An Allele of *RFA1* Suppresses *RAD52*-Dependent Double-Strand Break Repair in *Saccharomyces cerevisiae*

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ABSTRACT

An allele of *RFA1*, the largest subunit of the single-stranded DNA-binding complex RP-A, was identified as a suppressor of decreased direct-repeat recombination in *rad1 rad52* double mutants. In this study, we used two *LEU2* direct-repeat assays to investigate the mechanism by which the *rfa1-D228Y* allele increases recombination. We found that both intrachromatid and sister chromatid recombination are stimulated in *rfa1-D228Y* strains. In a *rad1 rad52* background, however, the majority of the increased recombination is caused by stimulation of deletion events by an intrachromatid recombination mechanism that is likely to be single-strand annealing. Studies in which an *HO* endonuclease cut was introduced between the two *leu2* copies indicate that the *rfa1-D228Y* mutation partially suppresses the *rad52* defect in recovering recombination products. Furthermore, molecular analysis of processing and product formation kinetics reveals that, in a *rad52* background, the *rfa1-D228Y* mutation results in increased levels of recombinant products and the disappearance of large single-stranded intermediates characteristic of *rad52* strains. On the basis of these results, we propose that in the absence of wild-type *Rad52*, the interaction of RP-A with single-stranded DNA inhibits strand annealing, and that this inhibition is overcome by the *rfa1-D228Y* mutation.

RECOMBINATION between repeated sequences is thought to be the major mechanism governing the evolution of multigene families, as well as alterations of genome structure (Edelman and Gally 1970). However, recombination between dispersed homologous sequences can also lead to deleterious genomic rearrangements such as deletions, inversions, translocations, and amplifications (Haluska *et al.* 1986; Fukuchi *et al.* 1989). These types of rearrangements have been suggested to be associated with several human diseases including hypercholesterolemia (Lehrman *et al.* 1986), type A severe hemophilia (Lakich *et al.* 1993), and the expansion of triplet repeats associated with many neurological diseases such as fragile *X* (Fu *et al.* 1991), myotonic dystrophy (Fu *et al.* 1992; Harley *et al.* 1992), spinal and bulbar muscular atrophy (La Spada *et al.* 1991; Caskey *et al.* 1992), and Huntington's disease (Huntington's Disease Collaborative Research Group 1993). In addition, increased genomic instability has been shown to be associated with several human maladies including ataxia telangiectasia, Fanconi anemia, and Bloom and Werner syndromes (Langlois *et al.* 1989; Monnat 1992; Meyn 1993; D'Andrea and Grompe 1997).

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In *Saccharomyces cerevisiae*, insight into the mechanisms involved in generating rearrangements has been obtained by analysis of recombination between directly repeated sequences (for review see Klein 1995). Genetic analysis of direct-repeat recombination has defined two pathways for deletion events. These alternate pathways are defined by the *RAD1* and *RAD52* genes. The *RAD1* gene, which was identified through a UV-sensitive mutation, is involved in the nucleotide excision repair pathway (Game and Cox 1971; Reynolds and Friedberg 1981). The Rad1 protein has been shown to form a complex with Rad10 that functions as a single-stranded DNA (ssDNA) endonuclease (Bardwell *et al.* 1992; Tomkinson et al. 1993). This endonuclease function is thought to be required for the removal of nonhomologous sequences in direct repeat recombination (Fishman-Lobell and Haber 1992). A mutation in the *RAD52* gene results in X-ray sensitivity (Resnick 1969), and the Rad52 protein has been shown to be involved in the repair of many kinds of double-strand break-induced recombinational repair events (Resnick and Martin 1976; Malone and Esposito 1980; Orr-Weaver *et al.* 1981). It has also been demonstrated that the Rad52 protein can promote strand annealing *in vitro* (Mortensen *et al.* 1996) as well as stimulate strand exchange by the Rad51 protein (Sung 1997a; Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998). In direct-repeat recombination, neither *rad1* nor *rad52* mutants significantly affect deletion events. However, *rad1 rad52* double mutants are dramatically decreased for this type of recombination (Klein 1988;

Schiestl and Prakash 1988; Thomas and Rothstein 1989b). Although the double mutants exhibit a large decrease in recombination rates, a low level remains, suggesting that additional pathways of recombination exist.

To identify alternate pathways of recombination that function in deletion formation, mutations that suppress the decreased levels of recombination in *rad1 rad52* strains were isolated (Smith and Rothstein 1995). Interestingly, the single mutation that was isolated is an allele of *RFA1*, the large subunit of the ssDNA-binding complex RP-A (Heyer et al. 1990; Brill and Stillman 1991). The mutation isolated is a missense allele that alters a conserved residue of the protein (D228Y). Biochemical and genetic analysis suggests that the phenotype observed in *rfa1-D228Y* mutants may be in part caused by the decreased levels of the RP-A complex within the cell. Characterization of its recombination phenotype indicates that the *rfa1-D228Y* mutation restores wild-type levels of deletion formation in *rad1 rad52* strains. In addition, it displays a hyper-recombinogenic phenotype on its own that is independent of *RAD52* and slightly dependent on *RAD1*.

In direct-repeat recombination, deletion events can occur by recombination between repeats located on the same chromosome (intrachromatid) or by an interaction between sister chromatids. Thus, the first approach taken to characterize the role of *rfa1-D228Y* in deletion formation was to determine whether intrachromatid, sister chromatid, or both types of recombination events were responsible for the elevated levels of recombination observed in an *rfa1-D228Y* background. Because the direct-repeat assay originally used to identify the *rfa1-D228Y* mutation does not allow these two events to be distinguished, recombination was examined using a pair of *LEU2* direct-repeat assays that separately monitor intrachromatid and sister chromatid recombination. Second, to define the mechanistic role of *rfa1-D228Y* in deletion formation, recombination was analyzed molecularly using an HO-induced deletion assay that allows the kinetics of intermediate processing and deletion formation to be monitored.

MATERIALS AND METHODS

Media: YPD, synthetic medium supplemented with dextrose (2% w/v), and synthetic medium supplemented with glycerol and lactic acid (3% v/v each) were made as described previously (Sherman *et al.* 1986; Sherman 1991), with the exception that synthetic medium contains twice the amount of leucine (60 mg/liter). 5-Fluoro-orotic acid medium (5-FOA) is synthetic complete (SC) medium with 50 mg/liter of uracil and 0.75 g of 5-FOA per liter (Boeke et al. 1984). To induce the *HO* gene, galactose (2% w/v) was added as a 20% solution to SC-Trp glycerol lactate medium.

Yeast strains and plasmids: Standard procedures were used for mating, sporulation, and dissection (Sherman *et al.* 1986). All *S. cerevisiae* strains used in this study are derivatives of W303-1A and W303-1B (Thomas and Rothstein 1989a). W303 has recently been shown to contain an allele of *RAD5* (*rad5-G535R*) in which the amino acid residue at position 535 is altered from a glycine (G) to an arginine (R) (Fan *et al.* 1996). All strains used in this study are from a *RAD5* derivative of W303 (R823), kindly provided by Hannah Klein (Table 1).

