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A Mutation in the Gene Encoding the *Saccharomyces cerevisiae* Single-Stranded DNA-Binding Protein Rfa1 Stimulates a *RAD52*-Independent Pathway for Direct-Repeat Recombination

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In the yeast *Saccharomyces cerevisiae*, recombination between direct repeats is synergistically reduced in *rad1 rad52* double mutants, suggesting that the two genes define alternate recombination pathways. Using a classical genetic approach, we searched for suppressors of the recombination defect in the double mutant. One mutation that restores wild-type levels of recombination was isolated. Cloning by complementation and subsequent physical and genetic analysis revealed that it maps to *RFA1*. This locus encodes the large subunit of the single-stranded DNA-binding protein complex, RP-A, which is conserved from *S. cerevisiae* to humans. The *rfa1* mutation on its own causes a 15-fold increase in direct-repeat recombination. However, unlike most other hyperrecombination mutations, the elevated levels in *rfa1* mutants occur independently of *RAD52* function. Additionally, *rfa1* mutant strains grow slowly, are UV sensitive, and exhibit decreased levels of heteroallelic recombination. DNA sequence analysis of *rfa1* revealed a missense mutation that alters a conserved residue of the protein (aspartic acid 228 to tyrosine [D228Y]). Biochemical analysis suggests that this defect results in decreased levels of RP-A in mutant strains. Overexpression of the mutant subunit completely suppresses the UV sensitivity and partially suppresses the recombination phenotype. We propose that the defective complex fails to interact properly with components of the repair, replication, and recombination machinery. Further, this may permit the bypass of the recombination defect of *rad1 rad52* mutants by activating an alternative single-stranded DNA degradation pathway.

Multiple pathways for genetic recombination have been defined for several organisms (4, 6, 26, 76, 85). In *Escherichia coli*, extensive genetic analysis has characterized three pathways for conjugational recombination (for a review, see reference 75). The initial isolation of recombination-deficient mutants identified *recA* and *recBCD* (13, 86, 87). Although both *recA* and *recBCD* mutants exhibit a recombination defect, *recBCD* mutants display higher residual recombination: 1% of the wild-type level, compared with 0.05% for *recA* strains (44, 87). This differential effect on recombination led to the hypothesis that *E. coli* has additional pathways that function independently of *recBCD* (12). This view was substantiated by the isolation of *sbcA* and *sbcB*, which are suppressors of the recombination deficiency of *recBCD* (7, 40). Furthermore, recombination-deficient derivatives of these suppressor strains identified two additional recombination pathways, controlled by *recE* and *recF* (25, 34).

In *Saccharomyces cerevisiae*, double mutants for two recombination and repair genes, *RAD1* and *RAD52*, are synergistically reduced for direct-repeat recombination. The classic interpretation of synergy is that the genes are involved in different pathways (37, 69, 80). The *RAD1* gene, which was identified through a UV-sensitive mutation, is involved in the nucleotide excision repair pathway (23, 62). The Rad1 protein forms a complex with Rad10 that functions as a single-stranded DNA endonuclease (5, 8, 82). A mutation in the *RAD52* gene results in X-ray sensitivity (60), and the Rad52 protein has been shown to be involved in the repair of many kinds of double-strand breaks in *S. cerevisiae* (47, 55, 61). In direct-repeat recombination, neither *rad1* nor *rad52* mutants individually display significant effects on deletion events. However, *rad1 rad52* double mutants are dramatically decreased for recombination (37, 69, 80). Although the double mutants exhibit a large decrease in recombination rates, a low level remains. One possible explanation is that additional alternate pathways of recombination exist.

In an approach similar to that taken with *E. coli*, we searched for mutations that increase direct-repeat recombination in a *rad1 rad52* background. A single mutation that restores wild-type levels of recombination was identified. This mutation displays a *rad52*-independent hyperrecombination phenotype on its own as well as UV sensitivity and a slow-growth defect. Cloning and sequencing subsequently identified the mutation as a missense allele of the essential gene *RFA1* (10, 30). Biochemical and genetic analysis of the mutant strain suggests that its phenotype results from decreased levels of the mutant RP-A complex. In addition, we propose that the suppression of the *rad1 rad52* recombination defect results from the activation of an alternative single-stranded DNA degradation pathway.

It should be noted that the gene described by Heyer et al. was originally designated *RPA1* (30). This same designation has been used for genes encoding RNA polymerase subunits (50, 81) and an acidic ribosomal protein (54). Therefore, to avoid confusion, the designation *RFA1* as used by Brill and Stillman (10) is used for the gene, while the designation for the protein complex remains RP-A.

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MATERIALS AND METHODS

Media. YPD and synthetic medium were made as described previously (70, 71), with the exception that synthetic medium contains twice the amount of

TABLE 1. *S. cerevisiae* strains used in this study

Strain[a]	Genotype	Reference or origin
W303-1B	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	79
W790-2A	*MAT*α *can1-100,x rad1*::*LEU2 rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	Rothstein laboratory
W838-7D	*MAT***a** *can1-100,x rad1*::*LEU2 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	Rothstein laboratory
W1042-4D	*MAT*α *can1-100,x rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3 rfa1-D228Y*	This study
W1042-7B	*MAT***a** *can1-100,x SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	This study
W1042-7C	*MAT*α *can1-100,x SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3 rfa1-D228Y*	This study
W1042-11C	*MAT*α *can1-100,x rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	This study
W1042-16B	*MAT*α *can1-100,x rad1*::*LEU2 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3 rfa1-D228Y*	This study
W1042-16D	*MAT***a** *can1-100,x rad1*::*LEU2 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	This study
W1052	*MAT***a** *can1-100,x rad1*::*LEU2 rad52*::*TRP1* *sup4* *MAT*α *can1-100,x rad1*::*LEU2 rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3*	This study
W1072-6B	*MAT***a** *can1-100,x rad1*::*LEU2 rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-ura3*::*LEU2 rfa1-D228Y*	This study
W1092	*MAT***a** *can1-100,x rad1*::*LEU2 rad52*::*TRP1* *SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3* *rfa1-D228Y* *MAT*α *can1-100,x rad1*::*LEU2 rad52*::*TRP1* *sup4* *RFA1*	This study
W1096	*MAT***a** *can1-100,x rad1*::*LEU2 rad52*::*TRP1* *sup4* *rfa1-D228Y* *MAT*α *can1-100,x rad1*::*LEU2 rad52*::*TRP1 SUP4*-**o**::*HIS3*::pWJ317-*CAN1-URA3 rfa1-D228Y*	This study
W1112-19D	*MAT*α *can1-100,x SUP4*-**o**::*HIS3*::pWJ317-*CAN1-ura3*::*LEU2*	This study
W1188-2D	*MAT*α *can1-100,x SUP4*-**o**::pWJ317-*CAN1-URA3 rfa1-D228Y*	This study
W1298-1C	*MAT*α *can1-100,x leu2*Δ*Bst*EII	This study
W1298-8D	*MAT***a** *can1-100,x leu2*Δ*Bst*EII *rfa1-D228Y*	This study
W1442	*MAT***a** *can1-100,x leu2*Δ*Eco*RI *sup4*::*HIS3* *MAT*α *can1-100,x leu2*Δ*Bst* EII *sup4*	This study
W1446	*MAT***a** *can1-100,x leu2*Δ*Bst*EII *sup4* *RFA1* *MAT*α *can1-100,x leu2*Δ*Eco*RI *sup4*::*HIS3* *rfa1-D228Y*	This study
W1449	*MAT***a** *can1-100,x leu2*Δ*Eco*RI *sup4*::*HIS3* *rfa1-D228Y* *MAT*α *can1-100,x leu2*Δ*Bst*EII *sup4* *rfa1-D228Y*	This study

[a] All strains used are derivatives of W303-1B (79). The genotypes are identical to W303-1B except as noted. In most instances, several strains with the same genotype (except mating type) were used. However, only one strain is noted. Further, *can1-100,x* denotes an allele of *can1-100* in which an undefined secondary mutation prevents suppression by *SUP4*-**o**.

leucine (60 mg/liter). RZUC medium is synthetic minimal medium containing histidine, leucine, lysine, methionine, tryptophan, uracil, one-fourth the normal amount of adenine (5 mg/liter), and 60 μg of L-canavanine per ml. RZC is RZUC medium that lacks uracil. RZUC−Leu is RZUC medium which lacks leucine. OFAC medium is synthetic complete medium minus arginine and containing one-fifth the normal amount of adenine (4 mg/liter) and 60 μg of L-canavanine per ml (49). Medium containing limiting amounts of adenine allows *ade2* mutants to develop a red color more rapidly.

