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A Novel Allele of *Saccharomyces cerevisiae RFA1* That Is Deficient in Recombination and Repair and Suppressible by *RAD52*

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To understand the mechanisms involved in homologous recombination, we have performed a search for *Saccharomyces cerevisiae* mutants unable to carry out plasmid-to-chromosome gene conversion. For this purpose, we have developed a colony color assay in which recombination is induced by the controlled delivery of double-strand breaks (DSBs). Recombination occurs between a chromosomal mutant *ade2* allele and a second plasmid-borne *ade2* allele where DSBs are introduced via the site-specific HO endonuclease. Besides isolating a number of new alleles in known *rad* genes, we identified a novel allele of the *RFA1* gene, *rfa1-44*, which encodes the large subunit of the heterotrimeric yeast single-stranded DNA-binding protein RPA. Characterization of *rfa1-44* revealed that it is, like members of the *RAD52* epistasis group, sensitive to X rays, high doses of UV, and HO-induced DSBs. In addition, *rfa1-44* shows a reduced ability to undergo sporulation and HO-induced gene conversion. The mutation was mapped to a single-base substitution resulting in an aspartate at amino acid residue 77 instead of glycine. Moreover, all radiation sensitivities and repair defects of *rfa1-44* are suppressed by *RAD52* in a dose-dependent manner, and one *RAD52* mutant allele, *rad52-34*, displays nonallelic noncomplementation when crossed with *rfa1-44*. Presented is a model accounting for this genetic interaction in which Rfa1, in a complex with Rad52, serves to assemble other proteins of the recombination-repair machinery at the site of DSBs and other kinds of DNA damage. We believe that our findings and those of J. Smith and R. Rothstein (Mol. Cell. Biol. 15:1632–1641, 1995) are the first in vivo demonstrations of the involvement of a eukaryotic single-stranded binding protein in recombination and repair processes.

Studies of DNA recombination and repair in *Saccharomyces cerevisiae* have identified a large number of genes involved in these related pathways (reviewed in references 26 and 61). Most of the genes were identified by mutations that confer various degrees of sensitivity to UV and ionizing radiation (28, 55, 64). Three major epistasis groups have been identified for DNA repair: nucleotide excision repair (*RAD3*), error-prone repair (*RAD6*), and double-strand break (DSB) repair (*RAD52*) (for reviews, see references 25, 27, and 46).

Genetic analysis has shown that DSBs can be repaired in *S. cerevisiae* by a recombinational mechanism and that this process is impaired by mutations in various genes of the *RAD52* epistasis group (reviewed in reference 61). The finding that DSBs are highly recombinogenic (59, 65) led to the proposal of a general model for homologous recombination that explains the repair of DSBs (84).

To better understand the mechanisms involved in homologous recombination, we sought to isolate mutants of *S. cerevisiae* not on the basis of their radiation sensitivity but because of their impaired ability to carry out recombination between an *ade2* insertion allele maintained on a centromeric plasmid and an *ade2* deletion allele in the chromosome. Spontaneous mitotic nonreciprocal recombination (gene conversion) resulting in repair of either the plasmid or the chromosomal allele occurs with about equal frequency at the two sites but at too low a frequency for use in a simple screen for mutations affecting this process. Insertion of an HO endonuclease target sequence into the plasmid allele's mutant site permits the creation of targeted DSBs by the action of a galactose-inducible *HO* endonuclease gene (41, 57, 58, 69). In this format, the recombination frequency is markedly increased, permitting ready detection of mutants that are unable to perform the recombinational repair of the plasmid's broken *ade2* allele.

This screen led to the isolation of a large number of mutants variously affected in their abilities to perform DSB-induced recombination. Genetic analysis and measurements of their UV and X-ray sensitivities identified many of the mutants as new alleles of genes belonging to the *RAD3* and *RAD52* epistasis groups. One radiation-sensitive, recombination-deficient mutant showed an unusual complementation pattern. Although it was fully complemented when crossed with the parental strain and with many well-characterized *rad* mutants, that mutant was only partially complemented when crossed with certain *rad52* alleles. Moreover, when provided on a plasmid, wild-type *RAD52* suppressed the various phenotypes of that mutant in a dose-dependent manner. The mutation producing this phenotype was localized to a single base pair in the gene encoding the 69-kDa subunit (*RFA1*) of the heterotrimeric yeast single-stranded DNA-binding protein named RPA (10, 34). (Although the gene encoding the 69-kDa subunit of yeast RPA was assigned the designation *rpa1* [34], the use of that same three-letter designation for genes encoding RNA polymerase subunits [48, 85] and for an acidic ribosomal protein [52] engenders some confusion. We have, therefore, used the designations *rfa1* and *RFA1* for the mutant and wild-type alleles, respectively, as in reference 10. Accordingly, the designation *rfa1-44* refers to the mutant described herein. RPA, however, remains the consensus designation for the heterotrimeric protein complex.) The structure of the yeast heterotrimeric RPA (whose subunits have molecular masses of 69, 36, and 13 kDa) has been conserved in all eukaryotes (1, 10, 21).

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TABLE 1. Yeast strains used in this study[a]

Strain	Source (reference)	Genotype
JM116	John McCusker (75)	*MAT***a** *ura3-52 leu2-Δ1*
YME2	This study	*MAT***a** *ade2-Δ1 his3-Δ187 leu2-Δ1 ura3-52*
YME4	This study	*MAT*α, isogenic to YME2
YME5	This study	*MAT***a** *rad52*::*LEU2*, isogenic to YME2
YME6	This study	*MAT*α *rad52*::*LEU2*, isogenic to YME2
YME7	This study	*MAT***a** *rad1*::*URA3*, isogenic to YME2
YME8	This study	*MAT*α *rad1*::*URA3*, isogenic to YME2
YAF5	This study	*MAT*α *rad52-34*, isogenic to YME2
YAF5-**a**	This study	*MAT***a** *rad52-34*, isogenic to YME2
YAF21	This study	*MAT***a** *rad51-36*, isogenic to YME2
YAF22	This study	*MAT*α *rad52-38*, isogenic to YME2
YAF22-**a**	This study	*MAT***a** *rad52-38*, isogenic to YME2
YAF23	This study	*MAT***a** *rad54-34*, isogenic to YME2
YAF37	This study	*MAT***a** *rad55-37*, isogenic to YME2
YAF44	This study	*MAT*α *rfa1-44*, isogenic to YME2
YAF44-**a**	This study	*MAT***a** *rfa1-44*, isogenic to YME2
YAF56	This study	*MAT***a** *rad57-38*, isogenic to YME2
YAF70[b]	This study	*MAT***a** *RFA1-URA3-RFA1*, isogenic to YME2
YAF71[c]	This study	*MAT***a** *RFA1-URA3-RFA1*, isogenic to YME2

[a] All strains are heterothallic.
[b] Duplication of *RFA1* with pAF102 (i.e., integration at the *Mlu*I site; see Materials and Methods).
[c] Duplication of *RFA1* with pAF104 (i.e., integration at the *Eco*RI site; see Materials and Methods).

In this paper, we characterize the mutant *rfa1-44* with respect to its recombination deficiency, its radiation sensitivity, its reduced sporulation, and its unusual dose-dependent suppression by *RAD52*. We suggest that *RFA1* is an essential participant in DSB repair and recombination, perhaps by binding to single-stranded regions created at the site of DSBs and promoting the assembly of recombination-repair complexes through its genetic interaction with *RAD52*.

MATERIALS AND METHODS

Yeast strains. The designations, origins, and genotypes of the strains used in this paper are listed in Table 1. The parental strain from which the mutants were derived is YME2, itself a derivative of JM116 and S288C (75). YME2 has, in addition, a deletion of 219 bp between the *Hpa*I and *Stu*I restriction sites in the *ade2* gene and a 187-bp deletion between adjacent *Hin*dIII sites in the *his3* gene. YME4 is the isogenic *MAT*α strain. YME5 and YME6 are the isogenic *MAT***a** and *MAT*α *rad52*::*LEU2* disrupted strains, respectively, and YME7 and YME8 are the isogenic *MAT***a** and *MAT*α *rad1*::*URA3* disrupted strains, respectively. YAF70 and YAF71, each of which has a duplication of the *RFA1* locus, were obtained by integrative transformation of YME2 with *Mlu*I-linearized pAF102 and *Eco*RI-linearized pAF104, respectively (see below for the construction of pAF102 and pAF104); in each strain, the *RFA1* duplication flanks the *URA3* gene. Haploid strains carrying disruptions in known *rad* genes were obtained either from Rodney Rothstein's laboratory (Columbia University) or from the Yeast Genetic Stock Center (Berkeley, Calif.).

