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RAD51 Is Required for the Repair of Plasmid Double-Stranded DNA Gaps from Either Plasmid or Chromosomal Templates

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DNA double-strand breaks may be induced by endonucleases, ionizing radiation, chemical agents, and mechanical forces or by replication of single-stranded nicked chromosomes. Repair of double-strand breaks can occur by homologous recombination or by nonhomologous end joining. A system was developed to measure the efficiency of plasmid gap repair by homologous recombination using either chromosomal or plasmid templates. Gap repair was biased toward gene conversion events unassociated with crossing over using either donor sequence. The dependence of recombinational gap repair on genes belonging to the *RAD52* epistasis group was tested in this system. *RAD51*, *RAD52*, *RAD57*, and *RAD59* were required for efficient gap repair using either chromosomal or plasmid donors. No homologous recombination products were recovered from *rad52* mutants, whereas a low level of repair occurred in the absence of *RAD51*, *RAD57*, or *RAD59*. These results suggest a minor pathway of strand invasion that is dependent on *RAD52* but not on *RAD51*. The residual repair events in *rad51* mutants were more frequently associated with crossing over than was observed in the wild-type strain, suggesting that the mechanisms for *RAD51*-dependent and *RAD51*-independent events are different. Plasmid gap repair was reduced synergistically in *rad51 rad59* double mutants, indicating an important role for *RAD59* in *RAD51*-independent repair.

Transformation of fungi with plasmid DNA has yielded important insights into the mechanisms of recombination and double-strand break repair (DSBR). Early studies by Hinnen et al. (17) provided evidence for integration of circular nonreplicating plasmids by homologous recombination into the yeast genome. In a subsequent study, Orr-Weaver et al. (38) elaborated more thoroughly the way in which circular and linear nonreplicating DNA molecules recombine with homologous chromosomal sequences. They showed that DNA ends are highly recombinogenic and interact directly with homologous sequences. If two restriction cuts are made within a plasmid region homologous to chromosomal DNA, thereby producing a double-strand gap, the resulting deleted linear plasmids transform at a high frequency and are faithfully repaired during the integration process. Using linear replicating plasmids, Orr-Weaver and Szostak (37) reported the recovery of approximately equal numbers of integrated and nonintegrated plasmids and concluded that gene conversion by double-strand gap repair can occur either with or without crossing over. These studies formed the basis for the DSBR model (65). They also observed circularization of linear plasmid DNA, suggesting the presence of additional, recombination-independent repair pathways. Subsequent studies of plasmid gap repair in *Saccharomyces cerevisiae* and *Ustilago maydis* indicated a lower association of crossing over with gene conversion (12, 44). Studies in *Drosophila* and mouse cells have also shown a very low association of crossing over (<5%) during DSBR (35, 48).

When plasmids capable of autonomous replication are cut within regions that have no homology to yeast genomic sequences, repair of the break can occur by nonhomologous end joining (7). In yeast, the efficiency of this process is dependent on the types of ends produced. Cohesive ends are efficiently repaired by precise end joining in a reaction dependent on *HDF1*, *HDF2*, *MRE11*, *RAD50*, *XRS2*, *DNL4*, and *LIF1* (31, 32, 51, 69). Cohesive ends generated in genomic DNA by either HO or *Eco*RI endonucleases are also efficiently repaired by the end-joining pathway, indicating that plasmid and chromosomal breaks are repaired by similar mechanisms (23). Repair of blunts ends is inefficient in wild-type cells (7). This contrasts with mammalian cells, which show efficient joining of a variety of DNA ends (50).

DNA repair-deficient strains have proven useful for understanding the genetic control of end joining, and the ease of recovering plasmids has allowed a molecular analysis of the type of end-joining event. Although the effects of mutations in DNA repair genes on mating-type switching (28, 60), direct repeat (25, 30), and inverted-repeat recombination (1, 45, 46) have been studied extensively, very few studies have dealt with the role of *RAD* genes in homology-dependent plasmid double-strand gap repair. The initial studies of plasmid gap repair demonstrated an essential role for *RAD52* (38) and subsequent studies showed a requirement for *RAD50*, *RAD53*, *RAD54*, and *RAD57*, but the repair events were not analyzed in detail (14, 15, 42).

Although all of the genes of the *RAD52* epistasis group are required for the repair of ionizing radiation-induced DNA damage, the mutants show considerable heterogeneity in recombination assays. *RAD52* has a unique position within the group in that it is required for most spontaneous and induced mitotic recombination events, for the formation of joint molecules in the rDNA locus, and for single-strand annealing (46, 59, 73). The *rad51*, *rad54*, *rad55*, and *rad57* mutants form a subgroup with similar phenotypes. In these mutants, joint molecules are still detected at the rDNA locus, and they are proficient at several double-strand break (DSB)-initiated and spontaneous mitotic recombination events (45, 60, 73). For

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example, although natural mating-type switching is lethal in *rad51* mutants, repair of an HO endonuclease-induced DSB can occur under certain circumstances if the donor sequence is unsilenced and on a plasmid (60). Furthermore, a DSB introduced into one allele of the *MAT* locus in *rad51* diploids can be efficiently repaired by strand invasion and replication primed from the invading strand to restore the chromosome arm (27). Single-strand annealing is also independent of *RAD51*, *RAD54*, *RAD55*, and *RAD57* (19).

Rad51 has significant homology to bacterial RecA proteins and catalyzes DNA strand exchange in vitro (54, 64). Rad54 and the Rad55-Rad57 heterodimer enhance the efficiency of the Rad51-mediated strand exchange reaction, consistent with genetic studies indicating similar phenotypes of the respective mutants (20, 43, 45, 63). Rad52 stimulates the Rad51-promoted strand exchange reaction by overcoming the inhibitory effects of replication protein A (6, 36, 55, 62). This is consistent with studies showing physical interactions between these proteins (40, 54) and genetic analysis indicating that *RAD52* is epistatic to *RAD51* (46). The observation that high levels of certain types of recombinational repair can occur in the absence of *RAD51* suggests that alternate mechanisms for homologous pairing and strand invasion exist in *S. cerevisiae*. *RAD59* was identified by its requirement for *RAD51*-independent mitotic recombination of inverted repeats (4). However, Rad59 shows 28% identity to Rad52 instead of homology to the RecA family of proteins. Although RecA-like proteins have formed the paradigm for homologous pairing and strand exchange, recent studies suggest that a different class of proteins, exemplified by bacteriophage lambda β protein, provide an alternate pathway for recombinational repair (71, 72). Rad52 shows no primary sequence homology to β protein, but both proteins form ring structures and catalyze strand annealing in vitro (22, 34, 41, 56).

Two alternative hypotheses for *RAD51*-independent recombination have been suggested. First that *RAD51*, *RAD54*, *RAD55*, and *RAD57* gene products do not play a direct role in recombination but instead are required to facilitate DNA strand invasion into otherwise inaccessible sequences (60). This hypothesis was put forward to explain the occurrence of *RAD51*-independent DSBR when the donor for repair was expressed and plasmid borne. If the hypothesis presented by Sugawara et al. (60), is correct, we would expect *rad51* mutants to be defective in all assays that involve repair from a chromosomal donor but not when the donor sequences are expressed and on a plasmid. Second, an alternative explanation to the donor accessibility model is that recombination is *RAD51* independent when the event can be resolved as a crossover. Based on evidence obtained from inverted-repeat recombination experiments (45, 46), we proposed that the *RAD51* pathway, including *RAD54*, *RAD55* and *RAD57*, leads primarily to noncrossover recombinants and is not involved in the recombination pathway that results in crossovers. A system for intermolecular plasmid gap repair was developed to test these hypotheses.

MATERIALS AND METHODS

Plasmids. The source of the gene encoding *O*-acetylhomoserine-*O*-acetylserine sulfhydralase, *MET17* (21), was plasmid pGC3 (ATTC 87440), which contains a 2.5-kb *Spe*I fragment of the genomic *MET17* locus cloned into pRS414 (57). To obtain a restriction fragment length polymorphism nonsense mutation marker (*met17-s*), oligonucleotide-directed insertion mutagenesis was performed on the unique *Sna*BI site in *MET17* of plasmid pSB99 (see Fig. 1, nucleic acid position 943) using the Quik Change site-directed mutagenesis kit (Stratagene). Plasmid pSB99 was constructed by cloning the 1.8-kb *Xba*I *MET17* fragment from pGC3 into the *Xba*I site of pBluescript (Stratagene). Plasmid pSB99 was used as the template and oligonucleotides oSB2 (5′-AGAACAATCCCCA*TAA CGTA*TCTTGGGTTTC-3′) and oSB1 (5′-AAACCCAAGA*TACGTTA*TGGG GATTGTTC-3′) were used to direct the sequence change. The nucleotides in the oligomers that caused the PCR-mediated mutation of the *Sna*BI site (*TAC GTA*) are underlined. Plasmids derived from the mutagenesis were screened for the absence of a *Sna*BI site and candidates were further analyzed by DNA sequencing to confirm that a stop codon was introduced (see Fig. 1). The mutagenized plasmid pSB99 was named pSB99-1. Plasmid pSB112 was constructed by replacing the *Xba*I-*Xba*I *MET17* fragment in pGC3 with the mutagenized *MET17* of pSB99-1. To create plasmid pSB115, used for the two-step replacement of the chromosomal *MET17* locus by *met17-s*, a *Bam*HI-*Not*I *met17-s* fragment was isolated from pSB112 and ligated to the *Bam*HI-*Not*I fragment of the integrating vector pRS406 (57).