Yeast strains and genetic methods. Standard procedures were used for mating, sporulation, and dissection (71). All *S. cerevisiae* strains used in this study are derivatives of W303-1A and W303-1B (79), and a single representative of each genotype is listed in Table 1. Construction of W303 derivatives containing *SUP4* direct repeats, *rad1*::*LEU2*, *rad52*::*TRP1*, or *can1-100,x* mutations has been described previously (49, 63, 79). The *SUP4* duplication strain U770, which contains the *ura3*::*LEU2* disruption within the plasmid sequences, was kindly provided by John McDonald. It was constructed by transforming a wild-type strain containing the *SUP4* duplication (49) with a *Hin*dIII *ura3*::*LEU2* DNA fragment. This fragment was created by inserting a 2.0-kb *Hpa*I-*Sma*I *LEU2* fragment (the *Sma*I site came from a polylinker adjacent to the *Sal*I site near the 3′ end of *LEU2*) into the unique *Stu*I site within the *URA3* gene after the addition of *Mlu*I linkers to both the *LEU2* fragment and the *Stu*I site in *URA3*.

A W303 derivative containing an allele of *LEU2* in which the unique *Bst*EII site was filled in (*leu2*Δ*Bst*EII) was created from pLS32, kindly provided by Lorraine Symington. A *Spe*I-*Sal*I fragment from pLS32, containing the *leu2*Δ*Bst*EII allele, was cloned into the *Nhe*I-*Sal*I sites of YIp5, an integrating vector containing *URA3*. This plasmid (pWJ543) was linearized with *Kpn*I and transformed into a *LEU2* strain, creating a chromosomal duplication of the *LEU2* locus which flanks the *URA3* marker. To obtain a strain containing only the *leu2*Δ*Bst*EII allele, several Ura^+ transformants were plated on 5-fluoroorotic acid, and the resistant colonies were screened for leucine auxotrophs. The presence of *leu2*Δ*Bst*EII was verified by genomic blotting. This results in a frameshift mutation at nucleotide 33 of the coding sequence. A W303 derivative (J539) containing an allele of *LEU2* in which the unique *Eco*RI site was filled in (*leu2*Δ*Eco*RI) was constructed in a similar fashion and was kindly provided by Adam Bailis. This results in a frameshift mutation at nucleotide 633 of the coding sequence.

Segregation of *rfa1-D228Y* was scored in two ways: a segregation test or a restriction site polymorphism. For the segregation test, the presence of the wild-type or mutant allele was determined by scoring the recombination phenotype in *rad1 rad52* segregants generated after a cross to an appropriate tester. For the restriction fragment length polymorphism analysis, the allele present was determined by the restriction pattern generated by *Acc*I digestion of a 600-bp PCR amplification product with the primers 5′-CAGAGCATCCAAATGAA ACC-3′ and 5′-TTTGGATAATACCGAGGACG-3′, since the G-to-T transversion in *rfa1-D228Y* creates a new *Acc*I site.

Plasmids and recombinant DNA. Standard methods were used for recombinant DNA manipulations (67). *E. coli* TG1 was transformed by a calcium chloride protocol (67). Yeast cells were transformed by the lithium acetate method (24). Plasmids were isolated from yeast strains by the method of Hoffman and Winston (33).

The *rfa1-D228Y* complementing clone (pWJ579) was isolated from a *Sau*3A partially digested yeast genomic library cloned into YCp50 (64). The following subclones were made from pWJ579. The 8.0-kb *Bam*HI-*Cla*I fragment was inserted into the corresponding sites of YIp5 (78), an integrative vector. The 9.0-kb *Bam*HI-*Sph*I fragment was cloned into the corresponding sites of YEp352 (31). The 3.0-kb *Xho*I-*Sph*I fragment was cloned into the *Sal*I and *Sph*I polylinker sites of YEp352. The 2.0-kb *Mlu*I-*Xho*I and 4.0-kb *Bam*HI-*Mlu*I fragments were cloned into the corresponding sites of pWJ512 (kindly provided by Adam Bailis), a derivative created by ligating a *Mlu*I linker to *Sma*I-digested pRS416 (74). The plasmid pJM125 was provided by Steve Brill and contains the 2.6-kb *Pst*I-*Hin*dIII fragment from the *RFA1* locus inserted into the corresponding polylinker sites of pRS413 (74).

A plasmid containing the *rfa1-D228Y* allele was obtained by gap repair (56). The wild-type *RFA1*-containing plasmid pJM125 was digested with *Aat*II and *Eco*RV, creating a linear fragment that deletes 720 bp of the *RFA1* gene. After transformation into W1188-2D, an *rfa1-D228Y* strain containing the *SUP4* duplication, several gap-repaired transformants were assayed for recombination. A plasmid (pWJ581) was rescued from the strain that still exhibited *rfa1-D228Y* levels of recombination, and PCR analysis, described above, confirmed the presence of the mutation. For the overexpression analysis, subclones of *RFA1* and *rfa1-D228Y* were made in 2μm-based plasmids. The 2.6-kb *Pst*I-*Hin*dIII fragments from pWJ581 and pJM125 were subcloned into the corresponding polylinker sites of pRS425 (74) to generate the 2μm-based plasmids, pWJ583 and pWJ585, containing *RFA1* and *rfa1-D228Y*, respectively.

Mutagenesis and mutant isolation. An overnight culture of a *rad1 rad52* strain containing the *SUP4* duplication (W790-2A) was mutagenized with 0.3% ethyl methanesulfonate to 22% survival, diluted, plated on YPD, and incubated at 30°C for 3 days (71). Colonies were replica plated to RZUC plates and incubated at 30°C for 7 days. Those exhibiting increased levels of red papillae were further analyzed.

Growth curves. Saturated cultures of wild-type (W1298-1C) and *rfa1-D228Y* (W1298-8D) strains were used to inoculate 50-ml YPD cultures at a density of approximately 10^6 cells per ml. Cultures were incubated at 30°C, and every 30 to

60 min the density of the culture was monitored both by hemacytometer counts and by densitometry with a Klett-Summerson meter.

The *SUP4* direct-repeat recombination assay. Determination of recombination frequencies with the *SUP4* direct repeats has been described previously (49, 63). Briefly, haploid strains containing the *SUP4* duplication were streaked on medium lacking adenine and uracil (−Ade−Ura) to select against mitotic recombination prior to plating. Single colonies were inoculated into 5 ml of liquid −Ade−Ura medium and grown overnight at 30°C to a cell density of 1×10^7 to 4×10^7 cells per ml. Cells were sonicated briefly and counted, and appropriate dilutions were plated on OFAC to select for canavanine-resistant (Can^r) cells and on synthetic complete (SC) medium to determine the plating viability. The SC plates were incubated at 30°C for 3 days, while the OFAC plates were incubated for 5 to 7 days at 30°C. To discriminate forward mutations in the *CAN1* gene (Can^r Ura^+) from *SUP4* deletion events (Can^r Ura^-), all Can^r colonies were replica plated to SC−Ura. The deletion frequency was calculated from the sum of the red and white Can^r Ura^- colonies divided by the total CFU plated. Deletion frequencies for at least two segregants of each genotype were determined between three and five times, and the mean values obtained from these trials are reported. The median test (43) with a chi-square contingency table statistic was utilized to determine whether the recombination frequencies were significantly different between strains.