Plasmid constructions. The plasmids used in this study are listed in Table 2, and their construction and use are summarized here. Standard cloning techniques were used (70). The *ade2*::*Y/Z* allele was constructed as follows. First, a 3.58-kbp partial *Bgl*II genomic *ADE2* fragment with *Bam*HI adapters (a gift from Christine Alfano) was cloned into the *Bam*HI site of pUN50 (18) to yield pUN50-*ADE2*. A *Bst*XI-*Not*I-*Xho*I 25-bp linker fragment (the plus-strand oligonucleotide, 5′-ATGGGCGGCCGCTCGAGCCACGTTA-3′, annealed to the minus-strand oligonucleotide, 5′-GTGGCTCGAGCGGCCGCCCATTAAC-3′) was inserted into the unique *Bst*XI site at position +148 of the *ADE2* coding region to yield pUN50-*ade2-25*. Next, the 135-bp fragment (*Hin*cII to *Hpa*II) containing the *HO* recognition site (*Y/Z* region) from the *MAT*α locus was isolated, and its ends were blunted and ligated to *Xho*I linkers and then inserted into the *Xho*I site of the *ade2-25* allele, yielding pUN50-*ade2-25*::*Y/Z*, also referred to as pME119. A plasmid (YCp-*his3*::*Y/Z*, also referred to as pME137) containing a *his3*::*Y/Z* allele was constructed in a similar way: the 145-bp *Xho*I fragment carrying the *HO* recognition site (from pME119) was cloned 58 bp into the *HIS3* coding region.

To construct the HO endonuclease expression vectors, a 2.7-kbp *Eco*RI-*Hin*dIII fragment containing the *HO* endonuclease gene under the control of the *GAL10* promoter (37, 57) was cloned into YCp*lac*111 (29), yielding YCp-*GAL-HO*. YCp-*GAL-HO* was cleaved at the *Hin*dIII site, and the ends were blunted and ligated to *Eco*RI linkers and then cloned into the *Eco*RI site of pUN50, yielding pUN50-*GAL-HO*. The same *Eco*RI fragment was inserted into

TABLE 2. Plasmids used in this study

Plasmid	Alternate name	Description and/or use
pAF30		*URA3* plasmid used for the sectoring assay (Fig. 1)
pAF35		*LEU2* plasmid used for the sectoring assay
pME119	pUN50-*ade2-25*::*Y/Z*	pAF30 without the *HO* gene under *GAL* control
pME137	YCp-*his3*::*Y/Z*	*LEU2* plasmid containing the *his3*::*Y/Z* gene (Fig. 1)
pUN50-*ADE2*		*URA3* plasmid used for detecting and screening mutations in the *ade1*, *ade12*, and *ade13* genes
pUN50-*GAL-HO*	p*GAL-HO.Ura*	*URA3* plasmid used for measuring repair of DSBs at the *MAT* locus
YCp-*GAL-HO*	p*GAL-HO.Leu*	Same as above, but with a *LEU2* marker
pJM125	pRS413-*RFA1*	*HIS3* plasmid (10) used for cross-complementation of mutant 44
pRFA1 CEN	λYES#3	*URA3* library plasmid (19) complementing mutant 44 and containing a genomic fragment spanning the full *RFA1* locus
pRFA1 2μm	pAF105 or pAF106, also pRfa1-G77 2μm	Wild-type *RFA1* sequence cloned into either YEp*lac*181 (pAF105) (29) or YEp*lac*195 (pAF106) (29)
pRfa1-G77 CEN	pAF131	*RFA1* wild-type allele cloned into LEU2 plasmid pRS315 (78)
pRfa1-D77 CEN	pAF120	*rfa1-44* mutant allele cloned into pRS315
pRfa1-D77 2μm	pAF128	*rfa1-44* mutant allele cloned into YEp*lac*181
pRfa1-D77G CEN	pAF130	Wild-type domain (*Spe*I fragment from pAF131) swapped into the mutant vector pAF120
pRfa1-G77D CEN	pAF132	Mutant domain (*Spe*I fragment from pAF120) swapped into the wild-type vector pAF131
pAF102	YIp-*RFA1*(λYES#3)	Integrative *RFA1-URA3* plasmid used to construct YAF70; uses an *RFA1* fragment isolated in this study
pAF104	YIp-*RFA1*(JM125)	Integrative *RFA1-URA3* plasmid used to construct YAF71; uses an *RFA1* fragment from pJM125 (10)
YEp13-*RAD52*		Original *LEU2-RAD52* plasmid (73) used in the subcloning of *RAD52* (see below)
pRAD52 CEN	pAF50 or pAF52	*URA3* and *LEU2* plasmids with *Eco*RI-*Sal*I *RAD52* fragment from YEp13-*RAD52* cloned into YCp*lac*33 (pAF50) and YCp*lac*111 (pAF52), respectively
pRAD52 2μm	pAF51	*URA3* plasmid with *Eco*RI-*Sal*I *RAD52* fragment from YEp13-*RAD52* cloned into YEp*lac*195 (pAF50)

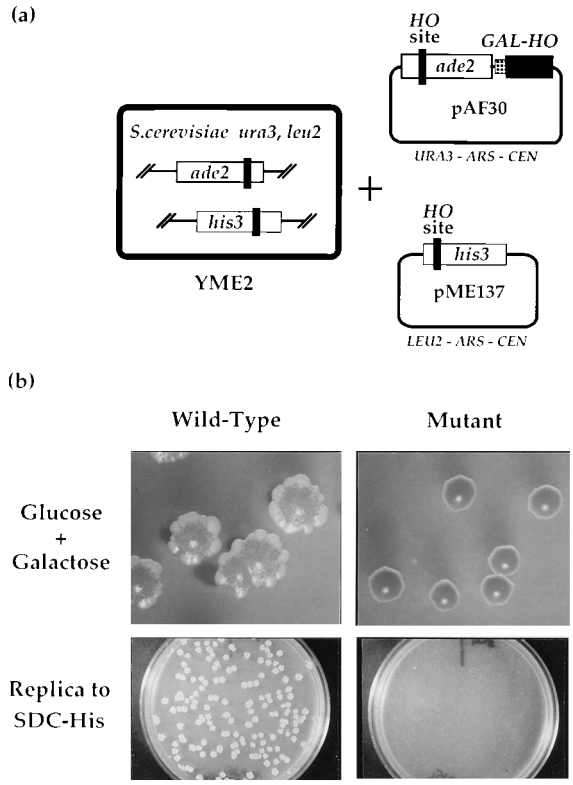

FIG. 1. Screen used to identify mutants affected in HO-induced plasmid to chromosome recombination. (a) Parental cells and plasmids. Wild-type YME2 cells, transformed with both pAF30 and pME137 plasmids, are grown on glucose plus galactose induction plates. (b) Papillation phenotypes of wild-type and representative mutant colonies after 5 days on induction medium, and the corresponding recombination phenotypes after replica plating to SDC−His medium.

pUN50-*ade2-25*::*Y*/*Z* (pME119) that was linearized at the *Eco*RI site to yield pAF30 (Fig. 1a). pAF35, which contains a *LEU2* marker instead of the *URA3* marker of pAF30, was made by swapping the plasmid backbones flanked by *Bgl*I sites between pAF30 and pRS315 (78).

pRAD52 CEN (pAF50) and pRAD52 2μm (pAF51) were constructed by cloning the 3.2-kbp *Eco*RI-*Sal*I fragment from YEp13-*RAD52* (73) into YCp*lac*33 and YEp*lac*195 (29) (Table 2). In an analogous way, vectors with complementing fragments from *RAD51*, *RAD54*, *RAD55*, and *RAD57* were constructed. A more complete description of their exact constructions and the plasmids themselves are available upon request.

An *S. cerevisiae* genomic DNA library (λYES), obtained from Sandy Ramer (19), was used to complement mutant 44's recombination deficiency by screening colonies for restoration of the papillation phenotype. The approximately 3.8-kbp *Xho*I genomic insert isolated from complementing clone 3 (which spans the entire *RFA1* locus) was subcloned into pRS306 (78), resulting in the integrative plasmid pAF102. Similarly, the 2.6-kbp *Bam*HI-*Hin*dIII fragment from pJM125 (pRS413-*RFA1*, obtained from Bruce Stillman [10]) was also subcloned into pRS306, resulting in pAF104. These two integrative plasmids were used to construct YAF70 and YAF71, respectively (see "Yeast strains" above).

Reagents, media, and growth conditions. Restriction enzymes were from New England Biolabs (Beverly, Mass.), and reagents were from Sigma Chemical Co. (St. Louis, Mo.), unless stated otherwise. *S. cerevisiae* was grown by standard techniques (75). Yeast-peptone-dextrose (YPD) plates were routinely supplemented with 40 μg of adenine per ml (such plates are referred to as YPAD plates) for spore germination and survival assays. To monitor papillation, cells were plated on minimal medium supplemented with 0.5% glucose, 1% galactose, 10 μg of adenine per ml, and 20 μg of histidine per ml; these plates are referred to as induction plates or induction medium.

Transformation procedures. Yeast transformation was achieved either by electroporation (4) or with polyethylene glycol and lithium acetate (72). When large numbers (≥10) of transformations were performed, a modification of the polyethylene glycol-lithium acetate procedure, with a microplate format, was used (24).