Both configurations of the *leu2* direct-repeat assay were constructed from pWJ567. This plasmid was created by the addition of *Bam*HI linkers to the *Pvu*II site of pWJ543, followed by the insertion of a 117-bp *Bst*YI-*Bam*HI fragment containing the *HO* endonuclease-cut site from pRK113 (kindly provided by R. Kostricken). pWJ543 contains the *leu2*Δ*BstEII* allele inserted between the *Nhe*I and *Sal*I sites of YIp5, an integrating vector containing *URA*3. To create the *leu*2 duplications, pWJ567 was linearized on either side of the *Bst*EII mutation by complete digestion with *Hpa*I or partial digestion with *Ase*I. The linearized fragments were gel purified and then transformed separately into J539, a strain containing a *leu2*Δ*EcoRI* allele. In this manner, strains containing both configurations of *leu2* direct repeats were obtained.

A W303 derivative containing *rad1::HIS5* was kindly provided by Naz Erdeniz. The *RAD1* gene disruption was created by inserting a 2.1-kb *Sal*I fragment containing *HIS5* into the *Cla*I-*Stu*I sites of *RAD1* by adding *Bgl*II linkers to both fragments. This replaces 2.1 kb of the *RAD1* reading frame with the *HIS5* disruption in the resulting plasmid, pWJ612. The *rad1* disrupted strain (U929) was then obtained by transforming a 2.9-kb *Sac*I-*Eco*O1091 fragment from pWJ612 into W1088-10D.

A *rad52* gene disruption was created by ligating the *HIS5* gene contained on a 2.0-kb *Sal*I (end-filled)-*Age*I fragment into the *Sph*I (end-filled)-*Age*I sites of the *RAD52* gene. This replaces 1.5 kb of the *RAD52* reading frame with the *HIS5* disruption in the resulting plasmid, pWJ600. The *rad52*-disrupted strain (U900) was then obtained by transforming a 3.8-kb *Sal*I fragment from pWJ600 into W1088-1A.

The *mat::HIS3* disruption was created by the PCR-based disruption method (Baudin *et al.* 1993; Smith *et al.* 1995). PCR primers were synthesized that contain 45 bp of target homology and 20 bp that is homologous to *HIS3*. The target homology of primer 1 is located within the W segment of the *MAT* locus, while primer 2 is homologous to sequences at the right boundary of the Z2 segment: primer 1, 5′-CCTCCAGG CGGAGTTAACAACTAGTAATACGGCATCCATGTTTGCG GATCCGCTGCACGGTCCTG-3′; primer 2, 5′-GATGCTAAG AATTGATTGTTTGCTTGAGTCTGAGTAATATCATATGCC TCGTTCAGAATGACACG-3′. PCR amplification of *HIS3* using these primers results in a 1.3-kb fragment that is used directly for target disruption. The presence of the *rad* and *mat* disruptions, as well as the configuration of the *leu2* duplications, was confirmed by analysis of genomic DNA blots (Southern 1975). Also, the presence of the *rad1* and *rad52* disruptions was verified by sensitivity of the mutant strains to UV or ionizing radiation, respectively.

The plasmid pJH132 (kindly provided by Jim Haber) contains the *GAL10::HO* fusion gene cloned into a *TRP1 ARS1 CEN4* vector, thus permitting galactose-regulated expression of the *HO* endonuclease (Jensen and Herskowitz 1984). Riboprobes were made from plasmids pWJ656 and pWJ644 containing the *Eco*RV-*Sal*I fragment of *LEU2* cloned into the *Sma*I-*Sal*I sites of pGEM-3Z and pGEM-4Z, respectively (Promega, Madison, WI).

DNA manipulations: Standard methods were used for recombinant DNA manipulations (Sambrook *et al.* 1989). *Escherichia coli* TG1 was transformed by a calcium chloride (Sambrook *et al.* 1989). Yeast cells were transformed by the lithium acetate method (Gietz *et al.* 1992). Genomic DNA from transformants and spontaneous recombinants was prepared from 5-ml YPD cultures according to the method of Hoffman and Winston (1987).

TABLE 1

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

Strain[a]	Genotype
W303-1B	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 rad5 trp1-1 ura3-1 rad5-G535R*
W1088-1A	*MAT***a** *his5*Δ
W1088-10D	*MAT*α *his5*Δ *lys2*Δ
W1479-11C[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII
W1479-30C[b]	*MAT*::*HIS3 lys2*Δ*Spe*I *RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rfa1-D228Y*
W1479-68C[b]	*MAT*::*HIS3 lys2*Δ*Spe*I *RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad1*::*HIS5*
W1479-18C[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad1*::*HIS5 rfa1-D228Y*
W1479-29A[b]	*MAT*::*HIS3 lys2*Δ*Spe*I *RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad52*::*HIS5*
W1479-80B[b]	*MAT*::*HIS3 lys2*Δ*Spe*I *RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad52*::*HIS5 rfa1-D228Y*
W1479-82A[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad1*::*HIS5 rad52*::*HIS5*
W1479-53D[b]	*MAT*::*HIS3 lys2*Δ*Spe*I *RAD5 leu2*Δ*Eco*RI::*URA3-HOcs*::*leu2*Δ*Bst*EII *rad1*::*HIS5 rad52*::*HIS5 rfa1-D228Y*
W1490-16A[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI
W1490-67D[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rfa1-D228Y*
W1490-82D[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad1*::*HIS5*
W1490-40B[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad1*::*HIS5 rfa1-D228Y*
W1490-83A[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad52*::*HIS5*
W1490-25C[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad52*::*HIS5 rfa1-D228Y*
W1490-79D[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad1*::*HIS5 rad52*::*HIS5*
W1490-241C[b]	*MAT*::*HIS3 RAD5 leu2*Δ*Bst*EII::*URA3-HOcs*::*leu2*Δ*Eco*RI *rad1*::*HIS5 rad52*::*HIS5 rfa1-D228Y*

[a] All strains used are derivatives of W303-1B (Thomas and Rothstein 1989a), and only genotypic differences are noted.

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

Neutral agarose gels were prepared and run in 0.5 × Tris-borate (Sambrook *et al.* 1989). Alkaline denaturing gels were run as described by McDonnell *et al.* (1977). Gels were transferred overnight to a Biotrans nylon membrane according to the alkaline technique described by the manufacturer (ICN). Radiolabeled DNA probes were prepared using a random priming kit (Pharmacia, Piscataway, NJ; Feinberg and Vogelstein 1983). RNA probes were prepared by the method of Melton *et al.* (1984) as modified by Promega. Hybridization and washing of filters were carried out according to standard procedures (Sambrook et al. 1989). Blots were analyzed and quantitated densitometrically on a Molecular Dynamics PhosphorImager 445SI.

Analysis of spontaneous *LEU2* direct-repeat recombination: Recombination rates and their standard deviations were calculated using the median method of Lea and Coulson (1949). Each rate was determined from trials of two to three different segregants, six to nine trials in total. Each trial represents a single colony from a YPD plate that was suspended in water, sonicated, and, after appropriate dilutions, plated on SC, synthetic medium lacking leucine (SC −Leu), and 5-FOA plates.

