^{32}P-labeled 48-mer oligonucleotide (oligo 25) was incubated with a complementary unlabeled 48-mer oligonucleotide (oligo 26) in 100 μl reaction buffer in the presence of the proteins indicated, and the products were monitored by PAGE. The final concentrations of Rad59, Rad52, and RPA were 50 nM, 20 nM, and 30 nM, respectively. (A) Reactions were started by the addition of oligo 26. (B) Reactions were started by the addition of the indicated proteins to preformed RPA-oligonucleotide complexes. (C–D) The percentages of DNA annealing in A and B, respectively, are plotted as a fraction of total radioactivity per lane.

by Rad52 using either plasmid or oligonucleotide substrates (MORTENSEN *et al.* 1996; SHINOHARA *et al.* 1998; SUGIYAMA *et al.* 1998). The annealing of plasmid length DNA molecules to form complex networks is promoted by Rad59, but unlike Rad52 is not stimulated by the addition of RPA (PETUKHOVA *et al.* 1999). We wished to determine if Rad59 mediates the annealing of complementary oligonucleotides to form duplex products and if this reaction is inhibited by RPA.

Complementary 48-mer oligonucleotides were incubated together with or without the protein of interest, and annealed products were monitored by gel electrophoresis (Figure 2; SUGIYAMA *et al.* 1998). In the absence of protein, 29% of the ^{32}P-labeled oligonucleotide annealed to the unlabeled complementary oligonucleotide within 2 min (Figure 2, A and C). Consistent with the results of SUGIYAMA *et al.* (1998), 100% of the DNA was annealed by 2 min when 20 nM Rad52 was included in the reaction. When 50 nM Rad59 was included in the reaction, 91% of the DNA was annealed at 2 min and 100% annealed at 4 min (Figure 2, A and C). This result shows that, like Rad52, Rad59 mediates annealing of complementary oligonucleotides *in vitro*.

The RPA complex plays both a stimulatory and inhibitory role in *in vitro* DNA annealing of plasmid length DNA molecules. RPA binds ssDNA, competing with Rad52 for ssDNA-binding sites, thereby inhibiting DNA annealing. RPA also removes secondary structure, which hinders annealing, thereby stimulating the DNA annealing reaction (SHINOHARA and OGAWA 1998; SUGIYAMA *et al.* 1998). This stimulation requires specific protein-protein interaction between Rad52 and RPA (SUGIYAMA *et al.* 1998). When using short oligonucleotide substrates, which have little or no secondary structure, RPA has only an inhibitory effect (SUGIYAMA *et al.* 1998). Consistent with the results of SUGIYAMA *et al.* (1998), RPA was found to inhibit spontaneous annealing with just 20% of the DNA annealed at 8 min (Figure 2, B and D). Rad52 promotes the annealing of the oligonucleotides when they have been preincubated with RPA with 47% of the DNA annealed at 1 min, 64% annealed at 2 min, and 95% annealed at 8 min (Figure 2, B and D). When 50 nM Rad59 was added to the complementary RPA-oligonucleotide complexes, 38% of the DNA was annealed at 1 min, 50% was annealed at 2 min, and 73% was annealed at 8 min (Figure 2, B and D), indicating that Rad59, like Rad52, mediates the annealing of complementary RPA-coated oligonucleotides. When a mixture of Rad52 and Rad59 was added to the complementary RPA-oligonucleotide complexes, the DNA was annealed in an additive manner, with 55% of the DNA annealed at 1 min, 75% annealed at 2 min, and 97% annealed at 8 min. Thus, under these conditions, Rad59 stimulates Rad52 in the annealing of complementary RPA-oligonucleotide complexes.