Centromeric and nonreplicating gap repair plasmids pSB103 and pSB101 were constructed by ligation of the *Bam*HI-*Eag*I *MET17* fragment isolated from pGC3 into the pRS416 (*CEN6 ARSH4 URA3*) and pRS406 (*URA3*) vectors, respectively (57). For the construction of the autonomously replicating gap repair plasmid pSB110, *ARSH4* (8) was first PCR amplified from the centromeric *ARS* plasmid pRS414. Primers oSB11 (5′-AGACTCTAGGGGGACGTCGATCGC CAACAA-3′) and oSB12 (5′-TTTCTTAGGACGGACGTCGATCGCTTGCC TG-3′) were designed in a way that *Aat*II sites (underlined) flanked the PCR-amplified *ARS* element. The PCR product was digested with *Aat*II and cloned into the *Aat*II site of pSB101. To obtain pSB118, the plasmid DNA donor in the plasmid by plasmid gap repair experiment, the *Bam*HI-*Eag*I *MET17* fragment from pGC3 was first cloned into pRS414 (*CEN6 ARSH4 TRP1*) to yield pSB104. The *MET17* in pSB104 was then replaced by the *met17-s* allele to generate pSB118.

All plasmids were amplified in *Escherichia coli* strain DH5αF′ [*F′ endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA gyrA96 relA1* Δ(*argF-lacZYA*)*U169*, P80 *lacZ*ΔM15).

Media and strains. All media were prepared as described previously (53) with minor modifications. Synthetic complete (SC) medium lacked cysteine, since this amino acid can be converted into methionine via *MET17*-independent pathways (10). Selective media lacking 1 amino acid (aa) are designated SC−aa, e.g., selection for *LEU2* disrupted genes was performed in SC−Leu which is SC with all the amino acids W303 needs for growth except leucine. Lead (Pb^{2+}) plates were prepared by dissolving 3 g of peptone, 5 g of yeast extract, 200 mg of ammonium sulfate, and 40 g of glucose and suspending 20 g of agar in 1 liter of water. The suspension was autoclaved, the agar solution was cooled to 55°C, 2 ml of lead nitrate, $Pb(NO_3)_2$ (0.5 mg/ml of water), was added, and the solution was mixed vigorously before the plates were poured (10). Standard genetic techniques were used to manipulate yeast strains (53).

All strains are derivatives of W303-1A and W303-1B (66) with the corrected *RAD5* allele (11) and are listed in Table 1. The *met17-s* mutant LSY693 was generated by two-step replacement (70) of *MET17* in strain YKH10a using the *URA3*-integrating plasmid pSB115. First, plasmid pSB115 was cut with *Bsp*EI to target integration at *MET17*. Then Ura^+ transformants were selected for 5-fluoroorotic acid (5-FOA) resistance and screened for the *met17-s*-derived brown phenotype when grown on Pb^{2+} plates (see Fig. 1). To obtain the *met17*::*ADE2* (P_{MET17}-*ADE2*) mutant strain LSY694, the complete *MET17* open reading frame (ORF) in W303-1A was replaced by the *ADE2* ORF using a microhomology-mediated one-step gene replacement method (5). For that experiment, two PCR primers were designed: a 60-mer oligonucleotide, in which the 5′ end was complementary to the 40-nucleotide proximal region of the *MET17* ORF and the 3′ portion was complementary to the first 20 nucleotides of the *ADE2* ORF, and a 61-mer oligonucleotide, in which the 3′ end was complementary to the distal 21 nucleotides of the *ADE2* ORF and the 5′ end was complementary to the sequence that extends 40 nucleotides from the *MET17* ORF. The *ADE2* ORF delineated by *MET17* sequences was PCR amplified from plasmid pL909, gel purified and transformed into strain W303-1A. Ade^+ transformants containing the correct disruption were identified by phenotype and confirmed by Southern blot analysis.

Strains W1588-4A and W1588-4C (Table 1) are *RAD5* derivatives of W303 (a gift of R. Rothstein) that were crossed to LSY693 and LSY694 to create *RAD5* strains containing the *met17-s* (LSY697) and *met17*::*ADE2* (LSY698) alleles, respectively. The *rad52*::*LEU2*-disrupted strain LSY715 (*MET17 rad52*::*LEU2 rad5-535*) was constructed by transformation of W303-1B with the *Bam*HI-digested disruption plasmid pSM20 (52). Leu^+ transformants containing the *rad52*::*LEU2* allele were identified by sensitivity to γ irradiation and confirmed by Southern blot analysis. Strains LSY697 and LSY698 were then crossed to W303 derivatives containing the appropriate *rad* gene mutation to create the strains listed in Table 1. Colony PCR was used to monitor the *rad5-535* missense allele in genetic crosses (29).

Plasmid DNA gap repair assays. Plasmids to be used as substrates in the gap repair assay were digested with *Bsp*EI and *Eco*NI (Fig. 1), and the linear DNA was gel purified. Transformation was performed by the lithium acetate transformation method (53) with 100 ng of gapped plasmid (gap repair substrate) or 100 ng of uncut plasmid (transformation efficiency control) in the presence of 50 μg of denatured salmon sperm DNA as carrier DNA. The transformed cells were diluted and plated onto SC−Ura−Met and SC−Ura media. The colonies on both plates were counted after incubation at 30°C for 3 days. The amounts of DNA used were determined to be in the linear range for uptake of DNA. The specific gap repair frequency was calculated as the number of Met^+ Ura^+ recombinants per microgram of transformed linearized DNA divided by the total

TABLE 1. *S. cerevisiae* strains used in this study[a]

Strain	Genotype	Source or reference
W303-1A	*MAT***a**	67
W303-1B	*MAT*α	67
YKH10a	*MAT***a** *ADE2*	K. Huang
W1588-4C	*MAT***a** *RAD5*	R. Rothstein
W1588-4A	*MAT*α *RAD5*	R. Rothstein
YHK595-1C	*MAT*α *rad51::LEU2 RAD5*	H. Klein
YHK598-8B	*MAT*α *rad57::LEU2 RAD5*med	H. Klein
LSY563	*MAT***a** *rad59::LEU2*	Y. Bai
B404-4D	*MAT*α *rad51::HIS3 rad59::LEU2 lys2*	Y. Bai
Y301	*MAT***a** *rad53-21*	S. Elledge
LSY693	*MAT***a** *ADE2 met17-s*	This study
LSY694	*MAT***a** *met17::ADE2*	This study
LSY695-2B	*MAT*α *ADE2 met17-s RAD5*	This study
LSY695-7D	*MAT***a** *ADE2 met17-s RAD5*	This study
LSY696-1C	*MAT*α *met17::ADE2 RAD5*	This study
LSY696-7A	*MAT***a** *met17::ADE2 RAD5*	This study
LSY715	*MAT*α *rad52::LEU2*	This study
LSY826	*MAT***a** *rad51::LEU2 met17-s ADE2 RAD5*	This study
LSY827	*MAT***a** *rad51::LEU2 met17::ADE2 RAD5*	This study
LSY718	*MAT***a** *rad52::LEU2 met17-s ADE2 RAD5*	This study
LSY723	*MAT***a** *rad52::LEU2 met17::ADE2 RAD5*	This study
LSY842	*MAT***a** *rad53-21 met17-s ADE2 RAD5*	This study
LSY843	*MAT***a** *rad53-21 met17::ADE2 RAD5*	This study
LSY838	*MAT***a** *rad57::LEU2 met17-s ADE2 RAD5*	This study
LSY839	*MAT***a** *rad57::LEU2 met17::ADE2 RAD5*	This study
LSY837	*MAT***a** *rad59::LEU2 met17-s ADE2 RAD5*	This study
LSY836	*MAT***a** *rad59::LEU2 met17::ADE2 RAD5*	This study
LSY841	*MAT***a** *rad51::HIS3 rad59::LEU2 met17-s ADE2 RAD5 lys2*	This study
LSY840	*MAT***a** *rad51::HIS3 rad59::LEU2 met17::ADE2 RAD5 lys2*	This study

[a] All strains are derivatives of W303 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 MET17 rad5-535*). Only differences in the genotype from the parental strains are indicated, except for B404-4D, which is also *ade2::hisG:URA3:hisG his3::ade2-5′Δ:TRP1:ade2-n*.

number of Ura^+ Met^+ transformants per microgram of appropriate circular transformation control DNA. As a control for undigested plasmid DNA contamination of the gapped substrate, a second yeast strain containing a complete deletion of the *MET17* ORF was used as a host for transformation. The rare Met^+ transformants arising from this strain were considered to be due to contamination of the gapped DNA with uncut plasmid, and this number was deducted from the number of Met^+ transformants obtained in the experimental strain. The gap repair experiments were repeated at least twice for each substrate and strain, and the mean gap repair frequencies are presented.