Independent recombinants were obtained by streaking colonies from a YPD plate onto SC −Leu medium and selecting a single colony from each streak. Recombinants that were unable to grow when replica plated to SC −Ura medium were not analyzed further and were categorized as deletion events. The remaining Ura^+ recombinants were subjected to molecular analysis to determine their configuration.

***HO*-induced recombination assay:** To monitor the efficiency of *HO*-induced deletions, cells of the appropriate genotype containing the proximal *LEU2* direct-repeat assay were transformed with the *HO* plasmid pJH132. Transformations were grown to midlog phase in −Trp glycerol lactate medium. This medium selects for the retention of the *HO* plasmid and derepresses the *GAL* structural genes. After the removal of an aliquot of cells for the zero time point, galactose was added to a final concentration of 2%, and the incubation was continued for 1 hr. Appropriate dilutions of the cells obtained both before and after *HO* induction were made in sterile water and plated on YPD and SC −Trp. The resulting colonies were replica plated to SC −Trp, SC −Ura, and SC −Leu media to monitor retention of the *HO* containing plasmid, deletion formation, and the number of Leu^+ recombinants, respectively. In addition, for each genotype, 20 Ura^- deletions were analyzed by genomic DNA blots to verify that they contained the proper molecular configuration (Southern 1975).

The procedure to monitor intermediates of double-strand, break-induced deletion formation was based on the protocol of Rudin and Haber (1988). Strains containing the *HO* plasmid pJH132 were grown to late log phase in −Trp −Ura medium, which selects for the retention of the *leu2* duplication and the *HO* plasmid. These cultures were diluted 1:100 in −Trp glycerol lactate medium and were grown at 30° to a final cell density of 0.7 to 1 × 10^7 cells per milliliter. After the removal of an aliquot of cells for the zero time point, galactose was added to a final concentration of 2%. The incubation was then continued with additional aliquots of cells removed at the given times. For the overnight time point samples, the final aliquot was centrifuged, resuspended in sterile water, and incubated overnight at 30°. DNA was prepared from each 30-ml sample (Hoffman and Winston 1987).

RESULTS

Spontaneous direct-repeat recombination

***LEU2* direct-repeat assays:** The original assay used to isolate *rfa1-D228Y* was limited in that only deletion events could be monitored. To extend the analysis, two

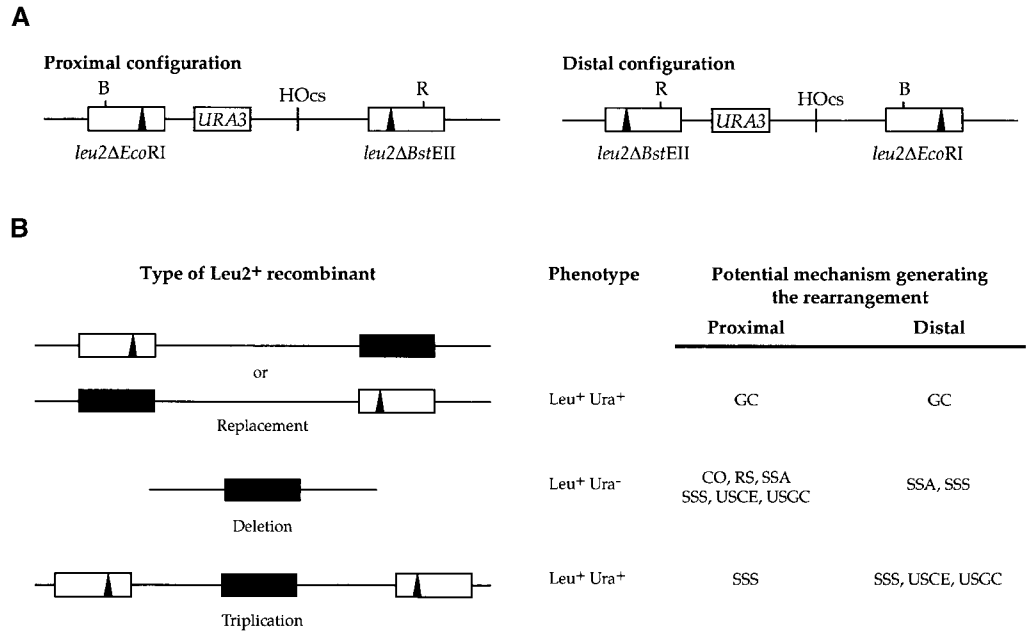


Figure 1.— (A) *LEU2* direct-repeat assays. Each construct contains a 2.4-kb duplication of the *LEU2* locus separated by 5.3 kb of plasmid sequences that include the *URA3* gene and the *HO* endonuclease-cut site. Restriction sites noted are *Bst*EII (B) and *Eco*RI (R). The *leu2*Δ*Bst*EII and *leu2*Δ*Eco*RI alleles are separated by ~600 bp. In the proximal configuration, the mutant sites are separated by the smaller distance, while in the distal configuration, the greater distance separates the sites. (B) Potential mechanisms generating Leu$^+$ recombinants. In this example, only the proximal configuration is depicted and the Leu$^+$ allele is denoted by a filled-in box. Replacement events, phenotypically Leu$^+$ Ura$^+$, contain the original parental configuration, with one of the duplicated genes containing wild-type information. In both configurations, these events result from gene conversion (GC). Deletion events, phenotypically Leu$^+$ Ura$^-$, contain a single wild-type copy of *LEU2*. Triplication events, phenotypically Leu$^+$ Ura$^+$, contain three copies of the repeated sequence, with the middle copy containing the wild-type information. The mechanisms contributing to deletion and/or triplication events include both intrachromatid [SSA, sister strand slippage (SSS), replication slippage (RS), and intrachromatid crossovers (CO)] and sister chromatid [unequal sister-chromatid exchange (USCE) and unequal sister chromatid gene conversion (USGC)] recombination events (see Klein 1995 for discussion of these mechanisms).

chromosomal inserts similar to those previously described (Klein 1988; Bollag and Liskay 1991; Sugawara and Haber 1992) were constructed to help differentiate between the multiple mechanisms that have been proposed to account for direct-repeat recombination (Figure 1A). Each insert contains a 2.4-kb duplication of the *LEU2* locus separated by 5.3 kb of plasmid sequences that includes the *URA3* gene and an *HO* endonuclease-cut site (Kostriken *et al.* 1983). One copy of *LEU2*, *leu2*Δ*Bst*EII, contains a 5-bp insertion at the *Bst*EII site at position 34, while the other *leu2*Δ*Eco*RI, results from a 4-kp insertion at the *Eco*RI site at position 637. Both mutations destroy the restriction site and create frameshift mutations that disrupt the open reading frame of *LEU2*. The two different configurations differ only in the relative positions of the *LEU2* alleles. In the "proximal" configuration, *leu2*Δ*Eco*RI is in the left repeat and *leu2*Δ*Bst*EII is in the right repeat; therefore, the mutant sites are closer (proximal). In the "distal" configuration, the alleles are reversed, resulting in a greater distance separating the sites (Figure 1A).