Genetic screen for recombination-deficient mutants. When YME2 cells carrying pAF30 are plated on medium containing 0.5% glucose and grown for 5 days at 30°C, they form uniformly red colonies. However, if the medium contains 1% galactose as well, the colonies develop a large number (≥8 per colony) of white papillae growing out of the red colony (Fig. 1b). This papillation phenotype, which results from recombinational repair of the ruptured plasmid-borne *ade2* gene, was used for the initial screen to identify mutants that are unable to perform this repair process.

YME2 cells carrying the recombination system (both pAF30 and pME137 plasmids [Fig. 1a]) were mutagenized by exposure to either ethyl methanesulfonate to about 50% survival, or UV irradiation to about 20% survival (30). A total of 309,000 ethyl methanesulfonate-treated colonies and 50,000 UV-irradiated colonies were screened. Colonies that failed to produce white papillae were picked for further study. Cells grown on induction medium were also replica plated on medium lacking histidine (SDC-His). Those that were unable to promote the formation of *HIS3* recombinants were scored as putative recombination-defective mutants (Fig. 1b). To eliminate mutations in the plasmids as the source of their apparent recombination deficiency phenotype, candidate mutants were tested for their abilities to produce white papillae or to produce *HIS3* recombinants after retransformation with unmutagenized pAF30 and pME137 plasmids. They were also shown to be able to grow on galactose as the sole carbon source in the absence of the plasmid carrying the *HO* gene. False positives due to novel mutant alleles of *ade1*, *ade12*, and *ade13* which passed the above-mentioned screens were eliminated on the basis of their inabilities to grow in the absence of adenine after transformation with a plasmid containing a functional *ADE2* gene (pUN50-*ADE2*). The remaining mutants were then tested for their sensitivities to X rays and UV by the qualitative spot test described below.

Complementation analysis and sporulation efficiency. Complementation analysis was carried out by crossing the putative mutants to one another and to a set of known *rad* mutants obtained from Rodney Rothstein or from the Yeast Genetic Stock Center. A description of the complete set of mutants isolated in this study and their characteristics will appear elsewhere. For sporulation and tetrad analysis (76), diploids were grown to mid-log phase under selective conditions, streaked in patches on YPAD, and grown at 30°C for 22 to 36 h. The cells were then replica plated from the YPAD plates onto sporulation plates (2% Bacto Agar, 2% potassium acetate, 0.22% yeast extract, 0.05% glucose, with all the necessary amino acid and base supplements). After incubation at 25°C for 7 days, the sporulation efficiency was determined by counting 1,000 to 5,000 cells on a glass slide under an Axiophot phase microscope (Carl Zeiss) and determining the number of tetrads. Tetrad analysis was carried out as described elsewhere (76). Briefly, a small loop of cells was treated with 10 μg of Zymolyase (ICN) in 100 μl of SP buffer (1 M sorbitol in sterile phosphate-buffered saline) for 5 min prior to being spread on the central section of a YPAD germination and dissection plate. Tetrads (usually 20 to 64 tetrads from each mating) were dissected, and the number of individual spores growing in the course of multiple (two to four) independent sporulation experiments was used as a measure of survival after germination.

Quantitative assays of X-ray and UV survival. Cells were grown to mid-log phase prior to sonication for 90 s in a Branson 1200 water bath sonicator to disrupt cell aggregates. Cell density was estimated by A_{560} measurements with a v_{max} kinetic reader (Molecular Devices). Appropriate volumes from the same dilutions in water were plated in duplicate or triplicate on YPAD plates and immediately irradiated with either a Stratalinker UV Crosslinker (Stratagene) or a Mark I model 23 irradiator (J. L. Sheperd and Associates) for X-ray irradiations. The dose rate from the ^{137}Cs X-ray source was 1,193 rads per min. Plates were incubated at 30°C for 2 to 3 days prior to being counted. Unirradiated control plates with the appropriate dilutions were included in every experiment as a reference.

Qualitative spot assay for UV and X-ray sensitivities and for gene conversion. Cells were grown overnight to mid-log phase and sonicated for 90 s. Of each culture, 100 μl was transferred to a microplate, and cell concentrations were deduced from their A_{560}. Dilutions were made so that the cell density of individual cultures fell within a ≤twofold range, and approximately 5×10^5 to 9×10^5 cells were spotted in series with an EDP Plus Motorized Microliter Pipette (Rainin) on 10-cm-diameter plates in a predetermined array. Immediately after the cells were spotted on YPAD plates, they were irradiated with UV or X rays. Gene conversion was tested by first growing the spots at 30°C for 5 days on induction plates and then replica plating the spots to minimal medium lacking either adenine (SDC-Ade) or histidine (SDC-His) (75) and incubating these plates for 2 additional days to determine the frequencies of *ADE2* and *HIS3* recombinants.

Determination of HO-mediated gene conversion levels. To measure the rates of gene conversion quantitatively, parental and mutant strains carrying the pAF30 or pAF35 plasmids were grown for 5 days on induction medium. About 40 individual colonies were pooled in about 2 ml of SP buffer, and after resuspension by vigorous vortexing and sonication, dilutions were made in 10 ml of sterile water. Appropriate volumes from the same dilution were plated as two to four replicates either on SDC−Ade plates (to monitor conversion at the *ade2* locus) or on YPAD (to count the total number of cells). Conversion frequencies usually fell within 15% of one another on replicate plates.

Determination of the abilities of strains to repair HO-induced DSBs. Parental and mutant strains carrying plasmids expressing the HO endonuclease under

galactose control (either pUN50-*GAL-HO*/YCp-*GAL-HO* or pAF30/pAF35) were grown to mid-log phase under selective conditions in glucose and sonicated for 90 s, and appropriate volumes from the same dilutions were plated either as two to four replicates on minimal medium containing 2% galactose lacking uracil (SGC−Ura) or leucine (SGC−Leu) or on replicate YPAD plates (to count the total number of cells). After incubation for 3 to 4 days at 30°C, the number of cells surviving the induced level of HO endonuclease expression was determined. For survival of DSBs inflicted at the chromosomal *MAT* locus only, pUN50-*GAL-HO* or YCp-*GAL-HO* (both lack an *HO* site on the plasmid) was used. pAF30 or pAF35 (both contain an *HO* site [Fig. 1a]) was used to monitor survival after the creation of DSBs at both the chromosomal and the plasmid-borne *HO* sites.

Cloning and sequencing of the *rfa1-44* mutant allele. The *rfa1-44* mutant allele was cloned directly from the genomic DNAs of two independent X-ray-sensitive haploid segregants derived from a cross between YAF44 and YME2. A total of 50 μg of DNA from each segregant was digested to completion with *Pst*I and *Hin*dIII, two restriction enzymes which are known to produce a 2.55-kbp fragment carrying the entire *RFA1* gene. Fragments ranging from 2.35 to 2.75 kbp were isolated by electrophoresis and digested further with another five restriction enzymes (*Apa*I, *Bgl*II, *Sph*I, *Xba*I, and *Xho*I) expected not to cut in the *rfa1* *Pst*I-*Hin*dIII fragment. The resulting fragments were ligated to *Pst*I-*Hin*dIII-purified Bluescript vector (pKS+; Stratagene) and transformed into DH5α bacteria. Colonies containing the expected 2.55-kbp fragment were identified by colony lift, essentially as described elsewhere (70). Positive colonies were detected by hybridization with a *Pst*I-*Hin*dIII fragment isolated from pRFA1 CEN and labeled with random primers (Prime-It kit; Stratagene). Two clones, one derived from each segregant, were picked for further analysis. Convenient restriction sites located within the *Pst*I-*Hin*dIII fragment were used to generate a series of overlapping subclones so that the entire 2.55-kbp region of both isolates was sequenced (PAN Facility, Beckman Center for Molecular and Genetic Medicine, Stanford, Calif.) by *Taq* dye-primer cycle sequencing (ABI).

Domain swaps between wild-type and mutant forms of *RFA1*. To construct the plasmids used for swapping domains between *rfa1-44* and *RFA1*, the 2.55-kbp *Pst*I-*Hin*dIII fragment from pRFA1 CEN (λYES#3) was subcloned into pRS315 (78) to yield pRfa1-G77 CEN; this plasmid expresses the wild-type Rfa1 protein which has a glycine at position 77. The same wild-type fragment was subcloned into the 2μm vector YEp*lac*181 (29) to yield pRfa1-G77 2μm (pAF105), also referred to as pRFA1 2μm. The 2.55-kbp *Pst*I-*Hin*dIII fragment recovered from the mutant *rfa1-44* strain was also subcloned in pRS315 and YEp*lac*181 to create pRfa1-D77 CEN (pAF120) and pRfa1-D77 2μm (pAF128), respectively; these vectors express the mutant form of the Rfa1 protein with an aspartate at position 77. The *Spe*I fragment from pRfa1-G77 CEN, carrying the first 115 amino acids of wild-type Rfa1, was then replaced with the *Spe*I fragment from pRfa1-D77 CEN, yielding pRfa1-G77D CEN. The reciprocal swap was carried out to construct pRfa1-D77G CEN, a vector which contains the wild-type *Spe*I fragment coding for a glycine at position 77 in place of the mutant fragment.