To test for the mitotic stability of the *URA3* and the *MET17* markers, all the resulting Ura^+ transformants were picked from one region of each transformation plate, transferred into water-filled 96-microtiter plate wells, and spotted onto SC−Ura plates. The cells were grown at 30°C for 3 days to confirm the Ura^+ phenotype. The spots were replica plated onto SC− Met plates to score for the Met^+ phenotype and, in parallel, onto YPD (plasmid by chromosome assay) or onto SC−Trp (plasmid by *TRP1* plasmid assay). Cells were grown under conditions nonselective for Ura^+ or Met^+ for 2 to 3 days at 30°C and subsequently replica plated onto 5-FOA and Pb^{2+} plates, respectively, to assess the mitotic stability phenotype. Confluent growth on 5-FOA indicated that the Ura^+ phenotype was mitotically unstable. Growth on SC−Met plates (diagnostic of Met^+) but then a Met^- phenotype (dark brown) when grown on Pb^{2+} indicated that the Met^+ phenotype was unstable.

Molecular analysis of gap repair recombinants. The mode of recombination during gap repair was determined by Southern blot analysis. Total DNA was extracted from 5-ml cultures of individual transformants (53) and digested with *Bam*HI-*Sna*BI, and fragments were separated by electrophoresis through 0.7% agarose. DNA was transferred to a nylon membrane (Hybond-N; Amersham) and hybridized with a 1.5-kb *Bam*HI-*Sna*BI *MET17* fragment isolated from plasmid pGC3 that was ^{32}P labeled for use as the probe.

RESULTS

Rationale for the gap repair system. A set of recombination reporter substrates was constructed by subcloning the *MET17* gene into plasmids of the pRS400 series containing the *URA3* marker (57). These plasmids were gapped within the *MET17* gene and used as recipients for gap repair during transformation into host strains. The gap was made in the plasmid by deleting a 238-bp fragment from the *MET17* ORF with the restriction enzymes *Bsp*EI and *Eco*NI (Fig. 1). The restriction enzymes produce overlapping but noncomplementary ends that should be poor substrates for end-joining ligation reactions. The donor sequences for DNA repair contained a nonsense mutation at the *Sna*BI site of the chromosomal *MET17* locus (*met17-s*). Since the *met17-s* mutation lies downstream of the region that covers the gap in the plasmid, Met^+ transformants can arise by repair of the gap using chromosomal information (Fig. 2). Plasmid-by-plasmid gap repair was studied with the same rationale except that the *met17-s* donor sequence was located on a circular centromeric *TRP1* plasmid that was introduced into a host strain in which the complete chromosomal *MET17* ORF was replaced by the *ADE2* ORF. Selection for the *TRP1* plasmid was maintained throughout the plasmid by plasmid gap repair assay. The use of both chromosomal and plasmid donors was to test the hypothesis that *RAD51* and *RAD57* are not required if the donor sequence for gap repair is expressed and plasmid borne.

All plasmids used for gap repair contain the *URA3* and *MET17* genes but differ in their ability to replicate in yeast (Fig. 2). The linearized (gapped) *ARS* plasmid can integrate into the chromosome but can also be maintained extrachromosomally upon repair of the plasmid gap. Integration of a gapped *CEN ARS* plasmid creates a dicentric chromosome that cannot be maintained in cells and leads to cell death, whereas gap repair without integration results in a viable product. Linearized plasmids with no origin of replication cannot be maintained in cells unless these sequences integrate into the genome. These events can be distinguished by monitoring the selective and mitotic stability phenotypes conferred to the recombinants by the *URA3* and *MET17* markers. The use of different replicons for gap repair substrates was to test the

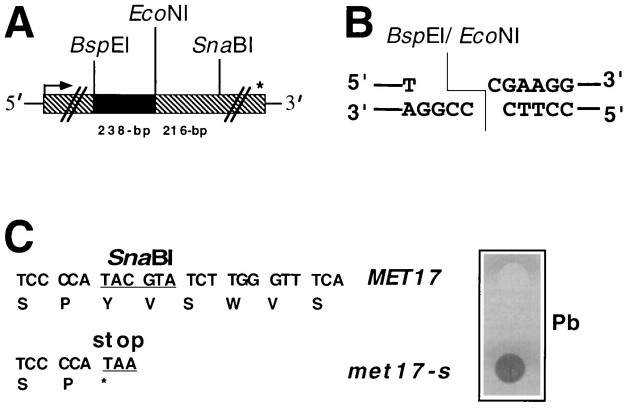

FIG. 1. Physical map of *MET17*, the molecular structure of the gap, and the phenotype conferred to cells by the *met17-s* mutation. (A) The hatched box indicates the 1.5-kb ORF (arrow, start codon; ∗, stop codon). *MET17* plasmids to be used as substrates in the gap repair assay were digested with *Bsp*EI and *Eco*NI to produce a 238-bp gap, indicated by a black box. The plasmid and chromosomal DNA donor sequences contain a nonsense mutation that destroyed a *Sna*BI site (*met17-s*) 216 bp downstream of the *Eco*NI site. (B) The gap produced by *Bsp*EI-*Eco*NI digests consists of noncomplementary 5′ overhangs that overlap in one nucleotide (C · C) and is expected to provide a poor substrate for ligation. However, degradation or melting of the *Eco*NI end could provide microhomologies (C · G and/or GG or CC) for annealing to the overhang produced by *Bsp*EI digestion. (C) The first sequence shows the *Sna*BI site in the *MET17* allele; the second line of sequence indicates the A insertion at the *Sna*BI site, which creates a stop codon and disrupts the recognition site for *Sna*BI. The *met17-s* mutation confers to cells a dark brown phenotype when grown on medium supplemented with lead (Pb).

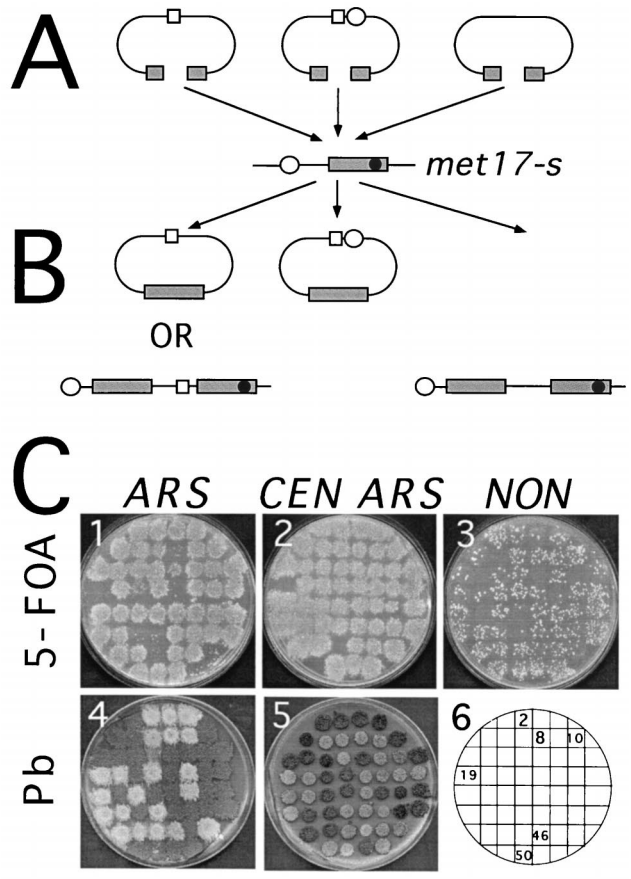

FIG. 2. Rationale of the gap repair assay. (A) The double-strand gap in *MET17* on either *ARS* (open square), *CEN ARS* (open square and open circle), or integrating (*NON*) plasmid is repaired from homologous chromosomal or plasmid *met17-s* sequences. (B) Repair of gapped *ARS* plasmids without a crossover produces a repaired *MET17* plasmid and an unchanged donor sequence (chromosome or plasmid); repair associated with a crossover results in an integrated *ARS* plasmid. Repair of a gapped *CEN ARS* plasmid has to occur by a noncrossover mechanism because integration results in a dicentric chromosome or plasmid, which is inviable. Repair of the gapped plasmid that contains no *ARS* element has to occur by integration to yield a stable transformant. The products were drawn based on the assumption that the gap in the plasmid is not extended by nucleases over the *MET17 Sna*BI site. (C) The products expected from gap repair unassociated and associated with crossover were distinguished by monitoring the selective and colony color phenotypes conferred by the *URA3* and *MET17* markers to the recombinants and by the mitotic stability phenotype of these markers. For the identification of single patches by numbers, a grid is included in C6. Patches on independent plates are shown; note that plates 4 and 5 are not from the same master plate. Confluent growth on 5-FOA medium indicated that the Ura^+ phenotype was mitotically unstable (i.e., C1 no. 2, and C2, no. 2). Secondary pop-out recombination between duplicated *MET17* alleles delineating *URA3* leads to the formation of papillae on 5-FOA due to the excision and loss of *URA3* (i.e., C1, no. 8 and C3, no. 2). Cells displaying a dark brown phenotype when grown on lead (Pb) plates indicated that the Met^+ phenotype was unstable in cells transformed with *ARS* plasmid (i.e., C4, no. 10) or absent (i.e., C5, no. 2). During further incubation of plate C5 for 4 to 5 days at room temperature, patches that were previously white turned a beige color and showed dark brown papillae, indicating the progressive loss of *CEN ARS* plasmids (i.e., C5, no. 8). White cells were diagnostic for a stable Met^+ phenotype (i.e., C4 no. 2, and C5, no. 46 and 50).

hypothesis derived from studies of *RAD51*-independent recombination of inverted repeats that crossing over is less dependent on *RAD51* function than is conversion. Based on this, we expected few recombinants when using the *CEN ARS* plasmid in a *rad51* strain, but recombinants were expected at close to wild-type frequency when using the nonreplicative gapped plasmid.