***RAD59* is not responsible for SSA in the absence of *RAD52*:** Repair of a DSB made within nonhomologous sequences between direct repeats occurs primarily by the SSA pathway. The ends of the break are processed by a 5′ to 3′ exonuclease, exposing complementary single-strand regions of the direct repeats that can anneal, resulting in a deletion of the unique DNA between the direct repeats (HABER 1995). This process is almost completely dependent on *RAD52* (SUGAWARA and HABER 1992) and partially dependent on *RAD59* (BAI *et al.* 1999; SUGAWARA *et al.* 2000), but it is independent of *RAD51*, *RAD54*, *RAD55*, and *RAD57* (IVANOV *et al.* 1996). The defect in SSA caused by *rad52* is partially suppressed by the *rfa1-D228Y* allele, which decreases the cellular levels of RPA (SMITH and ROTHSTEIN 1995, 1999). Rad52 is thought to be required for the removal of RPA from single-stranded DNA tails during strand

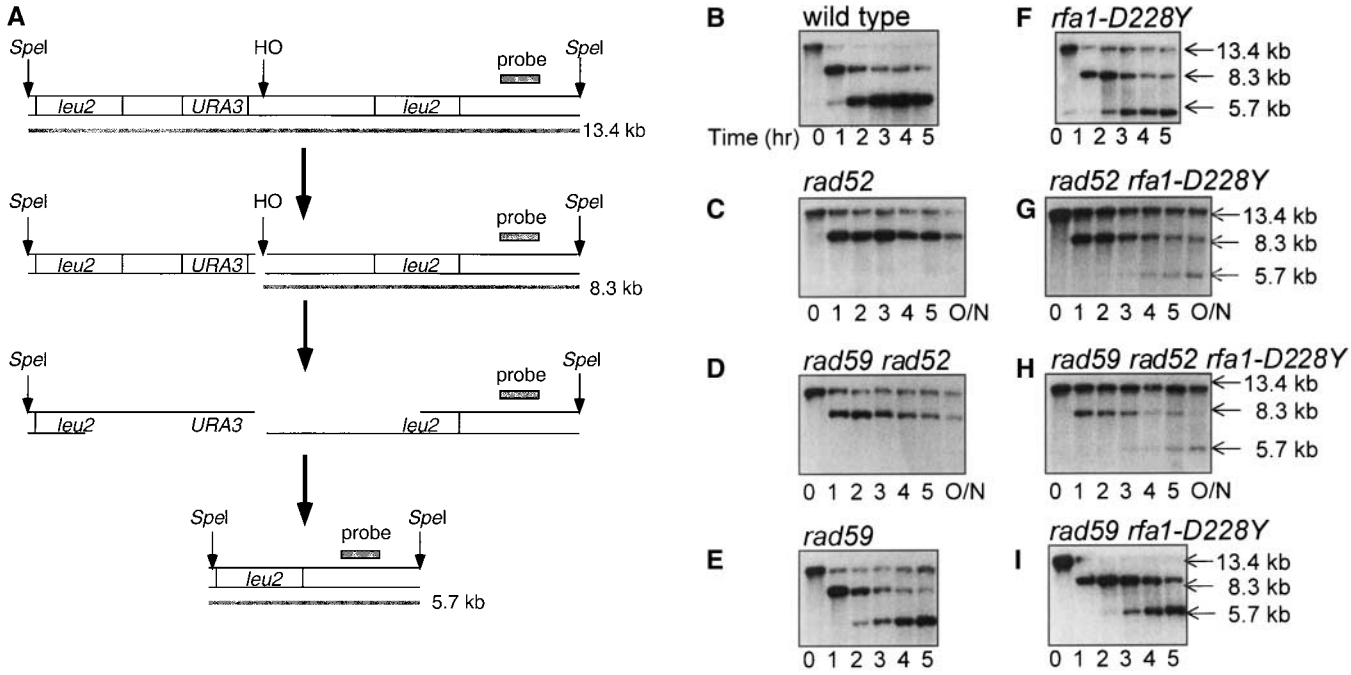

FIGURE 3.—Kinetics of HO-induced deletion formation. (A) Schematic representation of the *leu2* direct-repeat substrate showing the locations of the *Spe*I sites, the HO cleavage site, and hybridization probe. After cleavage by HO, an 8.3-kb fragment is produced from the 13.4-kb *Spe*I fragment. The single-stranded tails formed after resection from the DSB site can anneal to form a deletion product that is detected as a 5.7-kb *Spe*I fragment. (B–I) Deletion formation was monitored in eight backgrounds: (B) wild type; (C) *rad52*; (D) *rad59 rad52*; (E) *rad59*; (F) *rfa1-D228Y*; (G) *rad52 rfa1-D228Y*; (H) *rad59 rad52 rfa1-D228Y*; and (I) *rad59 rfa1-D228Y*. DNA was extracted at the times shown and digested with *Spe*I. The positions of parental, HO-cut fragment, and deletion products are shown to the right of the autoradiograms. O/N, overnight.