RESULTS

The screen used to detect mutants that are unable to carry out recombinational repair of double-strand breaks relies on the property of *ade2* mutants to produce uniformly red colonies when grown with limiting amounts of adenine (≤15 μg/ml), while *ADE2* colonies are uniformly white in the same media (23). Accordingly, we constructed a strain (YME2) which contains a chromosomal *ade2* allele with a deletion of 219 bp in the 3′-proximal part of its coding sequence. By introducing a *CEN-ARS* plasmid-borne *ade2* allele containing an insertion of 25 bp in the 5′ region of the coding sequence (*ade2-25*), we presumed that recombination between the two *ade2* alleles would yield *ADE2* cells that could be scored by the appearance of white sectors or papillae emerging from the red colonies. However, no papillation was evident during the growth of such cells, even though uniformly red colonies contained the expected *ADE2* recombinants as judged by replica plating to media lacking adenine (unpublished observations). Since DSBs are known to be recombinogenic (59, 65), the plasmid was modified both by the introduction of the *HO* endonuclease cleavage site at the *ade2-25* allele's insertion and by the addition on the same plasmid of the gene encoding the HO endonuclease under the control of the inducible *GAL10* promoter (37, 57) (pAF30 [Fig. 1a]). YME2 containing pAF30 produces uniformly red colonies when grown on glucose with limiting adenine. When grown on media containing both glucose (0.5%) and galactose (1%) with limiting adenine (10 μg/ml), a medium referred to henceforth as induction medium, YME2 containing pAF30 produces readily recognizable papillated colonies (Fig. 1b). Cells that are impaired in their capacities to repair the HO endonuclease-induced break in the plasmid *ade2* allele yield colonies that are either all red or only infrequently papillated (Fig. 1b).

Because YME2 also contained a disruption in the 3′ end of the chromosomal *his3* gene, putative mutants deficient in gene conversion could be screened further for failure to recombine at a second locus. In this test, the parental cells contained a plasmid (pME137) with a *his3* allele that can also be linearized at an *HO* cleavage site located at the 5′ end of the gene (Fig. 1a). Following growth on induction medium, recombination-proficient cells segregate progeny that can grow in the absence of histidine while recombination-deficient cells yield no or markedly fewer *HIS3* recombinants, as monitored by replica plating on SDC-His (Fig. 1b). Therefore, the phenotype we sought following mutagenesis was colonies that fail to produce white papillae after plating on induction medium and also fail to grow on media lacking histidine after induction of the HO endonuclease.

The key to the screening assay for recombination proficiency is the use of glucose plus galactose in the induction medium. In fact, when grown on galactose alone, the survival of recombination-proficient YME2 with pAF30 or pAF35 plasmids that are selected for is only about 10 to 20%. Recombination-deficient cells are several orders of magnitude less viable in the same medium (see below). When the medium contains glucose (0.5%) in addition to galactose (1%), all cells, whether recombination proficient or deficient, grow to visible colonies by preferential utilization of the glucose; under these conditions, the *GAL10* promoter is repressed and the HO endonuclease is not produced. As glucose is consumed and cells switch to galactose for growth, the HO endonuclease is induced, causing DSBs within the plasmid-borne *ade2* and *his3* alleles. Recombination-proficient cells restore the wild-type sequence in the broken, plasmid-borne genes and continue to grow as white papillae. Cells that cannot repair the disrupted plasmid alleles by recombination or that do so only by end-to-end joining retain the *ade2* phenotype, i.e., they produce only uniformly red colonies. Thus, the presence of glucose permits the recovery of cells that would otherwise be lost if grown in galactose alone.

Nature of the recovered mutants. Seventy-eight isolates which had diminished capacities in both the *ade2* and the *his3* recombination assays were obtained. A number of secondary screens (see Materials and Methods) had already eliminated mutations that either blocked the synthesis or utilization of adenine or prevented the induction of the HO endonuclease by galactose.

Because many of the gene products required for the repair of UV and X-ray damage are also involved in recombination (26, 61) and our assay relies on DSBs to promote recombination, each of the 78 isolates was tested for sensitivity to UV and X rays. Of the 78 mutants, 5 were strikingly more sensitive to UV mainly, 63 were overly sensitive to both kinds of damage, and 10 were only marginally sensitive to UV and X rays. Accordingly, all the mutants were tested to determine if their mutations affected genes that had previously been identified because of their radiation sensitivity. A more complete characterization of these mutants will be described elsewhere, but a brief summary of these results suffices here.

Each mutant was crossed to a set of strains bearing mutations in known *rad* genes, and the resulting diploids were tested for their abilities to restore both the recombination defect and the normal resistance to UV and X rays. Additionally, each of

TABLE 3. Complementation analysis of mutant 44

Cells	Recombination defect[a]	UV sensitivity[b]	X-ray sensitivity[b]
Haploid mutant 44	+++	+++	+++
Mutant 44 × YME2	0	0	0
Mutant 44 × 60 mutants or tester strains[c]	0	0	0
Mutant 44 × YME6 (*rad52*::*LEU2*)	0	0	0
Mutant 44 × *rad52-34*[d]	++	++	++
Mutant 44 × *rad52-38*[d]	0	0	0
Mutant 44 + pRAD52 CEN	++	++	++
Mutant 44 + pRAD52 2μm	+	0 to +	0 to +

[a] The extent of the recombination defect was estimated by replica plating patches of cells grown on induction medium for 5 days onto SDC−adenine medium. The extent of the defect is indicated by 0, +, ++, and +++ in the order of increasing severity.

[b] Measured by spot assay for survival after a UV dose of 150 J/m^2 or an X-ray dose of 30 to 60 kilorads. +++, almost no surviving cells per spot; ++ and +, decreasing sensitivity; 0, wild-type behavior.

[c] Complementation in diploids formed with 60 of the mutants from this study or with tester strains containing known *rad* mutations.

[d] The *rad52-34* and *rad52-38* alleles are two *rad52* mutants isolated during this study.

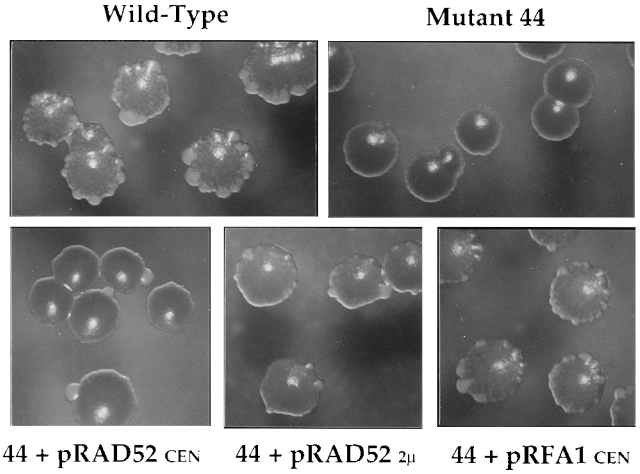

FIG. 2. Rescue of mutant 44's papillation phenotype by *RAD52* in a dose-dependent fashion and by its wild-type counterpart, *RFA1*. All strains contain the pAF35 (LEU2) induction plasmid. Panels: Wild-Type, parental YME2 cells with YCp*lac*33 control plasmid (29); Mutant 44, mutant 44 with YCp*lac*33; 44 + pRAD52 CEN, mutant 44 with the *RAD52* gene on a *URA3* centromeric plasmid (Table 2); 44 + pRAD52 2μ, mutant 44 with the *RAD52* gene on a *URA3* 2μm plasmid (Table 2); 44 + pRFA1 CEN, mutant 44 with plasmid carrying a complementing genomic fragment spanning the *RFA1* locus (Table 2).

the haploid mutants was transformed with plasmids bearing a particular wild-type *RAD* gene and then scored for its recombination proficiency and radiation sensitivity. Mutations generated in this study were then assigned to a particular *rad* gene on the basis of the genetic complementation test and the ability of that *RAD* gene to restore both normal recombination capacity and wild-type resistance to UV and X-irradiation. Most of the mutants were classified into the following complementation groups: *rad1*, *rad10*, *rad51*, *rad52*, *rad54*, *rad55*, and *rad57*. One group of 10 mutants, composing at least three complementation groups, showed marginal sensitivity to UV and X rays; none of these mutants, which are also slightly decreased in their HO-induced recombination, was complemented by the aforementioned genes. Last, one recombination-deficient, radiation-sensitive mutant, mutant 44, could not be related to any of the known *rad* genes, and its characterization is the subject of this paper.

Distinctiveness of mutant 44. The restoration of wild-type levels of recombination and sensitivity to UV and X-irradiation in diploids formed by the mating of mutant 44 to a representative group of 60 other mutants obtained in our screen as well as to each of the tester strains bearing known *rad* mutations suggested that mutant 44 contained a heretofore unrecognized defect in recombinational repair of DSBs (Table 3). When mutant 44 was backcrossed to the parental strain (YME2), the resulting diploid was wild type with respect to recombination competence and sensitivity to UV and X rays (Table 3), indicating that the mutation is recessive. After sporulation of these diploids, tetrads yielded two spores that were as recombination deficient and UV and X-ray sensitive as mutant 44 and two spores with corresponding wild-type properties. Perfect cosegregation of these three phenotypes indicates that they are likely to result from a mutation affecting a single gene.