The *MET17* gene provides the advantages of another widely used colony color marker, *ADE2* (47, 49) and excludes the two main disadvantages of using *ADE2* in recombination studies. First, in contrast to *ade2* mutants in which cellular growth is inhibited as a consequence of the toxicity of the colored byproduct, the pigmentation of cells with PbS does not appear to have a deleterious effect on viability (10). The color phenotype of a strain with the *met17-s* allele is shown in Fig. 1. Second, the *ADE2* gene harbors an autonomous replication sequence (*ARS*) upstream of the *ADE2* ORF that might interfere with replication and DNA repair (58). For *MET17*, no nearby *ARS* is known.

Classification of gap repair products. Genetic and physical tests were used to determine the distribution of gene conversion events associated or unassociated with a crossover. The gap repair products were grouped into categories based on the selective and mitotic stability phenotypes conferred to the recombinants by the marker genes. The Met^+ Ura^+ clones from the gap repair assay could have resulted from two different classes of recombination. (i) Repair of the gapped *ARS* plasmid by gene conversion without a crossover leads to an unstable Ura^+ Met^+ phenotype (Ura^{+u} Met^{+u}) due to loss of the plasmid under nonselective growth conditions. (ii) Repair of the plasmid followed by integration at the chromosomal *met17-s* locus results in a stable Ura^+ Met^+ phenotype (Ura^{+s} Met^{+s}). Transformed gapped *CEN ARS* and nonreplicating plasmids are constrained to remain episomal or to integrate into the genome during gap repair. Therefore, the majority of Met^+ transformants from the *CEN ARS* plasmid were expected to be Ura^{+u} Met^{+u} whereas only Ura^{+s} Met^{+s} should be detected in assays with nonreplicating plasmids. This separation of phenotypes allowed us to monitor exclusively either gene conversion events unassociated with crossing over or conversion associated with crossing over (integration). The classification of products based on stability of the markers was confirmed by Southern blot analysis. The nonsense mutation in the *met17-s* allele, used as the DNA donor, has destroyed a *Sna*BI restriction enzyme site in *MET17* and can be used to distinguish between plasmid and chromosomal alleles.

Gap repair proficiency of *rad51*, *rad52*, *rad53*, *rad57*, *rad59*, and *rad51 rad59* mutants. Initially, the gapped *ARS* plasmid was used to examine the role of plasmid versus chromosomal sequences as donors in gap repair and the dependence of plasmid-by-plasmid and plasmid-by-chromosome gap repair on *RAD* genes. In Rad^+ cells, the efficiency of gap repair using the plasmid and chromosomal donors was comparable (Table 2). The gap repair frequencies were substantially reduced in

TABLE 2. *ARS* plasmid gap repair frequencies[a] with chromosomal and plasmid donors

Relevant genotype	Transformation efficiency (10^5 CFU/μg of DNA)	Frequency (10^{-2}) with chromosomal donor	Fold decrease	Frequency (10^{-2}) with plasmid donor	Fold decrease
RAD	1.1	31	1	23	1
rad51	0.31	0.32	98	0.20	110
rad52[b]	0.16	0.06	550	0.02	1,000
rad53-21	1.1	12	3	11	2
rad57	0.63	0.39	80	0.34	67
rad59	0.42	1.5	21	0.40	57
rad51 rad59[b]	0.26	0.07	460	<0.03	>750

[a] Gap repair frequencies were determined from the number of Ura^+ Met^+ transformants obtained from the gapped plasmid divided by the number of Ura^+ Met^+ transformants from the uncut plasmid (see Materials and Methods).

[b] Gap repair frequencies for the *rad52* strain were estimated from the accumulated data from 12 independent transformations; only 3 transformations of the *rad51 rad59* strain were performed.

TABLE 3. Phenotypes of Ura$^+$ transformants derived from recombination between the gapped *ARS* plasmid and chromosomal *met17-s* donor

Relevant genotype	No. of Ura$^+$ transformants[a]	% of Ura$^+$ transformants[a] that were:					
		Ura^{+u} Met^{+u}	Ura^{+u} Met$^-$	Ura^{+s} Met^{+s}	Ura^{+s} Met$^-$	Others[b]	Met^{+c}
RAD	616	38	38	8	13	3	49
rad51	161	14	56	4	26	0	18
rad52[d]	34	0	88	0	12	0	0
rad53-21	91	37	44	8	9	2	47
rad57	224	20	50	8	20	2	30
rad59	288	38	38	11	11	2	51
rad51 rad59[d]	18	5	78	5	11	0	11

[a] Number of Ura$^+$ transformants analyzed; the other numbers are given as percentages of the total Ura$^+$.
[b] Percentage of transformants classified as Ura^{+s} Met^{+u} and Ura^{+u} Met^{+s}.
[c] Percentage of transformants that was Met$^+$.
[d] Low gap repair efficiency resulting in few Ura$^+$ transformants to analyze.

hosts containing *rad51*, *rad52*, *rad57*, and *rad59* mutations compared to wild-type cells, independent of the origin of the donor DNA (Table 2). The *rad52* mutant showed the greatest decrease in gap repair (>500-fold), the *rad51* and *rad57* mutants showed an intermediate decrease (67- to 110-fold), and the *rad59* strain showed a 21- to 57-fold decrease. Although not analyzed in detail, a *rad55* strain showed a similar reduction in gap repair efficiency to the *rad51* and *rad57* strains. *rad55* and *rad57* mutants showed more severe DNA repair defects at low temperatures; however, the defect in gap repair was apparent even at 30°C, the temperature used for the plasmid gap repair assay (16, 20, 26). In the *rad53-21* mutant, a moderate two- to fourfold reduction was observed, indicating a possible role of this DNA damage checkpoint gene in the regulation of gap repair. The epistatic relationship between *RAD51* and *RAD59* for gap repair was also assessed. The gap repair frequency in *rad51 rad59* double mutants was synergistically reduced compared to that observed in *rad51* or *rad59* single mutants. The transformation frequency of the *rad51 rad59* double mutants only slightly deviated from that observed in either single mutant alone. The highest reduction in the efficiency of transformation was observed in *rad52* mutants (0.16×10^5 CFU/μg of DNA), as reported previously (38).

Gap repair events are biased toward noncrossover products. In the previous experiment, Met$^+$ Ura$^+$ transformants were selected to determine the frequency of gap repair to restore the *MET17* gene. To determine all possible modes of plasmid repair, Ura$^+$ transformants were selected and then analyzed for the Met phenotype as well as mitotic stability. For example, Ura^{+s} Met^{+s} (integration) recombinants were distinguished from Ura^{+u} Met^{+u} (nonintegration) by growth on nonselective medium followed by replica plating either to medium supplemented with 5-FOA or to Pb plates (see Materials and Methods). As shown in Fig. 2, Ura^{+u} Met^{+u} cells formed dense patches on 5-FOA plates and cells grown on Pb-plates showed a dark brown colony color phenotype diagnostic for the loss of the *MET17* gene. Secondary pop-out recombination events in Ura^{+s} Met^{+s} cells would be indicated by the formation of papillae on 5-FOA plates due to the excision and loss of the integrated plasmid *URA3* marker by recombination of flanking homologous sequences.

In Rad$^+$ cells, 49% of the Ura$^+$ transformants were Met$^+$ (Table 3). Of the Ura$^+$ Met$^+$ transformants, 77% were unstable, indicative of gap repair unassociated with crossing over, and 16% were stable, indicative of gap repair associated with crossing over (integration). This distribution was confirmed by Southern blot analysis of DNA from these classes of transformants. The *Bam*HI-*Sna*BI digests of total DNA isolated from 18 Ura^{+u} Met^{+u} transformants produced a 6.7-kb *Sna*BI-*Sna*BI chromosomal fragment and a 1.5-kb *Bam*HI-*Sna*BI plasmid fragment (Fig. 3A, 8 transformants shown). In all transformants tested, the plasmid contained the wild-type *Sna*BI site while the chromosome retained the mutation. This pattern is diagnostic for gap repair events not associated with a crossover. In three transformants, an additional band of 7.2 kb was observed. This is most probably due to more than one plasmid entering the cell and repair to form a mixture of Met$^+$ and Met$^-$ products (see below). Analysis of eight stable Ura^{+s} Met^{+s} transformants showed more complex rearrangements (Fig. 3B). Three of the eight analyzed contained the expected fragments of 5.2 and 3.0 kb, indicating the presence of two *MET17* heteroalleles within the chromosomes separated by plasmid *ARS URA3* sequences. The upstream allele contained a functional *Sna*BI site, whereas the downstream allele retained the *met17-s* mutation. Four transformants contained multiple, tandemly integrated copies of the plasmids. One transformant had a restriction pattern consistent with a simple integration but with two *MET17* alleles.