For the experiments utilizing *LEU2* 2μm-based plasmids, recombination at *SUP4* was assayed as described above, with the modification that all media used lacked leucine to select for maintenance of the 2μm plasmid.

Complementation of the *rfa1-D228Y* mutation by subclones of the *RFA1* region was determined by measuring the recombination frequency at *SUP4* with colonies from plates. Transformants were streaked either on −Ade−Leu−Ura or −Ade−His−Ura medium, depending on the selectable marker present on the introduced plasmid. Single colonies were dispersed in water, and appropriate dilutions were plated on OFAC medium to select for canavanine-resistant colonies and on SC to determine the plating viability. Only those colonies that retained the plasmid, as determined by replica plating onto either SC−His or SC−Ura, were scored.

A modification of the *SUP4* assay was used to ascertain whether *rfa1-D228Y* is a dominant or a recessive mutation. The frequency of recombination in diploids heterozygous for the *SUP4* duplication was measured. In these heterozygous diploids, canavanine-resistant colonies can result not only from deletion events but also from chromosome loss or mitotic crossovers. Since *HIS3* is tightly linked to the *SUP4* duplication used in these strains, chromosome loss and mitotic crossovers were selected against by plating cells on canavanine-containing medium lacking histidine.

Mutation rate analysis. The forward mutation rate in the *CAN1* gene, located between the *SUP4* repeats, was determined from the number of Can^r Ura^+ colonies obtained during the *SUP4* deletion analysis. The median frequency for 6 to 17 experiments was used to determine the mutation rate by the method of Lea and Coulson (42).

Analysis of radiation sensitivity. UV and X-ray sensitivities were determined between two and five times for each genotype. Cells were grown to a density of 1×10^7 to 3×10^7 cells per ml in YPD liquid medium and sonicated briefly to disperse clumps, and appropriate dilutions were plated on YPD. The plates were exposed to the indicated doses of radiation, and surviving colonies were counted after 3 days of incubation at 30°C. Unirradiated plates with the appropriate dilutions were included to determine the number of viable cells plated.

Heteroallelic recombination. The procedure for determining mitotic recombination frequencies was essentially as described by Hoekstra et al. (32). Single colonies from recently constructed diploids were used to inoculate a 5-ml culture of YPD at a concentration of 10^4 cells per ml. The cultures were grown at 30°C to a density of 2×10^7 cells per ml. After being harvested by centrifugation, cells were washed with sterile water, sonicated briefly to disperse the clumps, and plated on SC−Leu to select for recombinants and SC to determine the plating viability. Plates were scored after 3 days of incubation at 30°C, and the median frequency for six to nine experiments was used to determine the recombination rate by the method of Lea and Coulson (42).

Mapping and sequencing of *rfa1-D228Y*. Plasmid gap repair was used to localize precisely the mutation within the *RFA1* locus (56). Several overlapping gaps extending over various portions of the *RFA1* locus were created by digesting the centromere-based plasmid, pJM125, with *Nde*I or the following pairs of enzymes, *Mlu*I and *Aat*II, *Aat*II and *Eco*RI, or *Mlu*I and *Eco*RI (see Fig. 3A). After transformation of the linear molecules into W1188-2D, an *rfa1-D228Y* strain containing the *SUP4* duplication, several transformants from each gap repair were assayed for recombination.

The following overlapping sets of PCR primers, covering an ~850-bp region (nucleotides 113 to 962) to which the *rfa1-D228Y* mutation was localized, were synthesized: p1A, 5′-TGGGGCTAACAGCAACAGAA-3′, and p1B, 5′-GCATTGCTGGCGTTTCGTTTG-3′; p2A, 5′-CAGAGCATCCAAATGAAACC-3′, and p2B, 5′-CTCGGATTTCTCCAGAGGTA-3′; and p3A, 5′-CGTGGCACAATCAAAGAGGT-3′, and p3B, 5′-TTTGGATAATACCGAGGACG-3′. PCR amplification of a *rfa1-D228Y* strain with p1A and p3B generated a fragment that was gel purified and used for sequencing. The double-stranded sequence of the entire fragment was obtained by using each of the six primers in an automated 373A DNA Sequencer (Applied Biosystems).

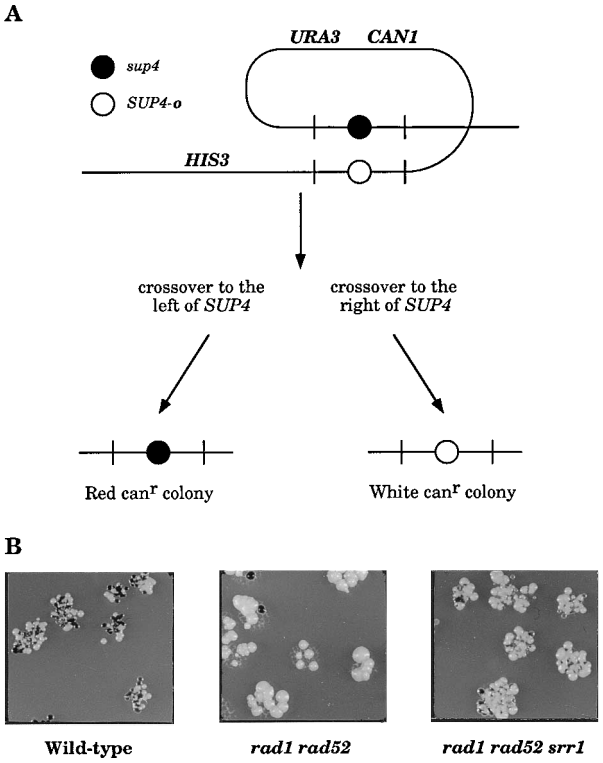


FIG. 1. *SUP4* direct-repeat assay. (A) The *SUP4* assay consists of a 2.4-kb duplication containing a yeast tyrosine tRNA gene, the *SUP4* locus. One copy of the tRNA gene is wild type (*sup4*) and the other is an ochre suppressor (*SUP4*-**o**). The repeats are separated by plasmid sequences containing two yeast selectable markers, *URA3* and *CAN1*. Recombinants that have lost the intervening plasmid sequences can be selected for on medium containing canavanine. Additionally, the presence of the ochre-suppressible red color marker *ade2-1* permits the visual detection of the *SUP4* allele remaining in the chromosome. Cells containing *sup4* form red colonies, while the presence of *SUP4*-**o** results in a white colony. (B) Papillation phenotypes of wild-type, *rad1 rad52*, and *rad1 rad52 srr1* colonies after replication to medium containing canavanine.

Biochemical analysis of RP-A. Initial purifications were performed as described previously by Brill and Stillman (9). For protein quantitation and immunoprecipitation, cells were lysed in buffer A (25 mM Tris-HCl [pH 7.6], 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol) containing 150 mM NaCl by a glass bead method (27). Protein concentrations were determined by a Bradford assay (Bio-Rad). Polyclonal antibodies against the 69- and 36-kDa subunits of RP-A (kindly provided by Steve Brill and Bruce Stillman) were used for immunoprecipitation (27).