In the course of this complementation analysis, we noticed that mutant 44 showed less than complete levels of complementation when crossed with certain *rad52* alleles (Table 3). In addition, when a wild-type *RAD52* gene was introduced into mutant 44 with a low-copy-number plasmid (pRAD52 CEN), the UV and X-ray sensitivities and the defective gene conversion were partially complemented (Table 3 and Fig. 2). Moreover, when wild-type *RAD52* was introduced on a multicopy plasmid (pRAD52 2μm), it provided near-complete complementation of mutant 44's deficiencies (Table 3 and Fig. 2).

To eliminate the possibility that mutant 44 was itself a partially dominant allele of *rad52*, mutant 44 was crossed to YME6, the parental strain carrying a disrupted *rad52* allele (Table 1). Sporulation of the diploids followed by dissection of the resulting tetrads (which were formed at a reduced efficiency) revealed a segregation pattern of one radiation-resistant spore to three radiation-sensitive spores in about 30% of the cases; this indicates unambiguously that the mutation affecting mutant 44 is not allelic to *RAD52*.

Deficiency of mutant 44 in HO-mediated gene conversion. The HO-mediated gene conversion deficiency displayed by mutant 44 and by characteristic *rad* strains derived from our screen was quantified by the papillation assay (see Materials and Methods) (Table 4). The deficiency displayed by mutant 44, a reduction of about 390-fold compared with that of the wild type, is less than that displayed either by the isogenic *rad52*::*LEU2* strain or by presumably missense alleles of *rad51*, *rad52*, and *rad54* (all of which are 1,000-fold or more reduced with respect to the wild type). However, mutant 44 is about as defective in HO-induced recombination as the *rad55* or *rad57* strains isolated in our screen.

The data in Table 4 substantiate the qualitative conclusions indicated earlier (Fig. 2): mutant 44's recombination defect is partially overcome by the presence of a wild-type *RAD52* gene on a low-copy-number plasmid (21- versus 390-fold) and the defect is virtually completely overcome when the *RAD52* gene is present on a multicopy 2μm plasmid. It should be noted that the disrupted *rad52* allele in YME5 and YME6 is fully complemented by pRAD52 CEN. And, as we shall discuss later, the wild-type counterpart of the mutant 44 allele, in the same centromeric plasmid (pRFA1 CEN), fully reverses mutant 44's recombination defect.

Radiation sensitivity of mutant 44. Figure 3a shows the comparative X-ray sensitivity of mutant 44 relative to YME2, its wild-type counterpart, and to YME5, whose *rad52* gene is

TABLE 4. Deficiency in HO-mediated gene conversion of strains used in this study

Mutant (strain) and plasmid	Gene conversion to ADE2^{+a}	
	Relative level	Decrease (fold) from wild-type level
RAD$^+$ (YME2)	1[b]	
rad1::*URA3* (YME7)	1.9×10^{-2}	53
rad52::*LEU2* (YME5)	$<2.2 \times 10^{-4}$[c]	>4,480[c]
rad51-36 (YAF21)	$<8.9 \times 10^{-4}$[c]	>1,123[c]
rad52-34 (YAF5)	$<6.0 \times 10^{-4}$[c]	>1,660[c]
rad52-38 (YAF22)	1.4×10^{-3}	730
rad54-34 (YAF23)	$<5.5 \times 10^{-4}$[c]	>1,818[c]
rad55-37 (YAF37)	2.7×10^{-3}	369
rad57-38 (YAF56)	3.5×10^{-4}	425
Mutant 44 + pControl	2.6×10^{-3}	390
Mutant 44 + pRAD52 CEN	4.8×10^{-2}	21
Mutant 44 + pRAD52 2μm	5.3×10^{-1}	2
Mutant 44 + pRFA1 CEN	9.5×10^{-1}	1

[a] All strains carried either the pAF30 or the pAF35 plasmid in addition to the plasmid given in the first column. Numbers are given relative to the levels observed with either plasmid in wild-type parental YME2 cells.
[b] Levels of gene conversion were 8 to 14% with pAF30 and 14 to 20% with pAF35.
[c] Minimal estimates are given when no recombinant colonies were obtained.

disrupted. At the low dose (15 kilorads), mutant 44 is about 75-fold-more sensitive than YME2, and at double the X-ray dose, the sensitivity increases to about 300-fold. YME5 (*rad52*::*LEU2*) is substantially more sensitive at all X-ray doses. The sensitivity of mutant 44 to UV radiation (Fig. 3b) is about 50- and 400-fold greater than YME2 at 80 and 160 J/m^2, respectively; YME7 (*rad1*::*URA3*), by contrast, is considerably more sensitive, especially at the low UV doses.

Just as was the case with HO-induced gene conversion, the introduction of the *RAD52* gene on a low-copy-number centromeric plasmid increases the resistance of mutant 44 to both X-ray and UV radiation and increases it even more so when the *RAD52* gene is on a multicopy 2μm plasmid. Here too, the wild-type form of the mutant 44 allele (see below) restores the responses to X rays and UV to normal levels.

Mutant 44 is defective in repairing HO-induced chromosomal DSBs. To obtain another measure of mutant 44's deficiency in repairing DSBs, we examined the consequences of cleaving the naturally occurring *HO* site at the *MAT* locus in chromosome III. Because a failure to repair a single chromosomal DSB may be lethal (5, 66), survival under conditions of continual expression of the HO endonuclease provides a measure of the recombinational repair process. Accordingly, after being grown in glucose medium, the same strains shown in Table 4 were plated on minimal medium containing galactose lacking either uracil (SGC−Ura) or leucine (SGC−Leu) depending on whether the plasmid was pUN50-*GAL-HO* or YCp-*GAL-HO*, respectively (Table 5). Under these conditions, the number of surviving colonies is a measure of the frequency with which cells repair the cleaved *HO* site at the *MAT* locus. Mutant 44 is some 500-fold-less able than the wild type to survive DSBs at the chromosomal *HO* site. As expected, the ability of a *rad52* disrupted strain, or of missense mutants in various members of the *RAD52* epistasis group, to repair the broken chromosomal *HO* site mirrors its ability to carry out HO-induced recombination at the *ade2* locus on a plasmid (Tables 4 and 5). In this assay also, mutant 44's repair deficiency is complemented partially by *RAD52* on a low-copy-number plasmid, virtually completely by the same gene on a 2μm plasmid or by a plasmid bearing the wild-type allele of mutant 44.

When plasmid pAF30 or pAF35 is used as the source of HO endonuclease, survival on galactose medium is assayed under somewhat more stringent conditions, as the *HO* site is present not only on chromosome III but also on the plasmid bearing the HO endonuclease (Table 5). This experiment mimics closely the conditions on an induction plate after the glucose supply has been exhausted. Under these conditions, the repair-deficient strains are even more vulnerable to the continuous presence of the HO endonuclease. Here, too, *RAD52* rescues mutant 44 in a dose-dependent fashion.

Unlinked noncomplementation between mutant 44 and *rad52-34*. The original complementation analysis revealed differential complementation of mutant 44's phenotypes when crossed to various *rad52* mutants obtained (Table 3). To analyze these differential effects further, eight different *rad52* alleles were serially backcrossed three or more times to the parental YME2 strain and then crossed to themselves (homozygous crosses), to mutant 44 (heterozygous crosses), or to YME2 (backcrosses). The resulting diploid strains were characterized for their abilities to carry out HO-induced gene con-

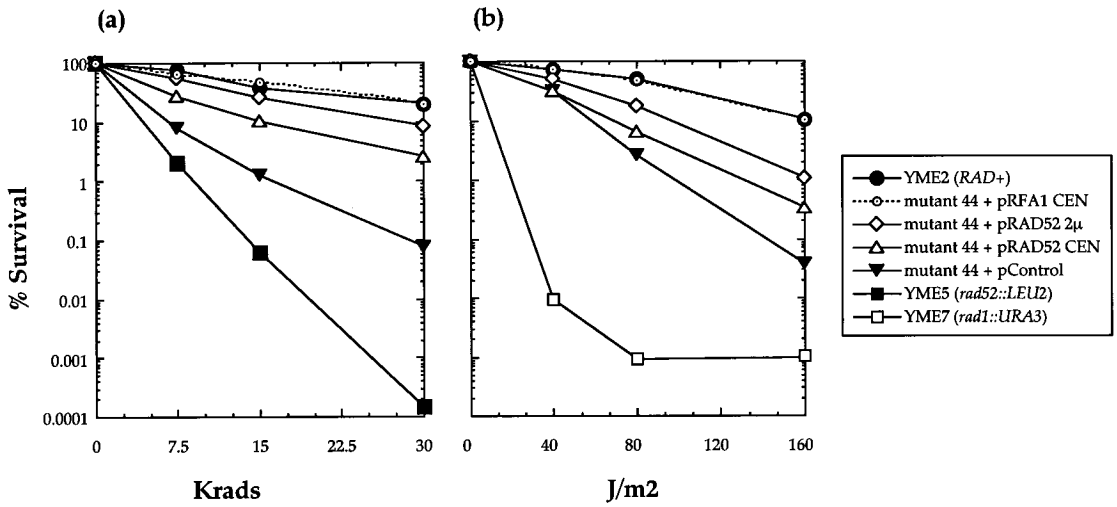

FIG. 3. X-ray (a) and UV (b) sensitivities of mutant 44 and suppression by *RAD52*.