Both configurations can detect three types of spontaneous Leu$^+$ recombinants: replacements (commonly referred to as gene conversions), deletions, and triplications (Figure 1B). All three can be distinguished by genomic DNA blots. Replacement events, which are likely to occur via gene conversion, are unaffected by the position of the mutant sites and, therefore, occur at the same rate in both configurations (Klein 1988). In contrast, the mechanisms that contribute to deletion and triplication events are influenced by the differential positioning of the *leu2* alleles (Figure 1B, see Klein 1995 for a discussion of mechanisms). In the proximal configuration, deletions result from both intrachromatid and sister chromatid recombination, while triplications are rarely observed. In the distal configuration, intrachromatid recombination produces deletions and sister chromatid events generate triplications.

The increased levels of recombination in *rfa1-D228Y* mutants result from a stimulation of both intrachromatid and sister chromatid recombination: The spontaneous rate of leucine prototroph formation was determined in both proximal and distal configurations for each strain. Also, the distribution of deletion, replacement, and triplication events was determined by the molecular characterization of 20–60 independent Leu$^+$ recombinants per genotype (see materials and methods).

Analysis of direct-repeat recombination in wild-type and *rfa1-D228Y* strains indicates that the rate of Leu$^+$ formation in *rfa1-D228Y* strains is elevated in both the proximal and distal configurations (12- and 3-fold, respectively) compared to wild type (see Tables 2 and 3). This increase is consistent with the hyper-recombination phenotype of *rfa1-D228Y* strains described previously (Smith and Rothstein 1995). In the proximal configuration, the majority of the stimulated recombination is caused by an increase in the rate of deletions, which

TABLE 2

Distribution of Leu$^+$ recombinants in the proximal configuration

	Deletions		Replacements		Triplications		
Genotype[a]	Events[b]	Rate[c]	Events[b]	Rate[c]	Events[b]	Rate[c]	Leu$^+$ rate[d]
Wild type	60 (24)	1.2	40 (16)	0.8	0.0	<0.05	2.0 ± 0.8
rfa1-D228Y	87 (35)	21	13 (5)	3.0	0.0	<0.05	24.0 ± 9.0
rad1	38 (15)	0.5	59 (23)	0.8	3 (1)	0.04	1.4 ± 0.5
rad1 rfa1-D228Y	51 (20)	1.8	49 (19)	1.7	0.0	<0.09	3.5 ± 1.0
rad52	95 (56)	0.1	3 (2)	0.003	2 (1)	0.002	0.1 ± 0.03
rad52 rfa1-D228Y	100 (60)	4.8	0.0	<0.08	0.0	<0.08	4.8 ± 1.4
rad1 rad52	68 (23)	0.006	32 (11)	0.003	0.0	<0.0003	0.009 ± 0.004
rad1 rad52 rfa1-D228Y	90 (36)	0.6	10 (4)	0.07	0.0	<0.02	0.7 ± 0.3

[a] The complete genotypes of the strains are listed in Table 1.
[b] Percentage of Leu$^+$ prototrophs for each genotype. The number within parentheses represents the actual number of Leu$^+$ prototrophs detected.
[c] The rate ($\times 10^{-5}$) is determined from the Leu$^+$ rate multiplied by the percentage of events in each class.
[d] Rates of total Leu$^+$ recombination ($\times 10^{-5}$) are given as the mean ± SD (Jinks-Robertson and Petes 1986).

is elevated 18-fold compared to wild-type strains. In the distal configuration, however, *rfa1-D228Y* strains display an increase in both deletion and triplication events, 30- and 10-fold, respectively. Thus, while the substantial increase in deletion events in both orientations indicates that an intrachromosomal recombination mechanism is stimulated, the increase in triplications in the distal configuration suggests that sister chromatid events also contribute to the elevated recombination observed in *rfa1-D228Y* strains.

The *rfa1-D228Y*-stimulated levels of intrachromatid and sister chromatid recombination display a differential dependence on the *RAD1* and *RAD52* genes: Analysis of recombination in the absence of *RAD1* or *RAD52* indicates that the increased rate of Leu$^+$ formation observed in *rfa1-D228Y* strains is partially dependent on both of these genes (Tables 2 and 3). In *rad1 rfa1-D228Y* double mutants, the rate of Leu$^+$ prototroph formation in the proximal and distal configurations is decreased seven- and twofold, respectively, compared to *rfa1-D228Y* strains. The decrease is largely caused by a reduction in deletion events (12- and 23-fold for the proximal and distal configurations, respectively). In *rad1 rfa1-D228Y* strains, deletion events occur at a level similar to that observed in *rad1* mutants, indicating that the intrachromatid recombination pathway(s) stimulated by *rfa1-D228Y* is largely *RAD1* dependent.

In *rad52 rfa1-D228Y* strains, Leu$^+$ prototroph formation is decreased in both configurations compared to *rfa1-D228Y*: 5-fold in the proximal and 36-fold in the distal configuration (Tables 2 and 3). The rate of deletion events in the proximal and distal configurations in

TABLE 3

Distribution of Leu$^+$ recombinants in the distal configuration

	Deletions		Replacements		Triplications		
Genotype[a]	Events[b]	Rate[c]	Events[b]	Rate[c]	Events[b]	Rate[c]	Leu$^+$ rate[d]
Wild type	3 (2)	0.03	90 (54)	1.0	7 (4)	0.08	1.1 ± 0.3
rfa1-D228Y	26 (15)	0.9	53 (31)	1.9	21 (12)	0.8	3.6 ± 1.3
rad1	2 (1)	0.01	81 (46)	0.4	17 (10)	0.09	0.5 ± 0.1
rad1 rfa1-D228Y	2 (1)	0.04	76 (45)	1.4	22 (13)	0.4	1.8 ± 0.7
rad52	15 (9)	0.005	17 (10)	0.005	68 (41)	0.02	0.03 ± 0.01
rad52 rfa1-D228Y	61 (34)	0.06	9 (5)	0.009	30 (17)	0.03	0.1 ± 0.06
rad1 rad52	0	<0.0001	59 (28)	0.005	41 (20)	0.003	0.008 ± 0.003
rad1 rad52 rfa1-D228Y	19 (10)	0.02	55 (29)	0.06	26 (14)	0.03	0.1 ± 0.05

[a] The complete genotypes of the strains are listed in Table 1.
[b] Percentage of Leu$^+$ prototrophs for each genotype. The number within parentheses represents the actual number of Leu$^+$ prototrophs detected.
[c] The rate ($\times 10^{-5}$) is determined from the Leu$^+$ rate multiplied by the percent of events in each class.
[d] Rates of total Leu$^+$ recombination ($\times 10^{-5}$) are given as the mean ± SD (Jinks-Robertson and Petes 1986).

rad52 rfa1-D228Y strains, although decreased compared to *rfa1-D228Y* (4- and 15-fold, respectively), is still significantly elevated compared to *rad52* strains (48- and 12-fold, respectively). In contrast, triplication events in the distal configuration in *rad52 rfa1-D228Y* double mutants are reduced 27-fold to approximately the same levels observed in *rad52* single mutants. This suggests that the sister chromatid recombination pathway(s) stimulated by *rfa1-D228Y* is *RAD52* dependent.