Protein samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)–14% polyacrylamide gels and electroblotted onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% nonfat dry milk and incubated with polyclonal antibody against either the 69- or the 36-kDa subunit of RP-A. Blots were washed, incubated with horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (Amersham), and developed with enhanced chemiluminescence detection reagents (Amersham) according to the manufacturer's instructions. The resulting signal was quantitated by densitometric analysis with a Computing Densitometer model 300A (Molecular Dynamics).

RESULTS

Isolation of a mutation that stimulates *rad1 rad52*-independent recombination. A chromosomal construct containing direct repeats (Fig. 1A) (49) was used to screen for mutations that increase *rad1 rad52*-independent recombination. This construct utilizes a duplication of the *SUP4* locus: one allele is a wild-type tRNA (*sup4*$^+$) and the other is an ochre suppressor (*SUP4*-o). The repeats are separated by plasmid sequences and two yeast markers, *URA3* and *CAN1*, which complement the chromosomal recessive alleles *ura3-1* and *can1-100,x*, respec-

tively. Cells containing the *CAN1* gene, which encodes the arginine permease, are unable to grow on medium containing canavanine, an arginine analog and cell poison. Recombination events that result in the loss of the intervening DNA between the two direct repeats generate Can^r Ura$^-$ cells. Additionally, the presence of the ochre-suppressible color marker, *ade2-1*, permits the identification of the *SUP4* allele that is left in the chromosome after recombination. Red colonies indicate the presence of the wild-type allele, and white colonies represent the presence of the *SUP4*-**o** allele. The frequency of deletions between the *SUP4* repeats can be estimated by the number of papillae generated after the colonies are replica plated onto canavanine-containing medium. Since there is a significant difference (35-fold) in deletion frequencies between wild-type and *rad1 rad52* strains (49), a decreased number of both red and white papillae are formed by *rad1 rad52* colonies after they are replica plated onto canavanine-containing medium (Fig. 1B). However, most of the white Can^r colonies in *rad1 rad52* strains result from forward mutations in the *CAN1* gene due to the elevated rate of mutation in these strains (38, 39, 80). Therefore, only the number of red papillae can be used to estimate levels of deletion formation from a replicated colony.

Over 7,000 mutagenized *rad1 rad52* colonies were screened, and two mutants that consistently exhibited increased levels of red papillae were identified. Genetic analysis was performed to determine if the phenotype resulted from a mutation in a single gene. Backcrossing the mutants to a *rad1 rad52* strain would have permitted all four spores from each tetrad to be analyzed for the mutant phenotype, that is, increased levels of recombination. However, diploids homozygous for *rad52* fail to sporulate. Therefore, each mutant was crossed to a *rad1* strain (W838-7D), and half-tetrad analysis was performed. One cross resulted in poor viability, and that mutant was not further analyzed. In the other cross, approximately 50% (16 of 28) of the *rad1 rad52* segregants exhibited increased papillae. This segregation pattern indicates that a mutation in a single gene is most likely responsible for the mutant phenotype. We named this mutation *srr1* for suppresses recombination in *rad* mutants.

***srr1* mutant strains grow slowly.** During the segregation analysis, we noticed that strains containing the *srr1* mutation on its own exhibit a slow-growth phenotype, as evidenced by smaller colonies. Measurement of the growth rate in liquid medium indicated that *srr1* haploids have a doubling time of 140 min compared with the 90-min doubling time of wild-type strains. To analyze further the growth defect of *srr1* strains, cells from wild-type and *srr1* cultures were synchronized in G_1 by α-factor arrest. Every 30 min after release from the block, samples were analyzed by flow cytometry for their nuclear content. The *srr1* cells maintained a 2C DNA content 30 min longer than wild-type cells (data not shown). This suggests that the increased doubling time can be attributed to a late-S/G_2 delay.

The *srr1* mutation results in an increase in deletions that is *RAD52* independent. To characterize the interaction of *srr1* with *rad1* and *rad52*, the deletion frequencies between the *SUP4* direct repeats in various mutant combinations were quantitated. As reported previously for this assay, we found that deletions are synergistically reduced 35-fold in *rad1 rad52* strains (49) (Table 2). The *srr1* mutation restores recombination in the *rad1 rad52* background to wild-type levels. When separated from *rad1* and *rad52*, the *srr1* mutation on its own results in a 15-fold increase in recombination compared with that of the wild type. This increase is partially dependent on *rad1*, since recombination in a *rad1 srr1* background is reduced but still slightly elevated compared with the level observed in *rad1* strains ($P \leq 0.01$). However, the deletion frequency in *rad52 srr1* double mutants is not statistically different ($P = 0.34$) from that of *srr1* on its own, indicating that the hyperrecombination phenotype of *srr1* is *RAD52* independent.

TABLE 2. Effects of *srr1* on *SUP4* direct-repeat recombination

Relevant genotype[a]	Recombination frequency (10^{-5})[b]	Relative frequency[c]	*CAN1* mutation rate (10^{-6})[d]
Wild type	13 ± 0.2	1	0.9
srr1	189 ± 54	15	4.7
rad1	28 ± 13	2	4.5
rad1 srr1	61 ± 32[e]	5	8.7
rad52	3.9 ± 1.4	0.3	3.3
rad52 srr1	150 ± 76[f]	12	6.7
rad1 rad52	0.4 ± 0.2	0.03	8.7
rad1 rad52 srr1	14 ± 4.9	1	8.6

[a] Complete genotypes of the strains used are listed in Table 1.
[b] Recombination frequencies and significant differences were determined as described in Materials and Methods. Standard deviations are $\sigma n - 1$ values.
[c] Frequency of recombination relative to that of the wild type.
[d] Determined as described in Materials and Methods.
[e] The recombination frequency for *rad1 srr1* is significantly different ($P \leq 0.01$) from that of *rad1*.
[f] The recombination frequency for *rad52 srr1* is not significantly different ($P = 0.34$) from that of *srr1*.

***srr1* strains are UV sensitive and display a mutator phenotype.** Since *srr1* suppresses the recombination defect of *rad1 rad52* mutants, we examined its effect on the UV sensitivities of *rad1* strains and the X-ray sensitivities of *rad52* strains. Figure 2 shows that the *srr1* mutation does not suppress the radiation sensitivity of either *rad1* or *rad52* strains. Additionally, *srr1*

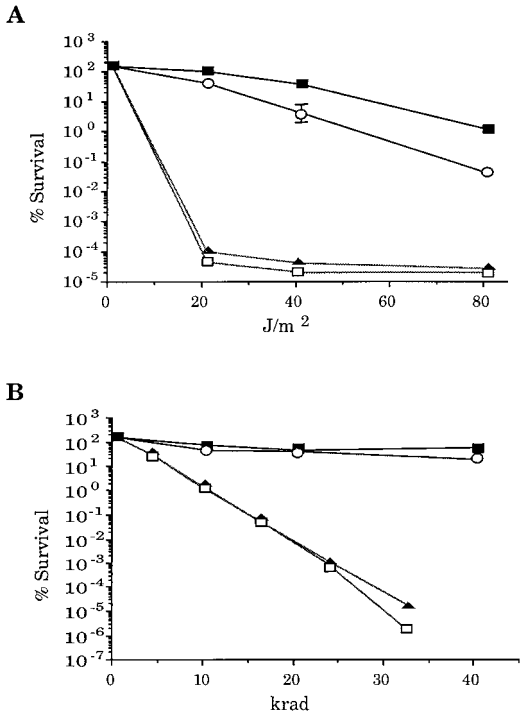


FIG. 2. Viability of wild-type (■) and *srr1* (○) strains after exposure to various amounts of radiation. (A) Survival curve after exposure to UV irradiation. □, *rad1*; ▲, *rad1 srr1*. (B) Survival curve after exposure to gamma irradiation. □, *rad52*; ▲, *rad52 srr1*.