TABLE 5. Deficiency in HO-mediated DSB repair of strains used in this study

Mutant (strain) and plasmid	Survival on galactose			
	Chromosomal DSBs only[a]		Chromosomal and plasmid DSBs[b]	
	Relative level	Decrease (fold) from wild-type level	Relative level	Decrease (fold) from wild-type level
RAD$^+$ (YME2)	1[c]		1[d]	
rad1::*URA3* (YME7)	5.7×10^{-2}	17.4	NT	NT
rad52::*LEU2* (YME5)	2.7×10^{-4}	3,750	NT	NT
rad51-36 (YAF21)	5.6×10^{-4}	1,771	2.4×10^{-4}	4,190
rad52-34 (YAF5)	$<4.8 \times 10^{-4}$[e]	>2,085[e]	$<2.4 \times 10^{-4}$[e]	>4,080[e]
rad52-38 (YAF22)	1.7×10^{-3}	581	1.9×10^{-3}	530
rad54-34 (YAF23)	6.5×10^{-4}	1,540	4.1×10^{-4}	2,416
rad55-37 (YAF37)	2.8×10^{-3}	361	1.9×10^{-3}	521
rad57-38 (YAF56)	1.5×10^{-3}	680	7.9×10^{-4}	1,260
Mutant 44 + pControl	1.9×10^{-3}	513	1.3×10^{-3}	758
Mutant 44 + pRAD52 CEN	2.0×10^{-2}	50	1.8×10^{-2}	55
Mutant 44 + pRAD52 2μm	4.1×10^{-1}	2.5	3.6×10^{-1}	2.8
Mutant 44 + pRFA1 CEN	6.7×10^{-1}	1.5	6.2×10^{-1}	1.6

[a] These experiments were done under selection for either the pUN50-*GAL-HO* or the YCp-*GAL-HO* plasmid, and values are relative to those observed with either plasmid in YME2 cells.
[b] These experiments were done with either pAF30 or pAF35, and values are relative to those observed with either plasmid in YME2 cells. NT, not tested.
[c] Survival was 40 to 50% with pUN50-*GAL-HO* and 30 to 40% with YCp-*GAL-HO*.
[d] Survival was 16 to 22% with pAF30 and 13 to 15% with pAF35.
[e] Minimal estimates are given when no recombinant colonies were obtained.

version and their sensitivities to UV and X-ray radiation. Although the *RAD52* gene itself can suppress mutant 44's phenotypes at a high-level gene dosage in haploids (see above), one of the eight *rad52* alleles tested, *rad52-34*, interferes with the complementation of mutant 44's phenotypes in heterozygous diploids (Fig. 4). Thus, although the strain with the *rad52-34* allele is nearly as X ray sensitive as cells with a disrupted *rad52* gene, diploids containing both mutant 44 and *rad52-34* alleles along with their respective wild-type alleles are still about 8- and 18-fold more sensitive to X rays than the wild type at 15 and 30 kilorads, respectively. By contrast, diploids containing both mutant 44 and *rad52-38* are virtually indistinguishable from wild-type diploid cells at these doses (Fig. 4), as are the heterozygous diploids of mutant 44 with the other six *rad52* alleles (data not shown). This nonallelic noncomplementation observed in mutant 44 × *rad52-34* diploids suggests the existence of a genetic interaction between *RAD52* and the gene affected in mutant 44.

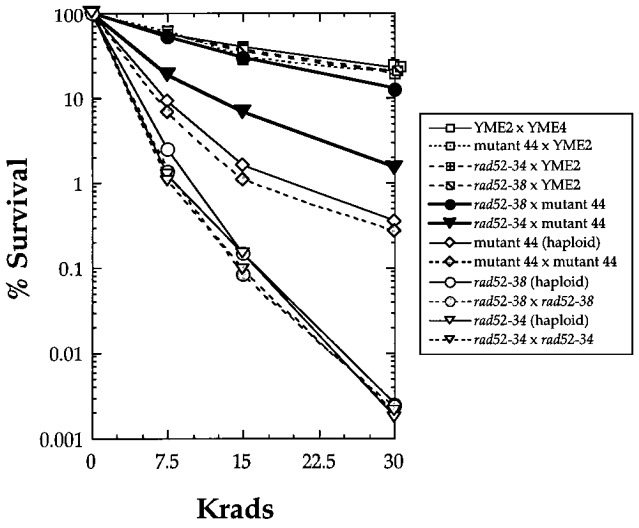

FIG. 4. Mutant 44's X-ray sensitivity fails to be complemented in diploids with *rad52-34*.

Meiosis and sporulation are defective in mutant 44. In view of the marked effects on recombination and DSB repair exhibited by mutant 44, we examined its ability to undergo sporulation and meiosis, processes which are accompanied by large numbers of DSBs (2, 11, 32, 60, 82). Indeed, we had noted in the course of our complementation studies that the sporulation efficiency of homozygous diploids of mutant 44 was significantly reduced. Table 6 shows that the yield of tetrads after

TABLE 6. Effects of *rfa1-44* on sporulation

Diploid strain sporulated	Tetrads[a] (%)	Decrease (fold) from wild-type level	Spore viability (%)	Asci with all four spores viable (%)
YME2 × YME4	17	1	91	78
YAF44 × YAF44-**a**	0.66	25	48	8
YAF44 × YME2	17	1	91	73
YAF5 × YAF5-**a**[b]	<0.03[c]	>553	NA[c]	NA[c]
YAF5 × YME2	14	1.2	85	65
YAF22 × YAF22-**a**[b]	0.03	553	(25)[d]	(0)[d]
YAF22 × YME2	16	1	88	70
YAF44 × YAF5-**a**[b]	2	9	51	20
YAF44 × YAF22-**a**[b]	17	1	84	70

[a] As observed by phase microscopy.
[b] The alleles *rad52-34* (strains YAF5 and YAF5-**a**) and *rad52-38* (strains YAF22 and YAF22-**a**) are two of the *rad52* mutant alleles isolated in our screen.
[c] NA, not applicable. Not a single tetrad was observed, of >3,000 cells. Therefore, the yield of tetrads is only a minimal estimate, and no estimate of strain survival after germination is possible.
[d] Only a single tetrad was found in several independent experiments, and this single tetrad yielded a single viable spore after dissection and germination.

homozygous diploids of mutant 44 were sporulated is reduced about 25-fold compared with that of wild-type diploids (YME2 × YME4) and that spores derived from such diploids showed reduced viability (48%) after germination. In fact, only 5 tetrads of a total of 64 produced four viable spores. By comparison, *rad52-34* and *rad52-38* homozygous diploids were even more impaired in their abilities to sporulate. These effects of *rfa1-44*, *rad52-34*, and *rad52-38* were recessive, as backcrossed diploids sporulated and produced viable spores at levels nearly identical to those of the wild type (Table 6).

Particularly interesting is the observation that diploids from a cross between mutant 44 and *rad52-34* sporulated about ninefold less effectively than the wild type. However, mutant 44 × *rad52-38* diploids sporulated as efficiently as the wild type (Table 6). Thus, the allele-specific interaction of *rad52-34* with mutant 44 in heterozygous diploids was observed not only for resistance to radiation and HO-induced gene conversion but also for sporulation, further suggesting a genetic interaction between *RAD52* and the gene affected in mutant 44.

Complementation of the mutant 44 gene by cloning. The function encoded by the wild-type allele of mutant 44 was identified by complementation of its nonpapillating phenotype with the λYES *S. cerevisiae* genomic library (19). The screening of about 50,000 transformants for papillated colonies yielded 24 positives. After retransformation of mutant 44 with plasmids recovered from these 24 colonies, 8 restored the wild-type papillation phenotype as well as normal levels of resistance to UV and X rays. Restriction enzyme analysis of the eight complementing cloned DNAs established that all shared a 2.1-kbp *Eco*RI-*Pst*I internal fragment and that all were derived from the same chromosomal locus. The sequence of the *Eco*RI-*Pst*I fragment was identical to the already identified gene encoding the large subunit of *S. cerevisiae* RPA single-stranded DNA-binding protein (10, 34). Plasmids containing the recovered gene fully complement all of the phenotypic defects of mutant 44 (Fig. 2; Tables 4 and 5). Moreover, plasmid pJM125, which contains the originally cloned *RFA1* gene (10), also fully complements the recombination defects and radiation sensitivity of mutant 44.