The *rfa1-D228Y* mutation alters the synergistic decrease in deletions and triplications in *rad1 rad52* strains only in the distal configuration: In *rad1 rad52 rfa1-D228Y* strains, the rate of Leu$^+$ recombinants is 78- and 13-fold higher than that observed in *rad1 rad52* strains in the proximal and distal configurations, respectively. This increase is partly caused by an increase in replacement and triplication events (see Tables 2 and 3). However, deletion events display the greatest stimulation, 100-fold in the proximal and ~200-fold in the distal configuration.

Previous analysis of direct-repeat recombination demonstrated that deletions are synergistically decreased in *rad1 rad52* strains (Klein 1988; Schiestl and Prakash 1988; Thomas and Rothstein 1989b). Here we find that triplications are also synergistically reduced in *rad1 rad52*. Interestingly, *rad1 rad52 rfa1-D228Y* strains exhibit a synergistic reduction in deletions in the proximal configuration compared with *rad1 rfa1-D228Y* and *rad52 rfa1-D228Y* double mutants, while in the distal configuration, neither deletion nor triplication events display a similar reduction. Thus, in the distal configuration, the synergistic decrease of deletion and triplication events in *rad1 rad52* strains is suppressed by the *rfa1-D228Y* allele.

HO-induced direct-repeat recombination

Analysis of *HO*-induced direct-repeat recombination indicates that the *rfa1-D228Y* mutation increases the efficiency of repair in a *rad52* background: Because deletions are the most stimulated events in an *rfa1-D228Y* background, we used an assay that specifically monitors deletion formation and permits a physical analysis of the intermediates and products. Using the proximal configuration of the *LEU2* assay described previously (Figure 1A), recombination was initiated by a double-stranded break (DSB) at an *HO* endonuclease-cut site located between the *LEU2* repeats. Subsequent repair of this DSB occurs by recombination between the direct repeats and specifically results in a deletion event via an SSA mechanism (Sugawara and Haber 1992). To regulate the induction of the DSB, a plasmid containing a galactose-inducible promoter fused to the *HO* endonuclease gene was introduced into each strain. Cells that fail to repair the DSB break die, while cells that repair the DSB delete the *URA3* gene located between the *LEU2* repeats. Therefore, to measure the efficiency of repair, we divided the number of Ura$^-$ cells after *HO* induction by the total number of cells containing the *HO* plasmid before induction. Last, the generation of Leu$^+$ prototrophs, which likely reflects mismatch repair events in this assay, was also monitored.

TABLE 4

Effect of *rfa1-D228Y* on the efficiency of DSB-induced deletion events

Relevant genotype[a]	% Ura$^-$[b]	% Leu$^+$[c]
Wild type	72 ± 6	22 ± 4
rfa1-D228Y	73 ± 5	18 ± 3
rad1	34 ± 9	19 ± 6
rad1 rfa1-D228Y	35 ± 7	20 ± 2
rad52	3 ± 1	19 ± 4
rad52 rfa1-D228Y	21 ± 4	18 ± 8
rad1 rad52	0.1 ± 0.05	ND
rad1 rad52 rfa1-D228Y	8 ± 1	15 ± 6

[a] Complete genotypes of the strains used are listed in Table 1.

[b] A comparison of the number of Ura$^-$ cells present 60 min after *HO* induction with the number of Trp$^+$ cells present at the zero time point. Percentages are given as the mean ± SD of three to six trials.

[c] The percentage of Ura$^-$ cells present after *HO* induction that also display a Leu$^+$ phenotype. Percentages are given as the mean ± SD of three to six trials.

In wild-type strains, after an *HO*-induced DSB, 72% of the cells have undergone a deletion event (Table 4). In an *rfa1-D228Y* background, deletion formation after *HO* induction was 73%, a level similar to wild type. In *rad1* and *rad1 rfa1-D228Y* strains, similar levels of deletions were observed after *HO* induction, 34 and 35%, respectively. The reduced levels observed in the absence of *RAD1* likely result from the requirement for the Rad1/Rad10 endonuclease in the removal of nonhomology during recombination (Fishman-Lobell and Haber 1992). Analysis of *rad52* strains indicates that only 3% of the cells have undergone a deletion event after *HO* induction. This decrease is consistent with the previously described requirement for the *RAD52* gene product in the repair of almost all DSBs, including mating type switching and transformation with linear fragments (Resnick and Martin 1976; Malone and Esposito 1980; Orr-Weaver et al. 1981). Interestingly, in *rad52 rfa1-D228Y* strains, deletion formation increases to 21%. These results demonstrate that the *rfa1-D228Y* mutation can partially rescue the DSB repair defect observed in *rad52* mutants. The partial rescue phenotype was also observed in *rad1 rad52 rfa1-D228Y* triple mutant strains, as we found an 80-fold increase in deletion events (8%) compared to *rad1 rad52* strains (0.1%). Finally, in all genetic backgrounds tested, the level of Leu$^+$ prototrophs was between 15 and 22%, consistent with the idea that these arise from mismatch repair of the heteroduplex formed during SSA.