invasion and SSA (SUNG 1997a ; NEW *et al.* 1998; SHINOHARA and OGAWA 1998; SMITH and ROTHSTEIN 1999). SMITH and ROTHSTEIN (1999) suggest that an annealing factor that does not normally have access to single-stranded DNA can gain access to the DNA to promote annealing when cellular levels of RPA are limiting, as they are in *rfa1-D228Y* mutants, thereby annealing the DNA in the absence of Rad52. Given the SSA defects of *rad59* mutants and the *in vitro* strand annealing activity of Rad59 protein (Figure 2; PETUKHOVA *et al.* 1999), we hypothesized that Rad59 is responsible for the SSA in *rad52 rfa1-D228Y* mutants and in *rad52* mutants. Strains containing 2.4-kb direct repeats of the *leu2* gene, separated by 5.7 kb of plasmid sequences containing a copy of the *URA3* gene and an *HO* endonuclease cut site (Figure 3A; SMITH and ROTHSTEIN 1999), were used to monitor SSA. Induction of HO results in the formation of a DSB between the *leu2* repeats and deletions are detected by the appearance of a novel 5.7-kb *Spe*I fragment. As described previously, the *rad59* strain showed a delay in formation of the deletion product, but the final product level was reduced only two- to threefold compared with the wild-type strain (Figure 3B). The kinetics and efficiency of SSA in the *rad59 rfa1-D288Y* strain were similar to that observed for the *rad59* strain. Deletion products were barely detectable in both the *rad52* and *rad52 rad59* strains. Analysis of the *rad52 rfa1-D288Y* and *rad52 rfa1-D288Y rad59* strains revealed similar levels of deletion products, indicating that *RAD59* is not required for SSA in the absence of *RAD52*. The reduced level of Rad59 protein observed in *rad52* mutants could possibly account for the inability of Rad59 to substitute for Rad52 *in vivo* (Figure 1). However, even when Rad59 was overexpressed in a *rad52* strain we were unable to suppress the defect in deletion formation (data not shown).

Elevation of rates of spontaneous deletion formation in *rfa1-D228Y* mutants is independent of *RAD59*: Rates of spontaneous deletion formation between 2.4-kb direct repeats were determined using the construct depicted in Figure 3A. Spontaneous deletions can occur by a variety of mechanisms, including SSA, intrachromatid reciprocal exchange, sister chromatid conversion or exchange, replication slippage, or sister strand exchange (reviewed in KLEIN 1995). Collectively, these events are identified as Ura$^-$ (5-FOA^R) colonies in this assay. The rate of deletion formation in a *rad59* mutant was not significantly different from the wild-type strain (Table 3). The rate of spontaneous deletion formation in *rad52* mutants was reduced 4.8-fold as compared to wild type, and the rate was reduced an additional 2.3-fold in *rad59 rad52* double mutants, consistent with a previous study (JABLONOVICH *et al.* 1999). The rate of spontaneous deletion formation in the *rfa1-D228Y* mutant was ele-

TABLE 3

Rates of spontaneous deletion formation

Relevant genotype	Rate $\times 10^{-5}$ (events/cell/generation)	Relative rate
RAD52 RAD59 RFA1	0.94 ± 0.44	1
rad59	1.08 ± 0.63	1.2
rad52	0.20 ± 0.09	0.2
rad52 rad59	0.09 ± 0.06	0.09
rfa1-D228Y	21.02 ± 10.2	22.4
rad59 rfa1-D228Y	18.48 ± 9.72	19.9
rad52 rfa1-D228Y	3.65 ± 1.85	3.9
rad52 rad59 rfa1-D228Y	6.44 ± 2.32	6.8

vated >22-fold as compared to wild type, and this elevation was partially dependent on *RAD52*, consistent with the findings of Smith and Rothstein (1999). The rate in the *rad59 rfa1-D228Y* mutant was not significantly changed from the rate in *rfa1-D228Y*, and the rate in the *rad59 rad52 rfa1-D228Y* mutant was not significantly different from the rate in *rad52 rfa1-D228Y* (Table 3). These results indicate that the elevation of the rate of spontaneous deletion formation in *rfa1-D228Y* mutants is independent of *RAD59*.