The Ura$^+$ Met$^-$ transformants could have arisen by several mechanisms. End joining of the gapped *ARS* plasmid would generate unstable Ura$^+$ Met$^-$ transformants; alternatively, gap repair events that extended to the *met17-s* marker (by nuclease resection or heteroduplex DNA extension) would give rise to unstable and stable Ura$^+$ Met$^-$ transformants. In 25 of the Ura^{+u} Met$^-$ recombinants analyzed, DNA fragments of 6.7 kb (chromosomal *met17-s* allele) and 7.2 kb were observed, indicative of gap repair to convert the plasmid *MET17* to *met17-s* (Fig. 3C, 10 recombinants shown). All of the Ura^{+s} Met$^-$ transformants analyzed had structures consistent with integration of the plasmid and duplication of the *met17-s* allele (Fig. 3D). Thus, all of the Ura^{+s} transformants (21% of the total Ura$^+$ transformants) were the result of recombinational repair to integrate the gapped plasmid. Importantly, the physical analysis demonstrated that the majority of Ura$^+$ transformants in Rad$^+$ cells were the result of recombinational repair rather than end joining.

In the *rad51* strain, only 18% of the Ura$^+$ transformants were Met$^+$, compared with 49% Met$^+$ obtained from the wild-type strain. Of these, 79% were unstable and 21% were stable and due to integration of the repaired plasmid. Physical analysis of six Ura^{+s} Met^{+s} transformants confirmed integration of the plasmid and the presence of *MET17* heteroalleles, but, unlike for the wild-type strain, no multiple integration events were observed (data not shown). Thus, the *rad51* mutation seemed not to significantly affect the ratio of crossover to noncrossover events, but the overall probability of correct repair to Met$^+$ was greatly reduced. Physical analysis of the Ura^{+s} Met$^-$ transformants revealed that 9 of 10 resulted from

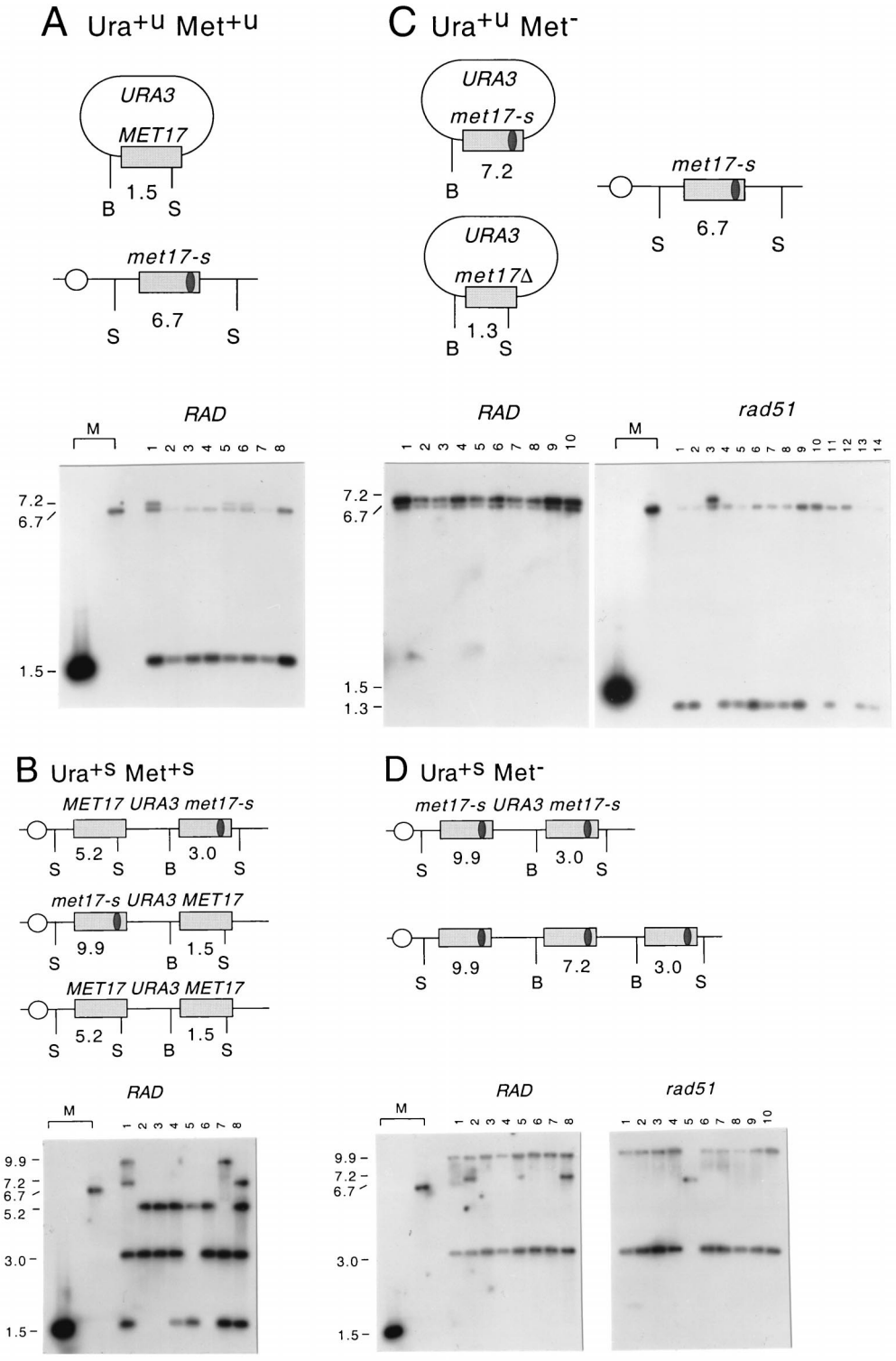

FIG. 3. Structural analysis of Ura$^+$ transformants. Yeast DNA isolated from Ura$^+$ transformants was digested with *Bam*HI (B) and *Sna*BI (S), and Southern blot analysis was performed with a *MET17* DNA fragment as the hybridization probe. (A) Schematic representation (not drawn to scale) of the expected DNA repair product to generate an unstable (u) Ura$^+$ Met$^+$ phenotype; also shown is the chromosomal *met17-s* allele. The sizes of DNA fragments that hybridize to the probe are shown. The lower panel shows a Southern blot of this class of events. (B) Schematic representation of integration events to produce a stable Ura^{+s} Met^{+s} phenotype. The three simplest classes are shown, although multiple integration events to produce fragments of 1.5 and/or 7.2 kb also occur. The lower panel shows a representative Southern blot of this class of events. (C) Schematic representation of events to produce a Ura^{+u} Met$^-$ phenotype. Conversion of the plasmid *MET17* to *met17-s* is monitored by the appearance of a 7.2-kb fragment. A 1.3-kb *Bam*HI-*Sna*BI fragment that hybridizes to the probe is diagnostic for a nonhomologous end joining of the gapped plasmid substrate. For both classes, the 6.7-kb *met17-s* allele is unchanged. Events from a wild-type strain (*RAD*) are shown on the left Southern blot, and those from a *rad51* strain are shown on the right. (D) Schematic representation of an integration event to produce a stable Ura^{+s} Met$^-$ phenotype. The 9.9- and 3.0-kb fragments are diagnostic of two copies of *met17-s*; multiple integration results in an additional fragment of 7.2 kb. Southern blots of DNA from *RAD* and *rad51* strains are shown below the schematic. Included as size markers (M) are *Sna*BI-*Bam*HI-digested plasmid pSB110 (*MET17*) and genomic DNA, isolated from an untransformed tester strain (*met17-s*), which produce signals of 1.5 and 6.7 kb, respectively. Fragment sizes are given in kilobase pairs on the left and were determined relative to *Hin*dIII-digested lambda DNA run as a standard.