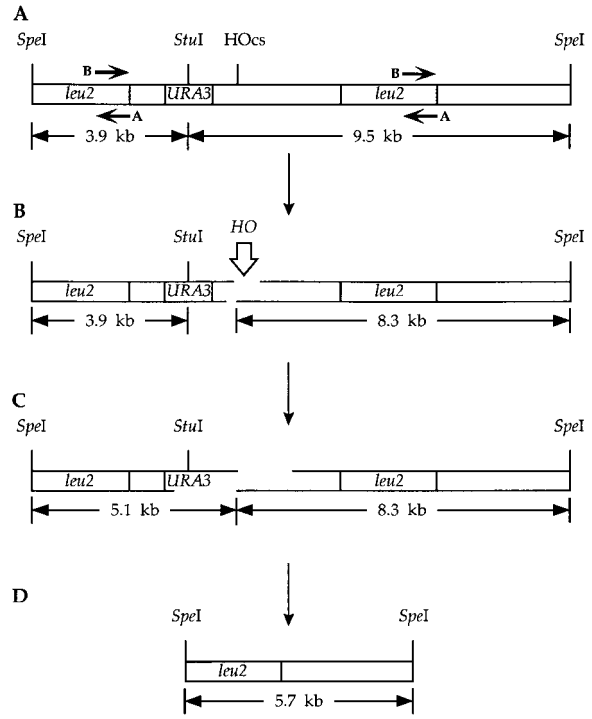


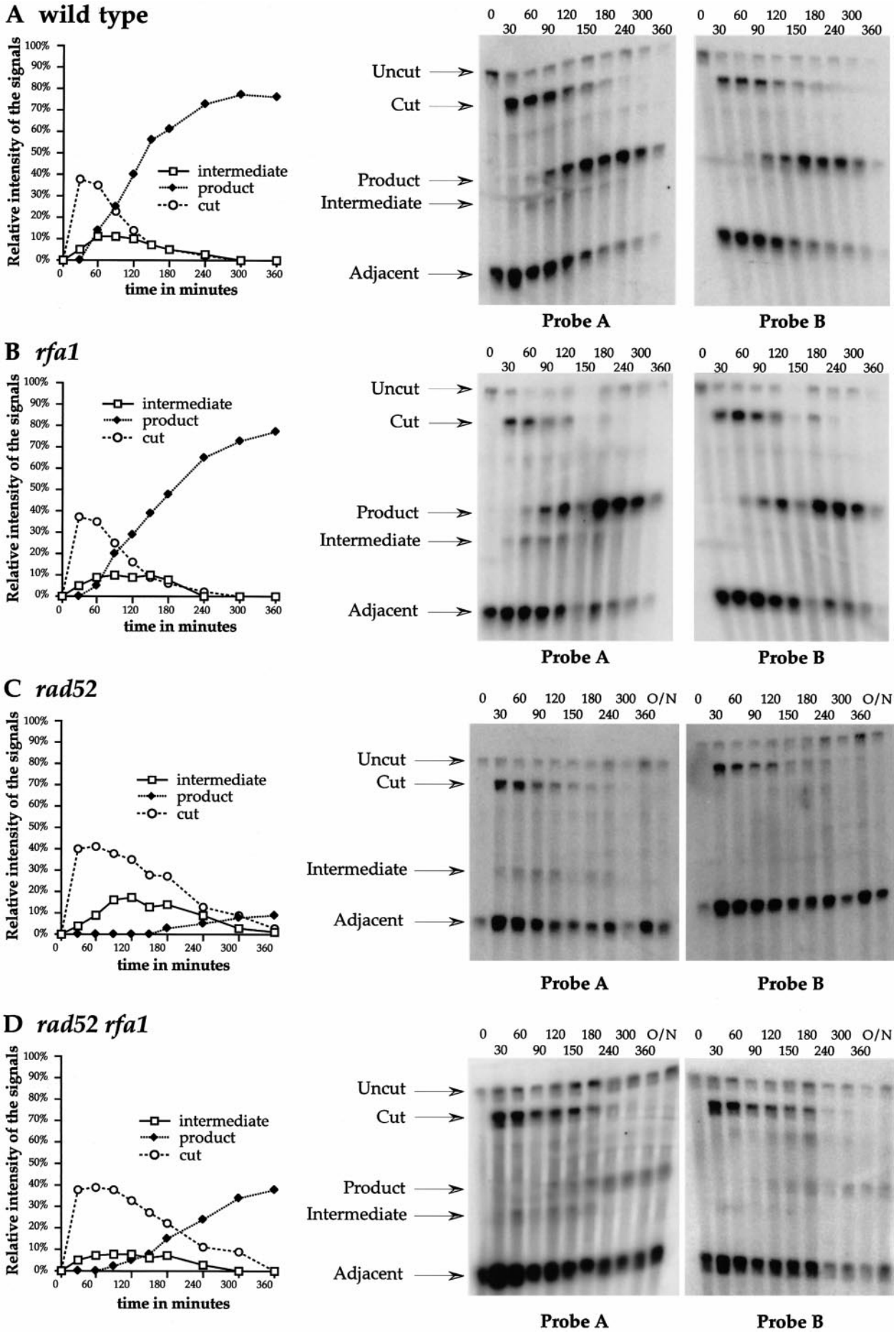
Figure 2.— Recombination intermediates generated during *HO*-induced deletion formation. (A) The arrows lettered A and B at *leu2* indicate riboprobes complementary to the top and bottom strands of DNA, respectively. The arrowheads indicate the direction of transcription. Before induction of the *HO* endonuclease, digestion of the DNA with the restriction enzymes *Spe*I and *Stu*I generates a 3.9-kb and a 9.5-kb fragment. (B) Induction of a DSB at the *HO*-cut site reduces the length of the distal fragment to 8.3 kb. (C) The subsequent processing of both sides of the DSB break by a 5′ to 3′ endonuclease results in the formation of extensive 3′ ssDNA tails. Because the restriction enzymes used in this analysis efficiently cleave only double-stranded DNA, a 5.1-kb ssDNA fragment is generated once the 5′ to 3′ exonucleolytic degradation has extended past the *Stu*I site. Also, because strand-specific probes are used for hybridization, the single-stranded intermediate is detectable only with probe A. (D) The formation of a recombinant product can be detected by the presence of a 5.7-kb fragment.

The *rfa1-D228Y* mutation alters processing of recombination intermediates in a *rad52* background: To examine the mechanism of *rfa1-D228Y* suppression of *rad52*-dependent recombination, we analyzed the processing of DSBs and the kinetics of deletion formation at the molecular level. After *HO* endonuclease induction, DNA was isolated from the cells at specific time points, digested with the restriction enzymes that flank the *HO* site, and electrophoresed on a denaturing gel. Hybridization of the resulting genomic blot with a *LEU2* sequence-specific probe allows the detection of fragments representing the intact assay, *HO*-cut intermediates, and the recombinant product (Figure 2). Previous studies of *HO*-induced recombination have demonstrated that after DSB formation, 3′ ssDNA tails are extended on both sides of the *HO*-cut site by 5′ to 3′ exonucleolytic degradation (White and Haber 1990; Sugawara and Haber 1992). As the degradation proceeds past the flanking restriction enzyme sites, cleavage is blocked and results in a longer single-stranded intermediate that is easily detected with a strand-specific probe (Figure 2).

DNA is isolated from wild-type strains before *HO* induction, and two fragments (3.9 and 9.5 kb) representing the intact, unrearranged assay are observed (Figure 3A). Thirty minutes after *HO* induction, an 8.3-kb *HO*-cut fragment is detected. This fragment is processed into a 5.7-kb recombinant product that is first detected after 1 hr. As a result of the loss of the adjacent *Stu*I site, a 5.1-kb single-stranded intermediate is observed at the 30-min time point. The level of this fragment reaches a maximum by 60 min and then slowly decreases. By 5 hr after induction, the reaction is complete, with the final amount of product reaching 77%. Virtually identical kinetics of deletion formation were also observed for *rfa1-D228Y* strains (Figure 3B).

In *rad52* strains, the level of product formation observed is significantly reduced, representing at most 10% of the total amount of signal detected (Figure 3C). The decreased efficiency of repair is also evidenced by the prolonged presence of both the ssDNA intermediate and the *HO*-cut fragment, which are detectable up to 6 hr after induction. In fact, the gradual decrease of these intermediates at later time points most likely results from degradation of ssDNA and/or double-stranded DNA. The kinetics of product formation are also delayed in *rad52* strains, as detectable levels of product are observed 2 hr later than in wild type (180 min). In contrast, repair of an *HO*-induced DSB is relatively efficient in *rad52 rfa1-D228Y* strains (Figure 3D). By 6 hr after induction, the product represents 40% of the total signal detected. However, there is a delay in processing in these double mutants because product formation occurs later (90–120 min after induction) and the intermediates persist longer.