TABLE 3. Interchromosomal recombination in *srr1* mutants

Relevant genotype[a]	Recombination rate (10^{-6})[b]	Relative rate[c]
Wild type	6.3	1.0
SRR1/srr1	5.0	0.8
srr1/srr1	1.0	0.16

[a] Complete genotypes of the strains used are listed in Table 1.
[b] Recombination rates were determined as described in Materials and Methods.
[c] Rate of recombination relative to that of the wild type.

mutant strains are not X ray sensitive. However, they are slightly UV sensitive, exhibiting a reproducible 10-fold decrease in viability at doses of 40 to 80 J/m^2.

Next, we measured the forward mutation rate in the *CAN1* gene for each of the genotypes described in Table 2. The number of forward mutations in *CAN1* was determined by counting Can^r Ura$^+$ colonies generated in each genetic background (see Materials and Methods). In *srr1* strains, the mutation rate is elevated approximately fivefold compared with those of wild-type strains (Table 2). This increase in mutation rate is similar to that observed in either the *rad1* or the *rad52* strain.

Heteroallelic recombination is decreased in *srr1* mutant strains. Having shown that the *srr1* mutation causes an increase in intrachromosomal recombination, we next examined its effect on interchromosomal recombination. The frequency of heteroallelic recombination between two *leu2* alleles in wild-type, heterozygous *srr1*, and homozygous *srr1* diploids was determined (Table 3). The rates of recombination in *srr1* diploids are reduced sixfold compared with that of the wild type. The rate observed in *srr1* heterozygous diploids is not significantly different ($P = 0.45$) from that of the wild type. Thus, a mutation that causes an increase in direct-repeat recombination has the opposite effect on interchromosomal recombination between heteroalleles.

The *srr1* mutation is partially dominant for direct-repeat recombination. Wild-type and heterozygous *srr1* diploids behave similarly in the heteroallelic recombination assay described above, suggesting that *srr1* is recessive. To determine whether *srr1* is recessive for direct-repeat recombination, deletion frequencies in diploids of the appropriate genotypes were measured (see Materials and Methods) (Table 4). Wild-type, heterozygous *srr1*, and homozygous *srr1* diploids were constructed. Additionally, each diploid was homozygous for *rad1 rad52* and heterozygous for the *SUP4* duplication. In diploid cells, canavanine-resistant colonies can result from deletion events, chromosome loss events, or mitotic crossovers between the *SUP4* duplication and the centromere. To select against chromosome loss and mitotic crossover events, we included the tightly linked *HIS3* gene adjacent to the *SUP4* repeats. Thus, only cells that become canavanine resistant as a result of direct-repeat recombination retain the *HIS3* gene and can grow on canavanine-containing medium that lacks histidine.

TABLE 4. Effects of *srr1* on *SUP4* recombination in diploids

Strain	Relevant genotype[a]	Recombination frequency (10^{-5})[b]	Relative frequency[c]
W1052	*rad1 rad52 SRR1* *rad1 rad52 SRR1*	0.4 ± 0.2	1
W1092	*rad1 rad52 SRR1* *rad rad52 srr1*	0.7 ± 0.2[d]	1.8
W1096	*rad1 rad52 srr1* *rad1 rad52 srr1*	17.0 ± 5.6	42

[a] Complete genotypes of the strains used are listed in Table 1.
[b] Recombination frequencies and significant differences were determined as described in Materials and Methods. Standard deviations are $\sigma n - 1$ values.
[c] Frequency of recombination relative to that of W1052.
[d] The recombination frequency for W1092 was significantly different ($P \leq 0.01$) from that of W1052.

The results in Table 4 show that *rad1 rad52* diploids homozygous for *srr1* display a 42-fold increase in recombination compared with that of wild-type *SRR1* homozygotes. This increase is similar to that observed in haploids (Table 2). Diploids heterozygous for *srr1* display recombination frequencies that are barely elevated (1.8-fold) compared with those of *SRR1* homozygotes. However, the difference between these two values is statistically significant ($P \leq 0.01$), indicating that the *srr1* mutation is slightly dominant in its effect on direct-repeat recombination.

***SRR1* is an allele of *RFA1*, a gene that encodes the major single-stranded DNA-binding protein in *S. cerevisiae*.** Although the *srr1* mutation is partially dominant, the 24-fold difference in deletion frequencies observed between the *srr1* heterozygous and *srr1* homozygous diploids suggests that the wild-type copy could be cloned by complementation. A centromere-based genomic DNA library (64) was introduced into a *rad1 rad52 srr1* strain (W1072-6B), and a total of 3,500 transformants were screened for decreased levels of red papillae by being replica plated to medium containing canavanine. Three transformants that resulted in a plasmid-dependent decrease in papillation were identified. Plasmid DNA was rescued from the three transformants, and restriction analysis indicated that they all contain the same 12-kb insert (Fig. 3).

To show that the insert identified by these plasmids encodes *SRR1*, we first demonstrated that it was genetically linked to *srr1*. An 8-kb *Bam*HI-*Cla*I internal fragment was subcloned into an integrating vector (YIp5) containing *URA3*. To target this plasmid to the homologous chromosomal region, it was linearized within the insert DNA by digestion with *Mlu*I. The linear plasmid was transformed into a wild-type strain (W1112-19D), resulting in an *SRR1* strain containing a duplication of the 8-kb *Bam*HI-*Cla*I fragment flanking *URA3* (55). Four *URA3*-marked transformants were crossed to a *rad1 rad52 srr1* strain (W1072-6B), sporulated, and dissected. The recombination phenotype was scored for 40 *rad1 rad52* segregants. Twenty-three exhibited the increased-recombination phenotype associated with *srr1*, and none was Ura$^+$. The remaining 17 segregants exhibited the decreased-recombination phenotype associated with *SRR1*, and all were Ura$^+$. This indicates that the insert DNA in the complementing plasmid is genetically linked to the *srr1* locus.

Next, the smallest fragment capable of complementing *srr1* was identified by constructing a series of subclones. The results in Fig. 3 show that a 4.0-kb *Bam*HI-*Mlu*I fragment is the smallest complementing clone. Hybridization of a yeast chromosome blot with a radioactively labelled probe from this fragment localizes *SRR1* to chromosome I (data not shown). Additionally, tetrad analysis indicates that *srr1* is centromere linked, since it exhibits a 65% first-division segregation frequency (the ditype-to-tetratype ratio is 47:25). The availability of an extensive restriction and transcript map of this region of chromosome I (77) permitted the localization of *srr1* to a single transcript (*FUN3*). This gene had been previously cloned and shown to encode *RFA1* (replication protein A) (9, 30, 35a).