TABLE 4. Gap repair efficiencies[a] with the *CEN-ARS* and integrating (*NON*) gapped plasmids

Relevant genotype	Gap repair frequency (10^{-2}) for *CEN-ARS* x chromosome	Fold decrease[b]	Gap repair frequency (10^{-2}) for *CEN-ARS* x plasmid	Fold decrease	Gap repair frequency (10^{-2}) for *NON* x chromosome	Fold decrease	Gap repair frequency (10^{-2}) for *NON* x plasmid	Fold decrease
RAD	17	1	37	1	8.1	1	7.2	1
rad51	0.66	26	0.32	120	0.05	150	0.04	180
rad57	0.97	18	1.2	30	0.08	99	0.14	51
rad59	3.8	4	2.9	12	0.39	21	0.32	22

[a] Gap repair frequencies were determined from the number of Ura^+ Met^+ transformants obtained from the gapped plasmid divided by the number of Ura^+ Met^+ transformants from the uncut plasmid (see Materials and Methods).
[b] Decrease relative to *RAD*.

gap repair to duplicate the *met17-s* allele associated with integration, as observed for the wild-type strain (Fig. 3D). Again, no multiple integration events were found. The other Ura^{+s} Met^- transformant had only the chromosomal 6.7-kb fragment and most probably resulted from conversion of the *ura3-1* allele (Fig. 3D, lane 5). Of 14 Ura^{+u} Met^- transformants analyzed from the *rad51* strain, 11 had a 1.3-kb fragment due to nonhomologous end joining (Fig. 3C). This class was absent from Rad^+ transformants. Of the 14, 2 had larger deletions and the plasmid sequences were detected only when vector sequences were used as a hybridization probe (lanes 10 and 12 and data not shown). Only 1 of the 14 Ura^{+u} Met^- transformants analyzed from the *rad51* strain had a restriction pattern consistent with conversion of the plasmid *MET17* to *met17-s* (lane 3). Thus, most of the Ura^+ Met^- transformants from the *rad51* strain were the result of end joining rather than recombinational repair. The transformants that were generated by recombinational repair occurred by integration 60% of the time. A similar pattern was observed in the *rad57* strain.

Of 34 Ura^+ transformants analyzed from the *rad52* strain, none were Met^+. Although some Met^+ transformants were generated in *rad52* strains (Table 2), these were extremely rare and gap repair frequencies were determined from only one or two Met^+ colonies obtained from multiple transformations. Most of the Ura^+ transformants were unstable. Analysis of five unstable Ura^+ Met^- transformants revealed that all occurred by end joining (data not shown). The stable Ura^+ transformants could have arisen by nonhomologous integration of the gapped plasmid or by conversion or reversion of the *ura3* gene but were not analyzed further.

For the *rad53* and *rad59* strains, the percentage of Ura^+ transformants that were Met^+ was comparable to that in the wild type and the distribution of gap repair events associated with crossing over was also similar to that in the wild type. As predicted from the low frequency of gap repair observed with the *rad51 rad59* double mutant, very few of the Ura^+ transformants obtained were Met^+. Six of the unstable Ura^+ Met^- transformants were analyzed by Southern blotting and shown to arise by end joining (data not shown). Thus, most of the Ura^+ transformants obtained from the *rad51 rad59* double mutant, like the *rad51* and *rad52* single mutants, resulted from end joining instead of homology-dependent gap repair. Although end-joining events were not expected to occur with high efficiency using the gapped substrate because the ends produced by *Bsp*EI and *Eco*NI are noncomplementary, these low-frequency events were recovered from recombination-deficient strains.

Gap repair of the *CEN/ARS* and nonreplicative plasmids. To further determine whether the *RAD51* pathway is involved in only a particular noncrossover pathway, *CEN ARS* and nonreplicating plasmids were used as substrates for gap repair. These plasmids are constrained to remain episomal (*CEN ARS*) or to integrate (nonreplicating plasmid) during gap repair. If the hypothesis that the *RAD51* pathway is required only for noncrossover events is correct, we would expect to obtain no Met^+ transformants in the *rad51* and *rad57* strains with the *CEN ARS* plasmid and wild-type levels of Met^+ transformants with the nonreplicating plasmid. The gap repair frequency from the chromosomal donor with the *CEN ARS* plasmid was lower than observed with the *ARS* plasmid (17×10^{-2}) and was reduced further when the nonreplicative plasmids were used in wild-type strains (8.2×10^{-2}) (Table 4). In contrast to our expectations, there was a greater reduction in gap repair when the nonreplicative plasmid rather than the *CEN/ARS* plasmid was used in all of the *rad* mutants tested. Thus, crossing over is dependent on *RAD51*, *RAD57*, and *RAD59*. As found for the *ARS* plasmid, gap repair of the *CEN/ARS* and nonreplicative plasmids was defective in these mutants when using either chromosomal or plasmid templates (Table 4).

As expected, most of the Ura^+ transformants obtained from the gapped *CEN/ARS* plasmid showed mitotic instability of the plasmid markers (Table 5). In all of the strains, approximately equal numbers of Met^+ and Met^- transformants were recovered. The rare stable Ura^+ Met^- transformants were probably due to conversion of the chromosomal *ura3-1* allele by the plasmid *URA3* marker. More than 99% of the Ura^+ transformants obtained from the gapped nonreplicative plasmid showed mitotic stability indicative of integration of the plasmid (Table 5). Again, approximately equal numbers of Met^+ and Met^- transformants were recovered from the wild-type and *rad59* strains, but there was a significant decrease in the number of Met^+ transformants recovered from the *rad51* and *rad57* mutants. Analysis of repair events between the gapped *ARS* substrate and plasmid donor indicated even fewer crossover events than were observed from the chromosomal donor (data not shown), but these could be the result of secondary recombination events. For this reason, the products of recombination between plasmid substrates and plasmid donors were not analyzed in detail because the backbones of the vectors are homologous and could give aberrant results due to secondary recombination events between direct repeats generated by integration.

DISCUSSION

An assay for plasmid gap repair during yeast transformation was developed to distinguish between homologous recombination events resulting in crossover (integration) or noncrossover products by genetic methods. The assay is based on the repair of a gap within a plasmid-borne *MET17* gene by a chromosomal or plasmid template containing a nonsense mutation near the 3′ end of the *MET17* ORF. A series of plasmids was constructed that contain the selectable/counterselectable marker *URA3* and the colony color marker *MET17* but differ in

TABLE 5. Phenotypes of Ura$^+$ transformants derived from recombination between the gapped *CEN ARS* plasmid or the gapped integrating plasmid and chromosomal *met17-s* donor

Relevant genotype	No. of Ura$^+$ transformants[a]	% of Ura$^+$ transformants[a] that were:					
		Ura^{+u} Met^{+u}	Ura^{+u} Met$^-$	Ura^{+s} Met^{+s}	Ura^{+s} Met$^-$	Ura^{+s} Met^{+u}	Met^{+b}
Gapped *CEN ARS* plasmid							
RAD	1,020	45	55	0	0.4	0	45
rad51	132	42	52	0	5	1	43
rad57	288	55	41	0	4	0	55
rad59	288	60	40	0	0.4	0	60
Gapped integrating plasmid							
RAD	1,153	0	0	51	49	0	51
rad51[c]	45	0	0	16	84	0	16
rad57[c]	173	0	0	31	69	0	31
rad59	288	0	0	50	50	0	50

[a] Number of Ura$^+$ transformants analyzed, the other numbers are given as percentages of the total Ura$^+$.
[b] Percentage of Ura$^+$ transformants that were Met$^+$.
[c] Significantly different from *RAD* as determined by chi-square analysis.

their ability to replicate in yeast. These features can be used to distinguish between crossover and noncrossover modes of gap repair by growth on various media (Fig. 2). The goals in the establishment of this assay were to assess the relationship between gene conversion and crossing over and to determine the basis for *RAD51*-independent mitotic recombination events. Several hypotheses have been put forward to explain *RAD51*-independent events. First, Sugawara et al. (60) suggested that *RAD51*, *RAD54*, and *RAD57* are not directly involved in recombination but instead are required to facilitate access to chromatin templates. Second, Rattray and Symington (45) proposed that *RAD51*, *RAD54*, *RAD55*, and *RAD57* are required for gene conversion but play a less important role in events that can be resolved as crossovers. As described in detail below, our results are inconsistent with either hypothesis. Instead, our results support the idea that strand invasion or strand annealing between repeated sequences can occur in the absence of *RAD51*.

***RAD51* is required for recombination from chromosomal or plasmid donors.** Using the *ARS* plasmid substrate, the gap repair frequency was reduced 98-fold with a chromosomal template and 110-fold with a plasmid template in *rad51* strains (Table 2). Similar reductions were observed in *rad57* mutants, consistent with other studies indicating that these genes function in the same pathway. This pattern was also evident with the *CEN*/*ARS* and integrating vectors (Table 4). Therefore, in this DSB repair system, *RAD51* is required for repair even when the donor locus is expressed and on a plasmid. This contrasts with the results of Sugawara et al. (60), who found efficient repair of DSBs from expressed plasmid donor templates in *rad51* mutants but a requirement for *RAD51* when the donor was chromosomal or transcriptionally silent and on a plasmid. Although in the plasmid gap repair system described here the recipient sequences are in the form of naked transformed DNA, the donors are in the same configuration as those used in the study by Sugawara et al. (60). Thus, donor accessibility is expected to be similar in the two systems. We cannot exclude the possibility that *RAD51* plays an additional role in protection of the broken ends and that this role is more important when the recipient DNA is naked instead of in chromatin.