The kinetics of strand degradation in *rad52* and *rad52 rfa1-D228Y* strains were examined in more detail by using an alternative restriction enzyme with a greater number of recognition sites around the *HO*-cut site (Figure 4). This permits the detection of a ladder of single-stranded intermediates caused by 5′ to 3′ degradation after the *HO*-induced DSB, which is characteristic of *rad52* strains (Sugawara and Haber 1992). At 30 min after an *HO*-induced DSB in a *rad52* strain, the first single ssDNA intermediate is observed (4.4 kb). By 2 hr after induction, a ladder of additional intermediates can be visualized. In *rad52 rfa1-D228Y* strains, the larger intermediates were not observed, and, throughout the time course, the only detectable ssDNA intermediate was the 4.4-kb fragment (Figure 4). This intermediate was first observed at the 30-min time point and persisted until ~5 hr after induction. The increased product formation seen in *rad52 rfa1-D228Y*, combined with the failure to observe large ssDNA intermediates in these


A wild type
Relative intensity of the signals
time in minutes
intermediate
product
cut
Uncut
Cut
Product
Intermediate
Adjacent
Probe A
Probe B
B rfa1
C rad52
D rad52 rfa1
O/N

double mutants, indicates that the *rfa1-D228Y* mutation suppresses both aspects of the *rad52* DSB repair defect.

DISCUSSION

In this study, we have used two approaches to define the mechanism by which direct-repeat recombination is stimulated in *rfa1-D228Y* mutant strains. First, we have examined spontaneous recombination using a pair of *LEU2* direct-repeat assays. This analysis indicates that the elevated levels of direct-repeat recombination observed in *rfa1-D228Y* strains result from an increase in both intrachromatid and sister chromatid recombination. In addition, suppression of the decreased recombination in *rad1 rad52* strains by the *rfa1-D228Y* mutation is mostly caused by stimulation of deletion events by intrachromatid recombination. Second, to determine the mechanistic role of *rfa1-D228Y* in intrachromatid recombination, processing of recombinants was monitored using an *HO*-induced deletion assay. This analysis demonstrates that the *rfa1-D228Y* mutation can partially rescue the DSB repair defect observed in a *rad52* background. Molecular characterization of intermediate and product formation in *rad52 rfa1-D228Y* strains indicates that in addition to increasing the efficiency of product formation, accumulation of large, single-stranded intermediates characteristic of *rad52* strains are no longer observed.

Analysis of spontaneous direct-repeat recombination using the distal and proximal configurations of the *LEU2* assay indicates that, in an *rfa1-D228Y* strain, both intrachromatid and sister chromatid recombination are stimulated. Additionally, these two types of recombination display a differential dependence on RAD1 and RAD52. Sister chromatid recombination in *rfa1-D228Y* strains is largely dependent on *RAD52*, while the elevated levels of deletion events display a strong dependence on *RAD1*. It is likely that the majority of the stimulated sister chromatid events observed in an *rfa1-D228Y* background are associated with gene conversions because it has been demonstrated previously that *RAD52* is required for most gene conversion events (Jackson and Fink 1981; Haber and Hearn 1985; Hoekstra *et al.* 1986; Malone *et al.* 1988; Gangloff *et al.* 1996). However, it is interesting to note that replacement events are not significantly increased in a *rfa1-D228Y* background; thus, if the *rfa1-D228Y* allele is stimulating gene conversion, this effect is limited to events associated with a crossover.

On the other hand, the stimulation of sister chromatid recombination may result from synthesis-dependent strand annealing (SDSA, Nassif *et al.* 1994; Pâques *et al.* 1998). In this process, a 3′ end created after a DSB invades the homologous sequences on the sister chromatid and primes DNA synthesis. Resolution subsequently occurs by unwinding the invading strand from the template and annealing with another DNA molecule from the other side of the DSB. In the case of direct repeats, triplications could be generated by repair of a DSB in the distal repeat that invades the proximal repeat on the sister chromatid. Although it has not yet been demonstrated, this mechanism is likely to depend on *RAD52*, as it involves strand invasion. Thus, SDSA can explain some of the stimulated events observed in a *rfa1-D228Y* background.

In contrast, the increased level of deletion events observed in an *rfa1-D228Y* background displays a strong dependence on *RAD1*, but only a partial dependence on *RAD52*. Previous studies have shown that *RAD1* is required for the removal of nonhomologous sequences in deletion formation, an important step in the recombination process of single-strand annealing (SSA, Fishman-Lobell and Haber 1992). It has also been demonstrated that SSA is only partially *RAD52* dependent (Ozenberger and Roeder 1991; Fishman-Lobell *et al.* 1992; Prado and Aguilera 1995). Thus, an SSA mechanism is most compatible with the stimulated deletion events observed in *rfa1-D228Y* strains.

The most dramatic effect of the *rfa1-D228Y* mutation was observed in a *rad1 rad52* background. Analysis of individual recombinants indicates that, in contrast to *rad1 rad52* strains, a synergistic decrease in triplications and deletions is not observed in *rad1 rad52 rfa1-D228Y* strains in the distal configuration. Of the two types of events, however, deletions display the greatest stimulation, 100–200-fold compared to *rad1 rad52* strains. This suggests that the suppression of decreased recombination in *rad1 rad52* strains by the *rfa1-D228Y* mutation is mostly caused by a stimulation of deletion events by an SSA mechanism.

To analyze further the role of *rfa1-D228Y* in deletion events, we examined the repair of an *HO*-inducted DSB located between the *leu2* direct repeats. Repair of this DSB occurs by recombination between the direct re-

Figure 3.— Kinetics of ssDNA and product formation. *HO*-induced deletion formation was analyzed in four backgrounds: (A) wild-type, (B) *rfa1-D228Y*, (C) *rad52*, and (D) *rad52 rfa1-D228Y*. Each strain contains the proximal configuration of the *LEU2* direct-repeat assay shown in Figure 1A. DNA was extracted at the times shown and digested with *Spe*I and *Stu*I, electrophoresed on a denaturing gel, blotted, and hybridized with a single-stranded probe, either probe A or probe B (see Figure 2). This analysis allows the detection of a 9.5-kb uncut fragment, on 8.3-kb *HO* endonuclease-cut fragment, a 5.7-kb product, a 5.1-kb ssDNA intermediate, and a 3.9-kb adjacent fragment. Because the intermediate is single-stranded, it is only detectable with one of the probes (probe A). The intensity of the *HO*-cut, product, and ssDNA intermediate bands are plotted as a fraction of the total intensity per lane. The mean values of two time courses are shown.