To verify that *srr1* is allelic to *RFA1*, two experiments were performed. First, a plasmid, pJM125, kindly provided by Steve

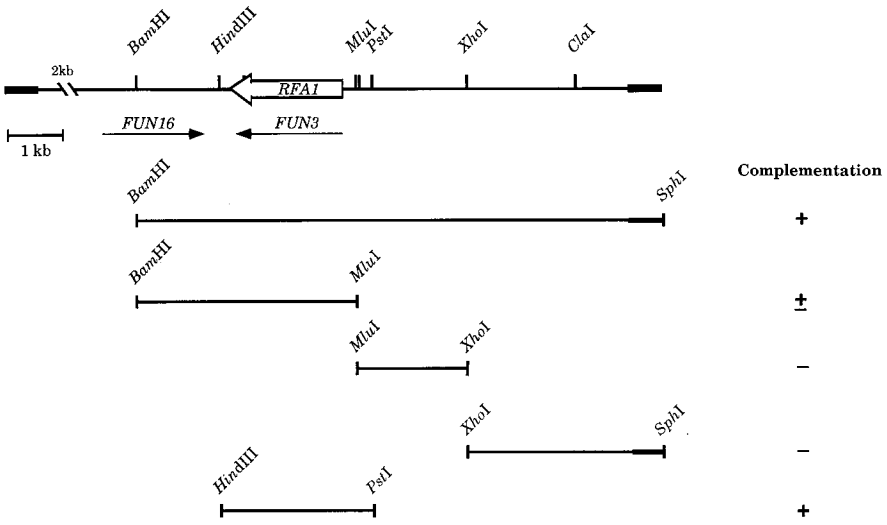
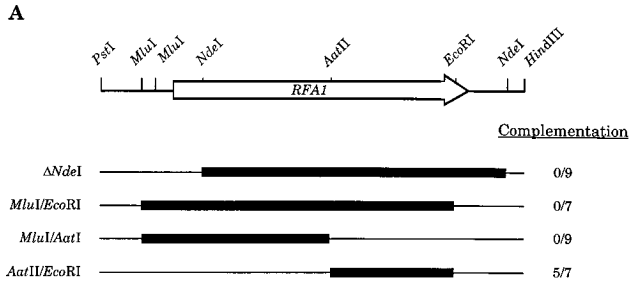


FIG. 3. Partial restriction map of the insert of pWJ579. The open reading frame of *RFA1* is depicted as an open arrow. The approximate locations of *FUN16* and *FUN3* transcripts, as previously determined by Steensma et al. (77), are also indicated. The subclones along with their abilities to complement the increased-recombination phenotype of *srr1* strains are depicted.

Brill, containing the entire coding sequence of *RFA1* on a 2.6-kb *Pst*I-*Hin*dIII fragment was introduced into an *srr1* strain (W1188-2D) and shown to complement the increased-recombination phenotype. Second, a 1.5-kb *Sca*I internal deletion within the open reading frame was shown to eliminate this complementation, demonstrating that *SRR1* and *RFA1* are alleles of the same gene. Subsequently, *srr1* will be referred to as *rfa1*.

The mutation in *RFA1* localizes to a conserved region of the protein. The location of the *rfa1* mutation was determined by gap repair (56). A centromeric plasmid (pJM125) containing wild-type *RFA1* was digested with restriction enzymes to create various-size gaps (Fig. 4A). These linear plasmids were transformed into an *rfa1* mutant strain (W1188-2D), and transformants were analyzed for their recombination phenotypes. When the mutation is located within the gapped region, the repaired plasmid duplicates the *rfa1* mutation, and increased levels of recombination are observed. On the other hand, when the mutation lies outside the gapped region, the repaired plasmid complements the mutation, and wild-type levels of recombination are observed. By this method, the *rfa1* mutation was localized to an 800-bp region flanked by *Nde*I and *Aat*II (Fig. 4A). This 800-bp region was amplified by PCR from the mutant strain, and the fragment was sequenced. A single nucleotide change, G→T, resulting in the replacement of aspartic acid 228 with tyrosine (D228Y), was detected at position 682 of the *RFA1* coding sequence. A comparison of the peptide sequences of the large subunit from *Homo sapiens*, *Xenopus laevis*, *Crithidia fasciculata*, and *S. cerevisiae* RP-A indicates that this residue is conserved in all four species (Fig. 4B) (2, 11, 17, 30).

The RP-A complex present in *rfa1-D228Y* mutant strains can bind to single-stranded DNA but is present in reduced amounts. To analyze the effect of the *rfa1-D228Y* mutation on the function of RP-A, the wild-type and mutant complexes were purified by the protocol of Brill and Stillman (9). Comparable amounts of total protein were loaded onto Affi-Gel Blue columns, washed, and eluted with 2.5 M NaCl–40% ethylene glycol. Fractions corresponding to the total protein peaks were combined, diluted to 0.5 M NaCl, and applied to single-stranded DNA-cellulose columns. Each column was washed first with 0.5 M NaCl, washed next with 0.8 M NaCl, and finally eluted with 1.5 M NaCl–50% ethylene glycol. Samples from each stage of the purification were separated by electrophoresis on an SDS–14% polyacrylamide gel, electroblotted, and



B

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Hs.rfa1      QNGS.SGMGS TVSKAYGASK TFGKAAGPSL SHTSGGTQSK VVPIASLTPY 193
Xl.rfa1      SAPP.PSMNR GTSKLPG... .....GGSL LNTPGGSQSK VVPIASLNPY 184
Sc.rfa1      SNAGVPDMLH SNSNLNANER KFANENPNSQ KTRP..... IFAIEQLSPY 193
Cf.rfa1      .......... .......... .......... MHQPGSHQ.. IQPIDSLTPF  18
Consensus    ---------- ---------- ---------- ---------- ---I--L-PY

Hs.rfa1      .QSKWTICAR VTNKSQIRTW SNSRAEGKLF SLELVDESGE IRATAFNEQV 242
Xl.rfa1      .QSKWTVRAR VTNKGQIRTW SNSRGEGKLF SIEMVDESGE IRATAFNEQA 233
Sc.rfa1      .QNVWTIKAR VSYKGEIKTW HNQRGDGKLF NVNFLDTSGE IRATAFNDFA 242
Cf.rfa1      LGGKWWIRAR VADKSDIRTW NKPTSQGKLF SFTLIDESAA IRATVFNDAV  68
Consensus    ----W-I-AR V--K--I-TW ------GKLF ---L-D-S-- IRAT-FN---