Linkage analysis demonstrates that mutant 44 is *rfa1-44*. To exclude the formal possibility that *RFA1* is an extragenic suppressor of mutant 44, genetic linkage analysis was performed on mutant 44, essentially as described elsewhere (68). In brief, the segment encoding the recovered *RFA1* gene was introduced into an integrating plasmid (pAF102). Transformation of YME2 with linearized pAF102 (cleaved at the *Mlu*I site in the 5′-flanking region) resulted in a haploid strain with a duplication of the wild-type *RFA1* sequence now linked to the *URA3* marker (YAF70). YAF70 was crossed to mutant 44, diploids were selected and sporulated, and the tetrads were analyzed (Fig. 5). The tetrads yielded the expected 2:2 segregation pattern, that is, two URA^+ and X-ray-resistant spores and two URA^- and X-ray-sensitive spores, confirming that mutant 44 is allelic to the *RFA1* locus (Fig. 5). In a similar analysis, the independently isolated *RFA1* gene (10) in pAF104 was cleaved at the *Eco*RI site within the coding sequence and used to construct YAF71. Tetrad analysis of diploids obtained from a cross between mutant 44 and YAF71 produced the same 2:2 spore segregation pattern (data not shown). We conclude that mutant 44 has a mutation in the gene previously referred to alternatively as *RPA1* (34) or *RFA1* (10). Accordingly, mutant 44 will be referred to as *rfa1-44*.

Cloning and characterization of the *rfa1-44* mutant allele. To identify the mutation in *rfa1-44*, the 2.55-kbp *Pst*I-*Hin*dIII fragments of two independent YAF44 X-ray-sensitive segregants were recovered by direct cloning of their *rfa1* loci, and these loci were then sequenced in their entireties. Both isolates had identical sequences, each having a single point mutation compared with the published wild-type *RFA1* sequence (10, 34). The substitution of an adenine for a guanine creates in *rfa1-44* a GAT triplet coding for an aspartate residue at position 77, instead of the GGT triplet coding for glycine in the wild-type protein.

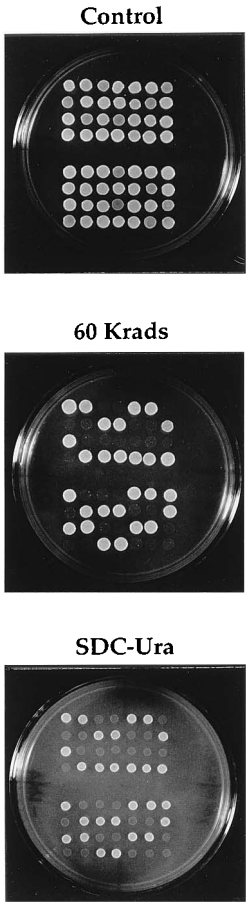

FIG. 5. Linkage analysis reveals that mutant 44 is allelic to *RFA1*. Tetrads obtained from YAF70 × mutant 44 diploids were dissected, and spores were grown for 3 days on YPAD plates and then briefly in liquid YPAD. Approximately equal numbers of cells were spotted on the appropriate plates. (Top panel) Control YPAD unirradiated plate; (middle panel) YPAD plate irradiated with 60 kilorads immediately after spotting; (bottom panel) growth on plates lacking uracil (SDC−Ura).

To test that the mutation identified in *rfa1-44* (Rfa1-D77) was responsible for the mutant's phenotypes, both wild-type YME2 (as a control) and YAF44 were transformed with a series of plasmids expressing various forms of the Rfa1 protein and then tested for their sensitivities to irradiation and their abilities to carry out HO-induced gene conversion (Fig. 6). As expected, plasmids expressing Rfa1 with an aspartate at position 77 (Rfa1-D77) were unable to rescue any of the defects characteristic of YAF44, whereas plasmids that expressed Rfa1 with a glycine at position 77 (Rfa1-G77) fully complemented the defects of YAF44. Overexpression of the mutant allele of Rfa1, with pRfa1-D77 2μm, caused a low level inhibition of gene conversion (Fig. 6). This partial dominant-negative effect of pRfa1-D77 2μm was also evident in additional experiments testing for radiation sensitivity (a three- to fivefold effect [data not shown]). Finally, the *rfa1-44* allele (Rfa1-D77) was able to

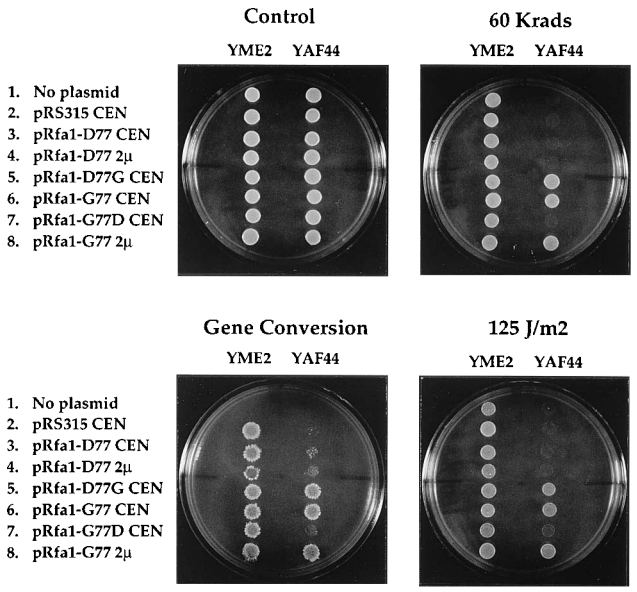

FIG. 6. Domain swaps between wild-type *RFA1* and mutant *rfa1-44* alleles. Both wild-type (YME2) and mutant strains (YAF44) were transformed with various plasmids expressing the Rfa1 protein with either an aspartate (D) or a glycine (G) at position 77. Only plasmids expressing the Rfa1-G77 protein were able to rescue YAF44. Control, unirradiated YPAD plate; 60 Krads and 125 J/m2, X-irradiated (60 kilorads) and UV-irradiated (125 J/m^2) plates, respectively; Gene Conversion, SDC−adenine replica from an induction plate that had been grown for 5 days (see Materials and Methods).

rescue the viability of spores derived from a W303 derivative carrying a disrupted form of *RFA1* (YSB89 [10]); these rescued cells were only slightly sensitive to radiation, suggesting that the phenotype of *rfa1-44* might vary in different genetic backgrounds. As expected, reconstitution of the *rfa1-44* allele in our S288C-derived strain disrupted for *RFA1* produced levels of sensitivity to X rays and UV essentially identical to those of YAF44 (23a).

DISCUSSION

A collection of mutants, each of which is defective in its ability to repair an HO endonuclease-induced DSB in an episomal *ade2* gene by recombination with an intact *ade2* segment in the chromosome, has been isolated. As anticipated, many mutants have mutations in genes that had previously been identified as being involved in the repair of UV- and X-ray-induced damage. Accordingly, most of our mutants provide new alleles of *rad1*, *rad10*, *rad51*, *rad52*, *rad54*, *rad55*, and *rad57*. One set of 10 mutants, distinct from those above and composing at least 3 complementation groups, is only slightly more defective than wild-type cells in HO-induced recombination and DNA repair.

This report focuses on one of the radiation-sensitive, recombination-deficient mutants (mutant 44) because it was clearly distinguishable by genetic tests from the *rad* mutants mentioned above. Mutant 44 is deficient in HO-induced gene conversion, DSB repair, and sporulation and is sensitive to X-ray and UV irradiation. However, these phenotypes are all suppressed by the *RAD52* gene product in a dose-dependent fashion. This behavior suggested an unusual genetic interaction between *RAD52* and the mutant gene or, perhaps, a physical interaction between the proteins they encode. We shall return to this point below.

Identification of the mutant allele. Complementation of the mutant's recombination defect with a yeast genomic DNA library and subsequent sequencing of the complementing cloned segment identified the affected gene as *RFA1*. *RFA1* encodes the 69-kDa subunit of the yeast heterotrimeric single-stranded DNA-binding protein RPA (10, 34). Although cloned on the basis of its ability to complement the mutant's recombination defect, the isolated gene also restores wild-type levels of resistance to UV, X rays, and chromosomal breaks induced by HO endonuclease. These mutant properties were also fully complemented by a plasmid expressing the previously cloned *RFA1* gene (10). The mutant allele, which we designate *rfa1-44*, codes for an aspartic acid residue in place of glycine at position 77. The mutation alters the sequence of an RGD tripeptide, an amino acid signature which has been implicated in a variety of protein-protein interactions in metazoans (35, 36). Because the mutation lies in a region of relatively low conservation between the organisms for which *RFA1* has been characterized (1, 10, 21), this protein domain may carry out functions or interactions specific to *S. cerevisiae.*

The role of Rfa1 in recombination. Before considering the possible role of Rfa1 in recombination, it is important to note that each of the constituents of RPA, *RFA1*, *RFA2*, and *RFA3*, is required for the survival of *S. cerevisiae*. Disruption of any one of the three coding sequences is lethal (10, 34), probably because the trimeric protein is essential for DNA replication. Interestingly, the large subunit alone possesses the strong single-stranded DNA-binding activity characteristic of the trimeric protein (1, 9, 39, 40, 80, 89).