Most of the *RAD51*-independent recombination events detected in previous studies occurred between plasmid-borne inverted repeats (4, 19, 60). Thus, an alternative explanation to donor accessibility for *RAD51*-independent recombination is that this pathway operates efficiently between closely spaced inverted repeats. Repair of the HO-induced DSB in an inverted-repeat plasmid could occur by one-ended strand invasion to prime DNA synthesis followed by strand annealing (Fig. 4). The two main features of this model, strain invasion to prime DNA synthesis to the end of a DNA duplex and strand annealing, are both known to occur in the absence of *RAD51* (19, 27). The prediction from this model is that *RAD51*-independent recombination of inverted repeats should be reduced in *rad1* or *rad10* strains because the Rad1/10 endonuclease is required to trim intermediates formed during SSA (18). The rate of spontaneous recombination of a chromosomal inverted repeat was reduced synergistically in a *rad1 rad51* double mutant, consistent with this prediction (45). Repair of the HO-induced DSB in one of the plasmid inverted-repeat substrates described by Sugawara et al. (60) was *RAD51* dependent. In that case, the donor DNA was the *HMR* locus, which is assembled in heterochromatin. We would argue that *RAD51*-independent strand invasion, DNA synthesis, or strand annealing is prevented by heterochromatin. Although repair of a chromosomal DSB is generally *RAD51* dependent, Sugawara et al. (60) reported *RAD51*-independent repair when the donor was expressed and on a plasmid. Since the donor plasmid in that particular experiment contained two *MAT* alleles, only one of which was *MAT*α*-inc* (refractory to HO cleavage), repair of the chromosomal break could have occurred by single-strand annealing from the linearized donor plasmid. When a donor plasmid with only the *MAT*α*-inc* allele was used, repair of the chromosomal DSB was *RAD51* dependent (N. Sugawara and J. Haber, personal communication). Although this supports the idea that intermolecular DSBR events require *RAD51*, an alternative explanation is that *RAD51* is not required if the donor sequence is linearized (N. Sugawara and J. Haber, personal communication).

The *RAD51*-dependent pathway is biased toward noncrossover products. Previous studies of spontaneous mitotic recombination with an *ade2* inverted repeat recombination substrate led to the suggestion that the *RAD51* pathway (including *RAD54*, *RAD55*, and *RAD57*) is primarily required for gene conversion. In this study, DSBR events associated or unassociated with crossing over were differentiated in two ways. First, repair of the *ARS* plasmid substrate results in integration (crossover) or the plasmid remains episomal (noncrossover). These two classes can be distinguished by genetic and physical tests. Second, repair of the nonreplicative plasmid can occur only by integration, and repair of the *CEN ARS* plasmid to yield stable transformants can occur only by a noncrossover

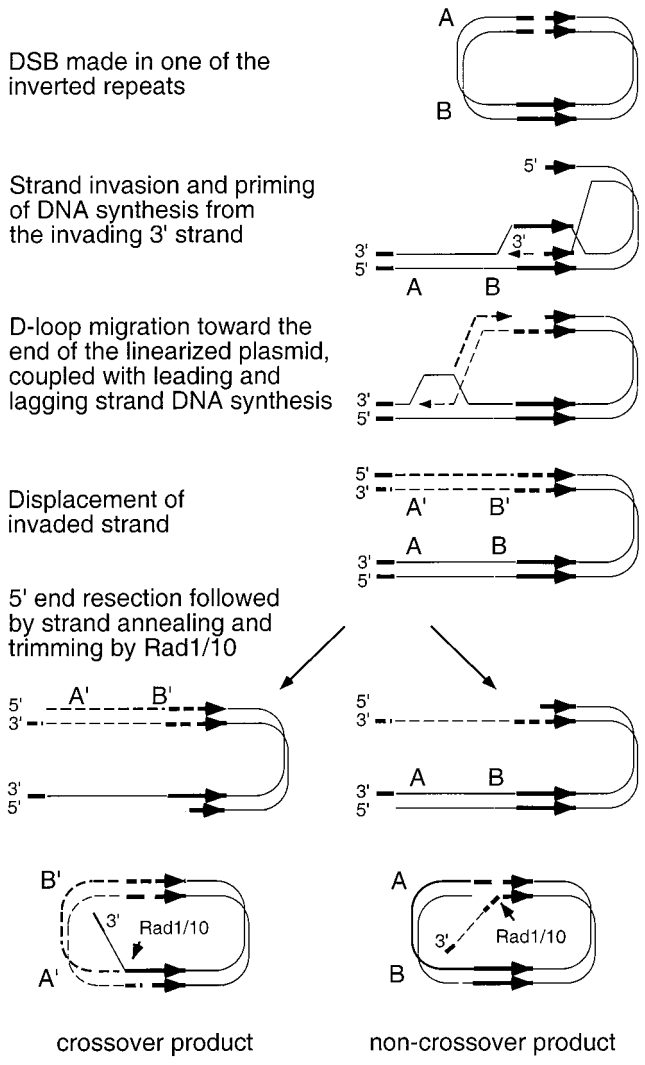

FIG. 4. Model for *RAD51*-independent recombination of inverted repeats. Following introduction of a DSB in one of the repeats, one end is resected to produce a 3′ single-stranded tail that invades the other repeat, possibly through the action of Rad52. DNA synthesis is primed from the invading strand and proceeds to the end of the DNA molecule (the other side of the break). The linear molecule formed contains short sequences corresponding to the 5′ end of the inverted repeat at its ends. If the linear intermediate is degraded by a 5′-3′ exonuclease so that complementary single-stranded regions are revealed, then strand annealing can occur to form two types of products. One has the same structure as a reciprocal exchange, and the other has the same structure as the parental plasmid. The inverted repeats are shown by thick arrows, DNA synthesized during repair is shown by dashed lines, and sequences between the inverted repeats are designated A and B.

mechanism. In wild-type strains, repair of the *ARS* plasmid yielded 76% episomal and 21% integration events (Table 3). While this is different from the 50% integration reported by Orr-Weaver and Szostak (37), it is consistent with recent studies of gap repair in *S. cerevisiae* and in other organisms (12, 35, 39, 44). Studies in *Drosophila* and *U. maydis* have led to the proposal of the synthesis-dependent strand annealing (35) and migrating D-loop (12) models to explain the low incidence of crossing over associated with DSBR. In these models, strand invasion and DNA synthesis primed from the invading 3′ end occur as described for the DSBR model, but instead of forming a Holliday junction intermediate, the invading strand is displaced and can then pair with the single-stranded tail on the other side of the break (Fig. 5). Crossover events can occur if the displacement loop pairs with the other side of the break, or by appropriate strand cleavages such that a Holliday junction is formed. To account for the level of crossing over observed in this study, we suggest that the strand invasion intermediate is converted to a double Holliday structure 40% of the time. The differences in the level of associated crossing over found in different studies could reflect locus-specific effects on the ratio of these intermediates.

Inversion events observed with the *ade2*-inverted repeat can be explained by a G2 conversion model for recombination (Fig. 6) (9). If sister chromatids pair such that one reporter is in the opposite orientation to the other, recombination by gene conversion would generate viable products but a single crossover would create inviable dicentric and acentric products. A sister chromatid gene conversion event initiated within one pair of repeats that terminated within the second pair would create an inversion of the intervening DNA even though no true crossover (by Holliday junction resolution) had occurred. It is possible that most of the inversion events observed in Rad^+ strains with the *ade2* system occur by this mechanism of long conversion tracts, and this class of events may predominate in *rad51* mutants, giving the observed bias in the products recovered (46). Alternatively, inversions might occur by *RAD51*-independent strand annealing of partially replicated sister chromatids during S phase.

Aberrant events in *rad51* mutants. The proportion of repair events associated with crossing over was increased in *rad51* and *rad57* mutants compared to the wild-type strain. Although this difference was not significant for the Met^+ events, the Met^- events showed a significant bias toward integration of the plasmid. Eighty percent of the Ura^+ Met^- transformants that occurred by recombinational repair of the *ARS* plasmid in the *rad51* strain were the result of integration of the plasmid and duplication of the *met17-s* allele. A similar alteration in the ratio of crossover to noncrossover recombinants was observed during plasmid gap repair in *rad51* mutants of *U. maydis* (13). Although crossover events were more frequently recovered when the *ARS* plasmid was used, integration of the nonreplicative plasmid was reduced more than 100-fold in the *rad51* mutant (Table 4), indicating an important role for *RAD51* in reciprocal exchange. The rare crossover events that occur in *rad51* mutants could potentially occur by two sequential break-induced replication events primed from the plasmid ends to duplicate the *met17-s* locus and both chromosome arms (27, 33).