Hs.rfa1      DKFFPLIEVN KVYYFSKGTL KIANKQFTAV KNDYEMTFNN ETSVMPCEDD 292
Xl.rfa1      DKFFSIIEVN KVYYFSKGTL KIANKQYTSV KNDYEMTFNS ETSVIPCDDS 283
Sc.rfa1      TKFNEILQEG KVYYVSKAKL QPAKPQFTNL THPYELNLDR DTVIEECFDE 292
Cf.rfa1      DTFEPLVVNG QVYYFSGGQV KNANRRFSNV NNDYELTFDR ASEVILARQD 118
Consensus    --F------- -VYY-S---- --A---F--- ---YE--F-- ---V------
```

FIG. 4. Mapping and sequencing of *rfa1-D228Y*. (A) The *Pst*I-*Hin*dIII insert of pJM125 is indicated. The open reading frame of *RFA1* is depicted as an open arrow. Gapped plasmids were constructed by treatment with the indicated restriction enzymes, and the repaired regions are depicted as thick bars. Complementation was determined by the ability of the gapped plasmids to restore wild-type levels of recombination to *rfa1-D228Y*-transformed strains. (B) Alignment of deduced amino acid sequences of the large subunit of RP-A from humans (Hs.rfa1), *X. laevis* (Xl.rfa1), *S. cerevisiae* (Sc.rfa1), and *C. fasciculata* (Cf.rfa1). Alignment and consensus were determined with the Genetics Computer Group programs (16). Gaps have been introduced to maximize the alignment. The consensus contains identical or conserved amino acids that are shared by all four sequences. The aspartic acid altered by the *rfa1-D228Y* mutation is indicated as a boldface D.

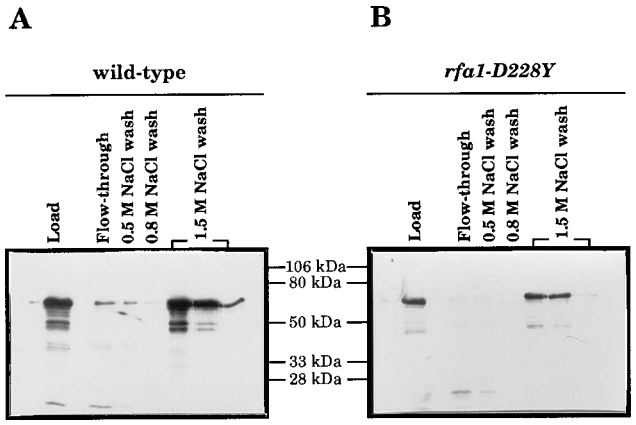

FIG. 5. Chromatography of RP-A from wild-type and *rfa1-D228Y* mutant strains. (A) Eluate from the wild type representing the pooled protein peak from an Affi-Gel column was diluted and applied to a single-stranded DNA column. The DNA column was washed with 2 volumes each of buffer A containing 0.5 M NaCl, 0.8 M NaCl, and 1.5 M NaCl–50% ethylene glycol. Samples from each stage of the purification were subjected to SDS-PAGE, immunoblotted, and probed with polyclonal antibody against p69. (B) A similar analysis was performed on total pooled protein from an *rfa1-D228Y* mutant strain. The positions of molecular mass standards are indicated.

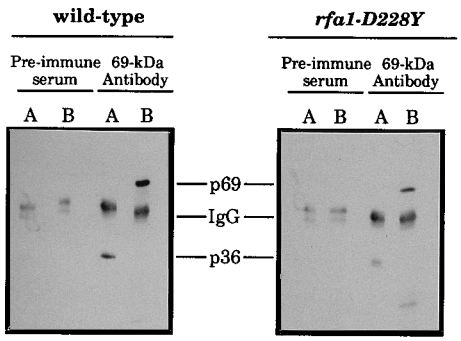

FIG. 6. Coimmunoprecipitation of the 69- and 36-kDa subunits of RP-A from wild-type and *rfa1-D228Y* mutant extracts. An equivalent amount of total protein from wild-type and mutant cell extracts was incubated with either preimmune serum or polyclonal antibody against the 69-kDa subunit of RP-A. In each case, the resulting immune complexes were precipitated with protein A beads and washed. The immunoprecipitates were then subjected to SDS-PAGE and electroblotted. Each sample lane was split in two and probed separately with antibodies against the 36-kDa subunit (A) and the 69-kDa subunit (B) of RP-A. The 55-kDa band present in all the lanes is antibody (immunoglobulin G [IgG]) that was precipitated with the protein A beads.

probed with polyclonal antibody against the 69-kDa (Fig. 5) and 36-kDa (data not shown) subunits of RP-A. In both strains, the majority of each subunit was detected in the eluate from the 1.5 M NaCl–50% ethylene glycol wash. This result indicates that the mutant RP-A complex, like the wild type, can bind single-stranded DNA. We did, however, consistently observe a reduced amount of RP-A subunits in the mutant compared with that in the wild type.

There are several possible explanations for the decreased amount of mutant RP-A detected after column chromatography. The decreased amount may directly reflect a lowered amount of the complex in the cell. Alternatively, it may be due to increased susceptibility of the mutant complex to degradation during purification, perhaps as a result of instability, and/or a decreased affinity of the mutant complex for the Affi-Gel blue resin. To distinguish amongst these possibilities, we determined the quantity of the p69 subunit present in crude extracts. An equivalent amount of total protein from wild-type and mutant cell extracts was separated by electrophoresis, blotted, and probed with polyclonal antibody against the 69-kDa subunit of RP-A (data not shown). Quantitation of the immunoblot revealed that the 69-kDa subunit was reproducibly reduced twofold in the mutant compared with the wild type. This result suggests that the lowered amount of RP-A complex observed in the mutant during purification results from decreased levels of complex in the cell.

To evaluate accurately the effect of the *rfa1-D228Y* mutation on the level of RP-A complex, the amount of 36-kDa subunit that coimmunoprecipitated with the 69-kDa subunit was determined. An equivalent amount of total protein from wild-type and mutant cell extracts was incubated with either preimmune serum or polyclonal antibody against the 69-kDa subunit of RP-A. The resulting immune complexes were precipitated with protein A beads and washed. Each immunoprecipitate was then subjected to SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted, and probed with antibodies against the 69- and 36-kDa subunits of RP-A (Fig. 6). Probing with antibodies against the 36-kDa subunit quantitates the amount of p36 associated with the 69-kDa subunit in the crude extract. Quantitation of the protein levels by densitometry revealed that the amount of p69 precipitated in the mutant extract is 47% of the amount obtained from the wild-type extract. Similarly, the amount of p36 coimmunoprecipitated from the mutant is 40% of the amount detected in the wild type. The same results were obtained when the reciprocal experiment was performed with antibody against the 36-kDa subunit to form the immunoprecipitate (data not shown). These results confirm that the total amount of RP-A complex in mutant extracts is reduced approximately twofold compared with that in the wild type.

Overexpression of *rfa1-D228Y*. The decreased amount of RP-A observed in the mutant extract may be responsible directly for the recombination and radiation phenotypes of *rfa1-D228Y* strains. To test this hypothesis, we measured the effect of overexpression of *rfa1-D228Y* on these processes. High-copy-number vectors based on the 2μm plasmid were used to overexpress the wild-type and mutant proteins. Mutant *rfa1-D228Y* and wild-type strains containing *SUP4* direct repeats were transformed with plasmids containing no insert (pRS425), wild-type *RFA1* (pWJ583), or mutant *rfa1-D228Y* (pWJ585). The transformants were used to measure deletion frequencies at *SUP4* or UV sensitivity.

Similar to the partially dominant phenotype we observed during our genetic studies (Table 4), wild-type strains overexpressing *rfa1-D228Y* exhibit a twofold increase in recombination (Table 5). However, the UV sensitivities of these strains are indistinguishable from that of the wild type. Next, we examined mutant *rfa1* strains, and as expected, overexpression of the wild-type allele completely complements the increased recombination frequency and the UV sensitivity phenotype. Additionally, overexpression of *rfa1-D228Y* in the same mutant strain suppresses hyperrecombination 2.4-fold (16×10^{-4} versus 6.7×10^{-4}). Moreover, when this strain is tested for UV sensitivity, overexpression of *rfa1-D228Y* completely suppresses the UV sensitivity phenotype. These results are compatible with the hypothesis that a decreased amount of RP-A contributes to the recombination and radiation phenotypes observed in *rfa1-D228Y* mutant strains. However, the slight semidominant effect on recombination observed in wild-type strains overexpressing *rfa1-D228Y* suggests that the mutant subunit has an altered function in addition to causing a decrease in the amount of complex in the cell.

TABLE 5. Effects of overexpression of *rfa1-D228Y* on *SUP4* recombination

Relevant genotype[a]	Plasmid[b]	Allele of *RFA1*[c]	Recombination frequency (10^{-4})[d]
Wild type	pRS425		1.1 ± 0.3
	pWJ583	*RFA1*	1.4 ± 0.2
	pWJ585	*rfa1-D228Y*	2.3 ± 1.1[e]
rfa1-D228Y	pRS425		16.0 ± 6.0
	pWJ583	*RFA1*	1.3 ± 0.3
	pWJ585	*rfa1-D228Y*	6.7 ± 2.6[f]

[a] Complete genotypes of the strains used are listed in Table 1.
[b] Denotes the 2μm-based plasmid present in the strain.
[c] Denotes the allele of *RFA1* contained on the 2μm-based plasmid present in the strain.
[d] Recombination frequencies and significant differences were determined as described in Materials and Methods. Standard deviations are $\sigma n - 1$ values.
[e] The recombination frequency for the wild-type strain containing pWJ585 was significantly different ($P \leq 0.05$) from that of the same strain containing pRS425.
[f] The recombination frequency for the *rfa1-D228Y* strain containing pWJ585 was significantly different ($P \leq 0.05$) from that of the same strain containing pRS425.

DISCUSSION