DISCUSSION

Genes in the *RAD52* epistasis group are required for the repair of ionizing radiation-induced DNA damage, but the mutants show considerable heterogeneity in assays for spontaneous or double-strand break-induced recombination (Paques and Haber 1999). This heterogeneity is due to the variety of pathways used for the repair of double-strand breaks. *RAD52* is required for all pathways of homology-dependent repair, including gene conversion, break-induced replication, and single-strand annealing (White and Haber 1990; Sugawara and Haber 1992; Rattray and Symington 1994; Malkova *et al.* 1996). In contrast, *RAD51*, *RAD54*, *RAD55*, and *RAD57* are essential for gene conversion, but dispensable for BIR and SSA (Ivanov *et al.* 1996; Signon *et al.* 2001). The *RAD59* gene encodes a protein with significant homology to Rad52 and appears to function in the same pathways of homology-dependent double-strand break repair (DSBR) as *RAD52*. However, *rad59* mutants show much less severe phenotypes in these assays than *rad52* mutants (Bai and Symington 1996; Bai *et al.* 1999; Jablonovich *et al.* 1999; Bartsch *et al.* 2000; Sugawara *et al.* 2000; Signon *et al.* 2001). Although Rad52 and Rad59 catalyze similar reactions *in vitro*, strand annealing shows greater dependence on *RAD52* than *RAD59 in vivo* (Figure 3). We propose that Rad59 functions primarily in the context of Rad52 to enhance the activity of Rad52 in gene conversion, BIR, and SSA.

We demonstrated physical interaction between Rad52 and Rad59 by the two-hybrid system and co-immunoprecipitation, which is central to this hypothesis. These results extend and solidify conclusions drawn from suggestive data showing suppression of the *rad59* radiation sensitivity by more than one copy of *RAD52* and the isolation of a non-null *rad52* allele with a phenotype similar to *rad59* that acts synergistically with *rad59* (Bai and Symington 1996; Bai *et al.* 1999). Rad52 is also known to form stable complexes with Rad51 and to interact with RPA (Park *et al.* 1996; Hays *et al.* 1998; Song and Sung 2000). The complex containing Rad52 and Rad59 appears not to contain Rad51, but could possibly include RPA. Rad52 forms ring structures when visualized by electron microscopy and binds to the ends of single-stranded DNA with the DNA apparently wrapped around the outside of the heptameric ring (Shinohara *et al.* 1998; Parsons *et al.* 2000; Stasiak *et al.* 2000). We currently have no evidence for an oligomeric form of Rad59. The GBD-Rad59 and GAD-Rad59 fusions failed to interact in the two-hybrid assay, and the GAD-Rad59 fusions were also defective for interaction with Rad52. Since the GAD-Rad59 and Rad59-GAD fusions complement the ionizing radiation sensitivity of *rad59* strains, and therefore retain Rad59 function, the failure in the two-hybrid assay could be due to the inability of GAD to activate transcription, the metric for interaction.

The high copy suppression of the *rad59* repair defect by *RAD52* could also be interpreted as evidence for overlapping functions of the two proteins. Biochemical characterization of Rad59 reveals several activities in common with Rad52, including DNA binding and annealing of complementary single-stranded DNA (Figure 2; Petukhova *et al.* 1999). Rad52 can overcome the inhibitory effect of RPA to annealing of complementary oligonucleotides. Similarly, Rad59 is able to partially overcome the inhibition to annealing by RPA (Figure 2), but the annealing of long molecules by Rad59 is not enhanced by RPA (Petukhova *et al.* 1999). In combined reactions we found a slight stimulation of Rad52-promoted annealing of RPA-coated oligonucleotides by Rad59. This result is consistent with the idea that Rad59 acts to enhance the activity of Rad52 in strand annealing.