Although there is no previous evidence for RPA's role in recombination or repair in vivo, Jessberger et al. (38) noted that RPA increases the efficiency of recombinational repair of DSBs in vitro, and Coverley et al. (14) reported that excision repair of DNA damaged by a bulky adduct is dependent on RPA in vitro. Considering that both repair processes require DNA synthesis, the *rfa1-44* mutation may impair RPA's activity in that phase of the repair process. However, this possibility is unlikely, because the rate of DNA synthesis in *rfa1-44* cells, as measured by direct incorporation (8, 63), is barely distinguishable from that of the wild type (an 8% decrease [23a]). Hence, *rfa1-44* appears to be the first known separation-of-function allele of a eukaryotic single-stranded DNA-binding protein.

We presume that DSBs induced by HO endonuclease cleavage and by X rays result in the production of single-stranded tails with 3′ hydroxyl ends, the kind that are formed at the DSBs associated with mating type switching (13, 81) and at the *HIS4* (11) and *ARG4* (83) loci during meiosis. RPA's role in the recombinational repair of such breaks could, therefore, result from its abilities to bind to and protect single-stranded ends and facilitate recombination and strand exchange, much as *Escherichia coli* single-stranded DNA-binding protein acts in concert with RecA (16, 44, 45, 49).

Conceivably, however, RPA could have another function in DNA repair reactions, one which follows from its ability to bind to single strands created at DSBs and during excision repair. In binding to single strands, RPA might recruit other proteins needed for repair to the site where they must act. In the simplest case, RPA could recruit the appropriate DNA polymerase, a property that has already been shown from its requirement for initiation of DNA replication in vitro (17, 20, 47, 86). A more elaborate model envisions that RPA, and perhaps more specifically *RFA1*, is responsible for assembling the essential components of the recombination and repair machinery. By this view, the proteins responsible for repairing

DSBs act as a complex consisting of multiple interacting polypeptides: a "DSB recombinosome."

Likely participants in such a recombinosome are proteins encoded by the *RAD52* epistasis group, and almost certainly the Rad52 protein itself. We presume that interactions between the Rfa1 and Rad52 proteins could initiate the assembly of the recombinosome and that additional interactions with other proteins participating in this repair pathway would result in the formation of a functional recombinosome. Our evidence to date implicates Rad52, but other studies have revealed the association of Rad52 with another member of its own epistasis group, the Rad51 protein (50, 77), as well as with itself (7, 50) and possibly with additional and as yet unidentified factors (50).

What has led us to this formulation? First, *RAD52* suppresses the DSB repair defect of *rfa1-44* in a dose-dependent fashion. Admittedly, we cannot rule out the possibility that increased levels of Rad52 promote repair of DSBs by a pathway wholly independent of RPA. However, both null and point mutations of *rad52* were found to be epistatic to *rfa1-44* (23a). Therefore, a more likely explanation is that elevated levels of Rad52 compensate for a defective interaction with Rfa1-D77 (the mutant protein encoded by *rfa1-44*), thereby increasing the likelihood of forming the recombinosome's precursor. Suppression of impaired protein-protein interactions through increased concentrations of one of the reactants is well documented. Thus, a mutation affecting one of the proteins needed for error-prone repair in *E. coli* (*umuC-36*) is suppressed by overexpressing the *UmuD′* gene (3). More to the point, Milne and Weaver (50) have shown that overexpression of *RAD51*, an essential participant in DSB repair and recombination, suppresses the dominant-negative effect of deletion alleles of *rad52*, in part through physical interactions between specific domains of each protein (50). They propose that the interactions of Rad52 with Rad51 and of Rad52 with itself or with still another protein(s) are necessary for repair and recombination. Our findings suggest that Rfa1 in RPA is one of the proteins with which Rad52 interacts.

Additional evidence for an interaction between Rfa1 and Rad52 stems from the results of the complementation analyses (Tables 3 and 6; Fig. 4). Specifically, each of the phenotypic defects of *rfa1-44* is fully complemented in diploids made with strains bearing various *rad52* alleles, but diploids with one particular allele, *rad52-34*, are only poorly complemented. Thus, the restoration of wild-type levels of recombination repair of DSBs expected in these diploids, which contain wild-type *RFA1* and *RAD52* alleles, is abrogated by the presence of the *rfa1-44* and *rad52-34* alleles. This incomplete complementation is not due to either *rad52-34* or *rfa1-44* being a partially dominant allele, because each is fully recessive in backcrosses with YME2 (Fig. 4 and Table 6). The finding that seven of the eight *rad52* alleles tested complement *rfa1-44* (data not shown) and that only one, *rad52-34*, complements poorly suggests that the interaction is allele specific, possibly involving discrete domains of the two proteins.

Of interest was the finding that the mutant phenotype of these eight different *rad52* alleles is not suppressed by overexpression of wild-type *RFA1* (23a). Thus, even though elevated levels of wild-type *RAD52* restore *rfa1-44*'s ability to carry out recombinational repair, the imputed interaction between wild-type *RFA1* and these eight *rad52* mutants does not restore their required function in recombination. Alternatively, overexpression of all three subunits of RPA may be necessary to effect suppression.

Our suggestion that the recombinational repair of DSBs requires the assembly of a complex of proteins at the site of the DSB is in keeping with a growing body of evidence that implicates multiprotein complexes in several coordinated synthetic and repair operations. For example, the single-stranded DNA-binding protein of bacteriophage T4 (gp32) participates in the formation of functional replication and recombination complexes (53, 54), the *ori* binding protein initiates the assembly of a DNA replisome in *E. coli* (42, 43), the binding of the TATA-binding protein component of TFIID near the site of transcription initiation promotes the assembly of a transcription complex (for a review, see references 62, 67, and 90), the binding of Int and integration host factor (IHF) at the *att* sites promotes the formation of the bacteriophage λ integration complex (56), and the interaction of UvrA(B) or of MutS(L) with damaged sites in DNA recruits other proteins that carry out excision repair (74, 87) and mismatch repair (51), respectively. Recent reports also suggest that the transcriptional and excision repair machineries share components (22, 31, 63, 71, 88), presumably to integrate the two processes (6, 33). How many and which of the proteins that have been implicated in DSB repair are included in the DSB recombinosome that we postulate remain to be determined.

Other activities of Rfa1. Rfa1 also appears to be involved in spontaneous mitotic recombination as evidenced by an approximately 10-fold-reduced efficiency of heteroallelic recombination between unbroken *ade2* alleles in the chromosome and on a plasmid and between unbroken *neo* alleles on stably maintained separate plasmids (24a).

The increased sensitivity of *rfa1-44* to UV-induced damage of DNA (Fig. 3b), to the UV mimetic agent *cis*-diaminedichloroplatinum, and to alkylating agents such as methyl methanesulfonate, ethyl methanesulfonate, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, and to the X-ray mimetic agent bleomycin (data not shown) implicates *RFA1* in cellular repair processes that respond to a wide variety of DNA damage. Thus, Rfa1, alone or as part of the trimeric RPA, may protect and stabilize single-stranded DNA at the site of damage and facilitate the initiation of repair by recruiting components of the repair machinery. Since Rfa1 has an increased binding affinity for single-stranded DNA treated with *cis*-diaminedichloroplatinum, it may participate in the recognition of structural aberrations caused by damage (12). Supporting this premise, the in vitro studies implicating RPA in excision repair indicate that RPA acts early, before the incision stage (15).

The identification and characterization of another allele of *RFA1*, *rfa1-D228Y*, by Smith and Rothstein (79) show that *RFA1* also participates in a recombination pathway that is independent of *RAD52*, thereby demonstrating *RFA1*'s involvement in multiple independent pathways of recombination. Moreover, their demonstration that *rfa1-D228Y* results in an unstable RPA complex suggests further that the interactions of various factors with RPA are crucial to its functions in recombination, repair, and replication.

This work identifies the large subunit of the heterotrimeric RPA complex as a key element involved in a variety of recombination and repair pathways. Taken together with the report by Smith and Rothstein (79), it provides the first in vivo demonstration that RPA participates in recombination and repair processes in eukaryotic cells. Moreover, the suppression by *RAD52* of the multiple phenotypes associated with *rfa1-44* and the nonallelic noncomplementation between *rfa1-44* and *rad52-34* both suggest the existence of a genetic interaction between *RFA1* and *RAD52*. We have proposed a model in which Rfa1 functions as an initiator or enhancer of multiprotein complex assembly. Despite the speculative nature of this model, it provides a useful framework for testing the multifaceted nature of Rfa1 both in vivo and in vitro.

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ADDENDUM IN PROOF

After this paper was submitted, M. P. Longhese, P. Levani, and G. Lucchini (Mol. Cell. Biol. **14:**7884–7890, 1994) implicated RF-A (RPA), and particularly Rfa1, in DNA repair and recombination because an *rfa1* mutant showed an increased sensitivity to UV radiation and a twofold decrease in their recombination assay. By contrast, recombination in the *rfa1-44* mutant described in this study is impaired by more than 400-fold.

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