Two basic kinds of mitotic recombination assays have been employed with *S. cerevisiae* to study the genes responsible for regulating this biological process. Classically, heteroallelic recombination between alleles on homologous chromosomes has been used to characterize recombination mutations (for a review, see reference 58). These events are likely mediated via gene conversion and are dependent on *RAD52* function (32, 47, 48, 59) as well as other genes in the *RAD52* pathway (66). Over the past several years, recombination assays involving direct repeats have also been used to assess known recombination mutations and to isolate new ones (35, 37, 49, 63). Both replacement (sometimes referred to as gene conversion) and deletions (referred to as pop-outs) can be detected. Replacements require *RAD52* function, while deletions mainly occur independently of *RAD52* (35, 37, 65, 69, 80). Paradoxically, mutations in several other *RAD52* pathway genes (e.g., *rad51*, *rad54*, *rad55*, and *rad57*) exhibit hyperrecombination when deletions are measured (49, 72). Multiple mechanisms, including intrachromatid exchange, gene conversion associated with a crossover, unequal sister chromatid exchange, unequal sister chromatid gene conversion, single-strand annealing, and replication slippage, have been invoked to explain the generation of deletions (see Fig. 4 in reference 49). This implies that multiple pathways exist. In fact, there is both physical and genetic evidence for multiple pathways controlling deletion formation (37, 69, 80). For example, unequal sister chromatid exchange, which generates deletions and triplications, has been observed but accounts for only 3% of the total deletion events (37, 63a). In addition, *rad1 rad52* double mutants are synergistically reduced for direct-repeat recombination, implying the existence of alternate pathways (37, 69, 80).

In the work described here, we used a direct-repeat recombination assay to identify an alternate pathway by searching for mutations that suppress the recombination defect of *rad1 rad52* mutants. One mutation that restores wild-type levels of recombination between *SUP4* direct repeats was isolated. The wild-type copy of the suppressor was cloned by complementation of the increased-recombination phenotype and shown to encode *RFA1*. Rfa1 was originally identified as a single-stranded DNA-binding protein that stimulates in vitro the activity of Sep1, a presumed strand-exchange protein (29, 30). *RFA1* is an essential cell cycle-regulated gene that encodes the largest subunit of the protein complex RP-A (10). The trimeric complex consists of 69-, 36-, and 13-kDa subunits with homologs in humans *X. laevis*, *Drosophila melanogaster*, and *C. fasciculata* that have been identified (2, 17, 18, 52, 74, 84).

The *rfa1-D228Y* mutation that we identified results in the alteration of a conserved residue that lies in a region of significant homology among the four homologs (Fig. 4B) (2, 17, 84). Judging from the ability of the mutant complex to bind single-stranded DNA, this mutation does not result in a gross defect in DNA binding. However, further experiments are required to determine whether there is a slight alteration in the ability of the mutant complex to bind DNA. When wild-type and mutant extracts are purified under identical conditions, there is a twofold reduction of the RP-A complex in the mutant (Fig. 5 and 6). Overexpression of the *rfa1-D228Y* mutant allele suppresses UV sensitivity and partially suppresses hyperrecombination. This suggests that increasing the level of the mutant subunit leads to a higher concentration of associated complex in vivo, supporting the argument that the decreased levels of *rfa1-D228Y* cause its mutant phenotype. In addition, increasing the amount of the defective complex by overexpressing the mutant protein in a wild-type background results in the same semi-dominant-recombination phenotype seen in diploids (Tables 4 and 5). These results suggest that the *rfa1-D228Y* mutation might also disrupt a function of the RP-A complex such as cooperativity for DNA binding or a specific interaction with another protein(s).

Clues for the role of the RP-A complex come from in vitro studies as well as the functional homology it shares with bacterial SSB protein. The complex itself was originally identified from extracts of human cells by a simian virus 40 in vitro replication assay and was subsequently shown to be involved in preinitiation and elongation of DNA replication (19, 36, 83, 88). Human RP-A has also been implicated in DNA repair, since extracts from cells depleted for RP-A are impaired for in vitro nucleotide excision repair (14, 15, 73). In bacteria, SSB protein is involved in both of these processes, interacting directly with components of replication and repair (45, 51, 68). In addition, bacterial SSB plays a role in recombination by stabilizing single-stranded regions generated during strand exchange (41).

The pleiotropic phenotype of *rfa1-D228Y* strains may be explained by the disruption of the proper functioning of RP-A. For example, UV sensitivity may result from defective excision repair caused by a faulty interaction between RP-A and the repair machinery. The mutator effect would follow from the subsequent stimulation of error-prone repair. Alternatively, the mutator phenotype may be explained by an inability of the RP-A complex to interact properly with the replication apparatus, causing an increase in the level of misincorporated nucleotides. Such a disruption of replication would also account for the slow growth of *rfa1-D228Y* strains. Similarly, an impaired replication apparatus may generate lesions that result in increased recombination.

There is precedent for elevated recombination in strains mutated for genes involved in DNA replication (*cdc9*-DNA ligase, *cdc2*-DNA polymerase δ, *cdc17*-DNA polymerase α) (3, 28, 46, 53). However, unlike *rfa1-D228Y* mutants, the increased direct-repeat recombination in these replication mutants is *RAD52* dependent (3, 28). In addition, the replication mutants increase heteroallelic recombination, while the *rfa1-D228Y* mutation causes a decrease. These differences make it unlikely that the recombination effect observed in *rfa1-D228Y* is due simply to a replication defect.

Alternatively, the recombination phenotype of *rfa1-D228Y* strains may be the result of a direct role for RP-A in recom-

bination rather than simply the generation of more lesions. For example, the mutant RP-A complex may interfere with the normal processing of recombination intermediates, shunting them to another pathway. Thus, the increase in deletion formation exhibited by *rfa1-D228Y* may be the result of recombination intermediates from a gene conversion (nondeletion) pathway being channelled to a deletion pathway (1, 22). A direct role for RP-A in recombination is supported by the recent identification of another *rfa1* point mutation by Firmenich et al. (20). Their mutation, *rfa1-G77D*, results in a deficiency for recombinational repair after the induction of DNA damage.

Finally, we return to the question of how *rfa1-D228Y* suppresses the recombination defects exhibited by *rad1 rad52* mutants. These double mutants are deficient for direct-repeat recombination because of blocks in alternate pathways. In the absence of *RAD52*, most direct-repeat recombination is thought to proceed via a single-stranded annealing pathway (57). This pathway requires *RAD1*/*10* endonuclease function to remove nonhomologous ends to permit ligation (21). It is likely that the extensive single-stranded tails in this intermediate are substrates for wild-type RP-A. We propose that the mutant RP-A complex is unable to bind efficiently these single strands. This would permit other endonucleases or an exonuclease to degrade the DNA and generate the homologous ends required to process the intermediate. Such an alternative degradation pathway would bypass the *rad1* block in the double mutant.

Activation of this alternative degradation pathway may also explain why heteroallelic recombination is reduced. The processing of interchromosomal events leading to heteroallelic recombination likely generates single-stranded regions, a substrate for wild-type RP-A, during strand invasion. In an *rfa1-D228Y* mutant, these single strands would be rendered more susceptible to nuclease attack. The resulting degradation would increase the length of the repair tract (gap) so that it more often encompasses the adjacent mutant heteroallele. Repair, with this mutant information as a template, effectively reduces the number of events that generate prototrophs. This would lead to the decreased heteroallelic recombination frequencies observed. To test this model, we are currently investigating the lengths of repair tracts in our mutant.

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