Integration of the gapped *ARS* and nonreplicative plasmids yielded Met^+ and Met^- products. Analysis of the stable Ura^+ Met^- transformants derived from the gapped *ARS* plasmid revealed that most had arisen by duplication of the *met17-s* allele (Fig. 3). These events could reflect exonuclease digestion from the *Eco*NI site beyond the *Sna*BI site, heteroduplex extension of a Holliday junction past the *Sna*BI site, or extensive DNA synthesis primed from the invading 3′ end at the distal side of the gap past the chromosomal *met17-s* mutation. Heteroduplex DNA resulting from any of these events could be repaired to duplicate the *met17-s* allele. In *rad51* and *rad57* strains, there was a significant increase in the number of stable Ura^+ Met^- transformants derived from the *ARS* and integrating gapped plasmids. This is consistent with more extensive degradation of the 5′ end of the DSB site in *rad51* and *rad57* mutants (60). Alternatively, it could reflect a difference in the strand invasion and replication step by the *RAD51*-independent pathway. This bias toward Met^- products was not observed in the *rad* mutants when the *CEN/ARS* plasmid was used. Repair of the gapped *CEN/ARS* plasmid is constrained to occur by a noncrossover mechanism, and this might be mech-

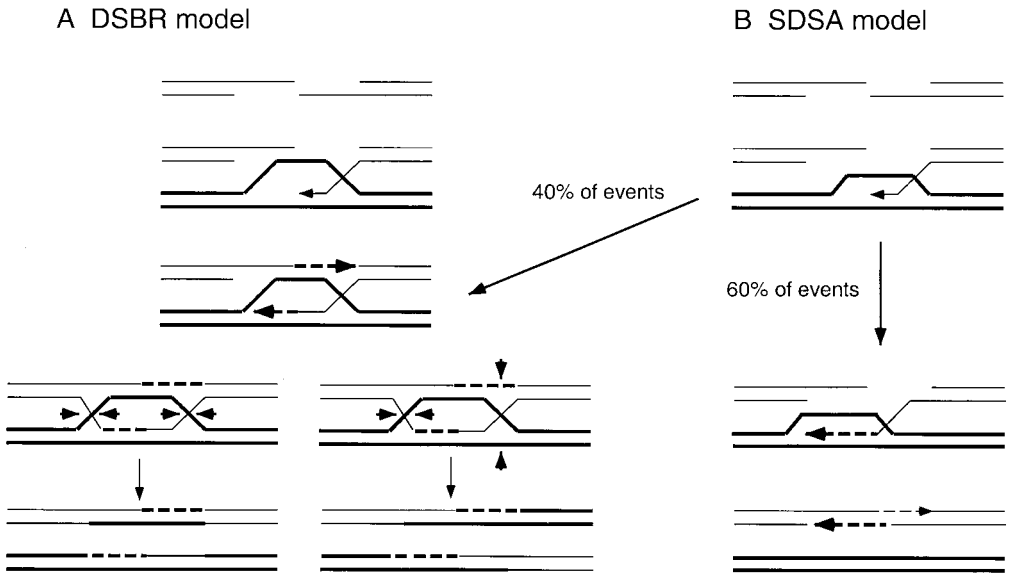

FIG. 5. Models for the recombinational repair of DSBs. (A) The DSBR model. After formation of a DSB, the 5′ ends are resected to form 3′ single-stranded tails. One of the 3′ single-stranded tails invades a homologous duplex and primes DNA synthesis. The displaced strand from the donor duplex pairs with single-stranded DNA at the other side of the break and is the template for DNA synthesis. After ligation, a double Holliday structure is formed and can be resolved to yield noncrossover or crossover products. (B) The synthesis-dependent strand-annealing (SDSA)/migrating D-loop model. The first two steps are the same as those in the DSBR model, but most of the time a double Holliday junction intermediate is not formed. Instead, the invading strand that has been extended by DNA synthesis is displaced from the donor duplex and can anneal to the single-stranded tail on the other side of the break. The resulting gaps are filled by DNA synthesis and ligation to yield a noncrossover product. To account for the crossover products recovered from plasmid gap repair, we propose that 40% of the events form a Holliday junction intermediate and, of these, 50% resolve to generate crossover products. Dashed lines represent newly synthesized DNA.

anistically different from events that result in integration. Conversion to the Holliday junction intermediate (Fig. 5) may involve different processing steps, giving rise to the altered distribution of Met^+ and Met^- products recovered from the *CEN/ARS* and nonreplicative plasmids.

Gap repair is partially dependent on *RAD53*. *RAD53* is classified as a member of the *RAD52* epistasis group but is thought to affect recombination indirectly by transducing signals induced by DNA damage and stalled replication complexes to downstream targets (2, 61, 68). *RAD53* encodes an essential, cell cycle-regulated protein kinase. *rad53-21* is a conditional mutation that confers defects in the replication checkpoint and damage-induced phosphorylation of the Dun1 protein kinase (2). Consistent with a previous study (14), we found a small (two to fourfold) reduction in the efficiency of gap repair in the *rad53-21* strain. However, recombination occurred at much higher frequencies than were observed for the other *rad* mutants, suggesting that phosphorylation of Rad proteins by Rad53/Spk1 is unlikely to play a significant role in recombinational repair.

Plasmid gap repair is dependent on *RAD52*. Repair of the gapped *ARS* plasmid occurred at very low frequency in the *rad52* strain, and no products were obtained that resulted from recombinational repair. This is consistent with other assays for mitotic recombination, which have shown a strong dependence on *RAD52* function, and with our suggestion that *RAD52* is an

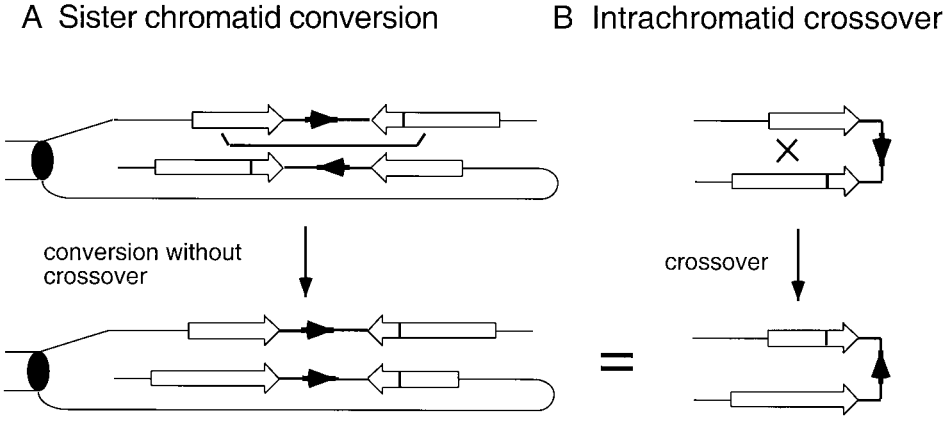

FIG. 6. Models for inversions between *ade2* inverted repeats (9, 46). (A) In G2 cells, the inverted repeats on different chromatids (open arrows) can pair such that the intervening DNA sequences (shown as solid arrows) are in an antiparallel configuration. A gene conversion event initiated within one repeat that extends to the other repeat could result in the inversion of the intervening DNA. (B) A reciprocal crossover between inverted repeats located on the same chromatid inverts the intervening DNA sequences, but the product is indistinguishable from a G2 conversion event. The inverted repeats are shown by open boxes with arrowheads, the short repeat corresponds to a truncation of the 5′ end, and the long repeat contains a point mutation shown by the vertical line.

essential component of *RAD51*-dependent and *RAD51*-independent recombination pathways (3, 4).

***RAD59* is required for *RAD51*-dependent and *RAD51*-independent recombination.** *RAD59* encodes a Rad52 homologue and was identified by its requirement for *RAD51*-independent recombination using an *ade2* inverted-repeat substrate (4). A synergistic decrease in spontaneous recombination of chromosomal inverted repeats was observed in *rad51 rad59* double mutants. In this study, we show a defect in repair of the gapped *ARS* plasmid in *rad59* mutants and a synergistic decrease in *rad51 rad59* double mutants. The observation that 95% of repair events require *RAD59* suggests that Rad59 plays an important role in the *RAD51* pathway, possibly as an accessory factor for Rad52. The synergistic decrease in gap repair efficiency observed in the *rad51 rad59* double mutant is consistent with observations obtained with the *ade2* inverted repeat and indicates an important role for *RAD59* in both spontaneous and DSB-induced recombination. Ninety-nine percent of the repair events involving the gapped *ARS* plasmid require *RAD51*, indicating that most repair events are mediated by the *RAD51* pathway. However, some repair can occur in the absence of *RAD51*, and these residual events require *RAD52* and *RAD59*. This observation supports the hypothesis that Rad52 or the Rad52-Rad59 complex promotes strand invasion in vivo, albeit inefficiently. This type of recombinational repair might be initiated by annealing of transiently single-stranded regions of the substrates. Such events are likely to be favored during replication or by transcriptional activation of sequences in close proximity, such as repeats. The idea that proteins without RecA homology can catalyze strand invasion is not without precedent. The bacteriophage lambda β protein is important for lambda recombination and catalyzes strand annealing and strand exchange in vitro (22, 24). Recent studies have shown that β protein and RecT, a homologue of β protein, can promote *recA*-independent gene replacement in *E. coli*, suggesting a role in strand invasion in vivo (71, 72). β protein forms ring structures on DNA similar to those formed by the yeast and human Rad52 proteins (41, 56, 67). The observation that Rad52 catalyzes single-strand annealing in vivo and in vitro is consistent with the structural analogy to lambda β protein (34, 59). Thus it seems reasonable to propose that Rad52, or the Rad52/Rad59 heterodimer catalyze an alternate pathway for strand invasion in vivo. However, this appears to be inefficient under most circumstances.

In summary, homology-dependent repair of plasmid DSBs from either chromosomal or plasmid donor sequences occurs predominantly by the *RAD51*/*RAD52* pathway. The low levels of repair that occur in the absence of *RAD51* are mechanistically different from repair events generated by the *RAD51* pathway and require *RAD52* and *RAD59*.

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