rad52 mutants are highly defective in SSA *in vivo*, suggesting that Rad59 is unable to substitute for the annealing activity of Rad52. Rad52 is known to interact with RPA and is thought to displace RPA from single-stranded DNA to promote strand annealing or to recruit Rad51 (Park *et al.* 1996; Hays *et al.* 1998; Smith and Rothstein 1999). The SSA defect of *rad52* strains is suppressed by the *rfa1-D288Y* mutation, which results in lower cellular levels of RPA (Smith and Rothstein 1995, 1999). However, even in the *rad52 rfa1-D288Y* strain, *in vivo* annealing was independent of *RAD59*. This suggests that another factor promotes annealing in the *rad52 rad59 rfa1-D288Y* strain or that, when RPA

levels are low, spontaneous annealing can occur with greater efficiency, eliminating the requirement for a dedicated annealing protein. The rate of spontaneous deletion formation between 2.4-kb direct repeats was unaffected by the *rad59* mutation, which is in contrast to a study by Jablonovich *et al.* (1999), who found a 1.5- to 3-fold reduction in rate. This may be due to the difference in the length of the repeats within the direct-repeat substrates. The direct repeats used by Jablonovich *et al.* (1999) are ~0.3 kb long and the direct repeats used in this study are 2.4 kb long. Results from the Haber lab (Sugawara *et al.* 2000) suggest that the *rad59* defect in SSA and DSB-induced gene conversion becomes less severe as the length of homology is increased. Thus, the weak effect of the *rad59* mutation on spontaneous and DSB-induced deletion formation in this study may be due to the use of long repeats. One interpretation of these results is that Rad52 alone can efficiently anneal long single-stranded regions, but when the sequences are short Rad52 becomes more dependent on Rad59. In *rad52 rad59* double mutants the rate of spontaneous deletion formation was slightly lower than observed for the *rad52* strain, consistent with the results of Jablonovich *et al.* (1999). We were unable to detect a similar decrease in the physical assay for SSA in the *rad52 rad59* double mutant. This could be due to the limitation of the method used (quantitation of weak bands by phosphorimaging) or could reflect the requirement for *RAD59* in a different pathway of spontaneous deletion formation.

In addition to SSA, *RAD52* and *RAD59* are both implicated in BIR. Diploid cells can repair a DSB induced at the *MAT* locus on one chromosome *III* homolog by gene conversion (the preferred mode of repair) or BIR. In *rad52* mutants only chromosome loss events occur due to the absolute requirement for *RAD52* in both repair processes (Malkova *et al.* 1996). By contrast, in *rad51* mutants where gene conversion is eliminated, repair can occur by BIR, albeit inefficiently (Malkova *et al.* 1996). Mutation of *RAD59* has no effect on gene conversion in a *RAD51* strain, but the *rad51 rad59* double mutant shows a sevenfold reduction in BIR events with a concomitant increase in chromosome loss events (Signon *et al.* 2001). The requirement for both *RAD52* and *RAD59* in BIR suggests that the initial strand invasion event to prime DNA synthesis is promoted by Rad52 or the Rad52/Rad59 complex. This could occur by annealing between the invading single strand and a region of the donor duplex that is transiently unwound, either by replication or transcription. If the initial annealing step is confined to short regions of DNA, this might explain the requirement for Rad59 acting together with Rad52. Alternatively, Rad59 or the Rad52/Rad59 complex might be important for recruiting components of the replication apparatus.

RAD52 is essential for homology-dependent repair of double-strand breaks in yeast, but in mouse and chicken bursal cells it appears to play a less important role in repair. Deletion of *MmRAD52* does not cause embryonic lethality, unlike *RAD51* (Lim and Hasty 1996; Tsuzuki *et al.* 1996), and cell lines deficient for mouse or chicken *RAD52* are resistant to ionizing radiation and exhibit only a slight decrease in homologous recombination (Rijkers *et al.* 1998; Yamaguchi-Iwai *et al.* 1998). The yeasts *S. cerevisiae, Kluyveromyces lactis*, and *Schizosaccharomyces pombe* each have two Rad52-like proteins (Bai and Symington 1996; Suto *et al.* 1999; van den Bosch *et al.* 2001a,b). In *S. cerevisiae*, *RAD52* is essential for DSBR and *RAD59* plays an auxiliary role (Bai and Symington 1996; Bai *et al.* 1999). The fission yeast Rad52-like proteins show more of a division of labor with *rad22A* playing the major role, but synergistic defects in radiation sensitivity and meiosis are found in *rad22A rad22B/rti1* double mutants (Suto *et al.* 1999; van den Bosch *et al.* 2001). We suggest higher eukaryotes will have other Rad52-like proteins and these will contribute to the repair of DSBs with the known Rad52 homologs.

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