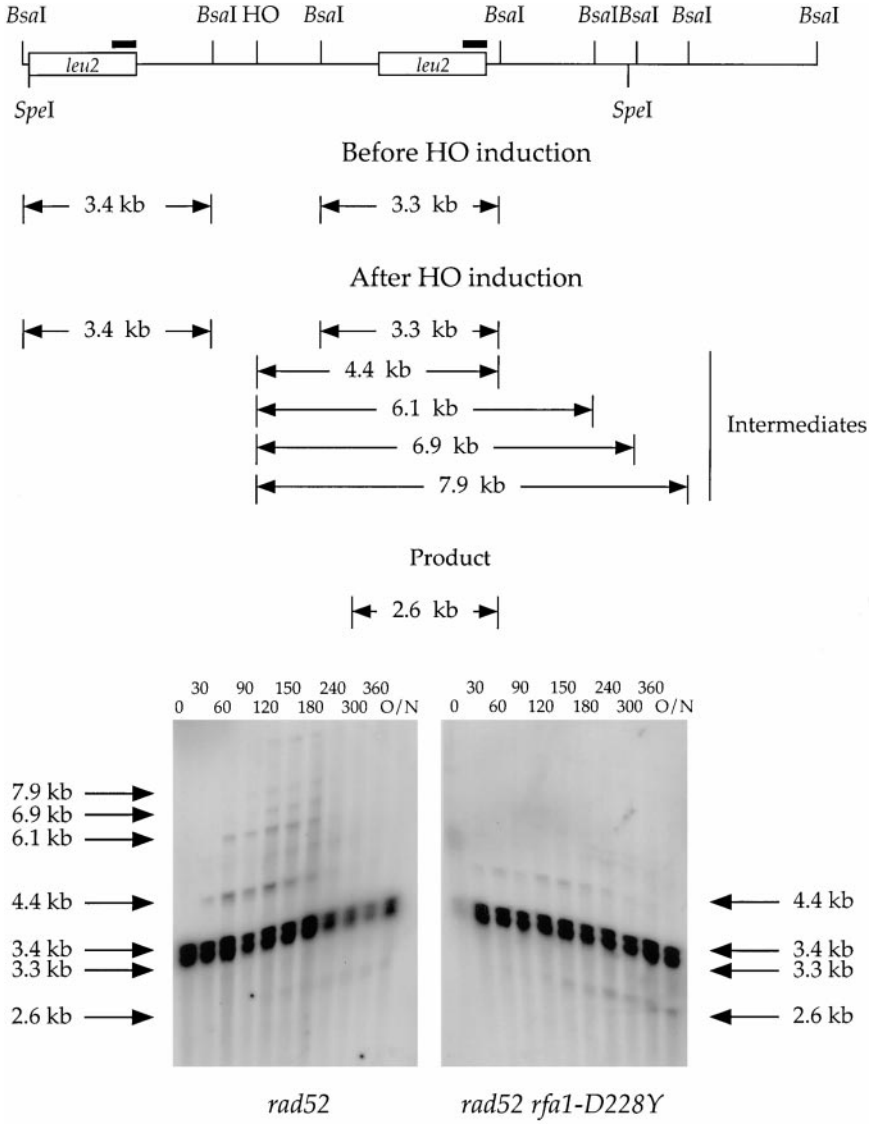


Figure 4.— Monitoring of extended 5′ and 3′ degradation in *rad52* and *rad52 rfa1-D228Y* strains. To allow the efficient detection of large ssDNA intermediates, DNA was digested with the restriction enzyme *Bsa*I. Before *HO* induction, 3.3- and 3.4-kb fragments are detected. Extensive 5′ and 3′ degradation after *HO* induction generates several ssDNA intermediates that range in size from 4.4 to 7.9 kb. Product formation can also be monitored and results in a 2.6-kb fragment. DNA was extracted at the times shown, digested with *Bsa*I, electrophoresed on a denaturing gel, blotted, and hybridized with probe B (see Figure 2). The numbers to the left and the right of the autoradiograms indicate the band sizes in kilobases.

peats and specifically results in a deletion event via SSA (Sugawara and Haber 1992). The *rfa1-D228Y* mutation partially rescues the DSB repair defect observed in *rad52* mutants, and it increases the efficiency of repair in a *rad1 rad52* background. These results suggest that the *rfa1-D228Y* mutation has a direct effect on recombination processing/resolution.

Molecular analysis of intermediate and product formation during processing of *HO*-induced DSB indicates that product is observed at an earlier time point in *rad52 rfa1-D228Y* strains than in *rad52* strains. In contrast to *rad52* strains, the double mutants do not display an accumulation of large ssDNA intermediates. One hypothesis to explain the decrease in ssDNA intermediates is that the *rfa1-D228Y* mutation directly inhibits the exonucleolytic activity responsible for its formation, perhaps via an interaction between an exonuclease and Rfa1. This explanation seems unlikely, however, because the formation of the 5.1-kb ssDNA intermediate does not appear to be delayed in *rad52 rfa1-D228Y* strains compared to *rad52* strains, suggesting that the exonuclease activity is not dramatically reduced in these strains. In addition, mutations in the *RAD50* gene decrease ssDNA formation in a *rad52* background without affecting the level of product formation (Sugawara and Haber 1992). Thus, the *rfa1-D228Y* mutation likely increases the efficiency of a processing step that occurs subsequently to ssDNA degradation, thereby preventing the appearance of the ssDNA intermediates seen in *rad52* mutants.

Previous studies have shown that *rad52* mutants display a significant decrease in viability after induction of a DSB (White and Haber 1990; Fishman-Lobell *et al.* 1992; Sugawara and Haber 1992). However, there are some cases where repair of a DSB occurs efficiently. Although Ozenberger and Roeder (1991) have demonstrated that an *HO*-cut site could be efficiently repaired when embedded in the tandemly arrayed rDNA cluster and, to a lesser extent, within a *CUP1* array, a DSB located between two repeats is only repaired

~10–20% of the time (Fishman-Lobell *et al.* 1992; Ivanov and Haber 1995; this study). Thus, the efficiency of repair appears to be dependent on the number of repeats surrounding the break. These results suggest that *rad52* mutants may possess a reduced ability to find homologous sequences. This hypothesis is supported by the observation that the Rad52 protein can promote the annealing of two complementary DNA strands *in vitro* (Mortensen *et al.* 1996).

A decreased efficiency of pairing in *rad52* mutants may account for both decreased level of product formation and increased ssDNA degradation. One explanation for the ability of *rfa1-D228Y* to partially suppress both decreased product formation and increased degradation is that the mutant protein increases the efficiency of homologous pairing. In fact, *E. coli* ssDNA-binding protein has been shown to stimulate DNA renaturation *in vitro* (Christiansen and Baldwin 1977). A direct role for RP-A in pairing seems unlikely, however, because the conditions required for *E. coli* ssDNA-binding protein activity are distinctly nonphysiological, requiring high concentrations of spermidine and magnesium ion. Alternatively, the role of wild-type RP-A may be to inhibit pairing, and the *rfa1-D228Y* mutant protein may relieve this inhibition. This view is supported by recent *in vitro* experiments in which ssDNA substrates preincubated with *S. cerevisiae* RP-A inhibited subsequent homologous pairing by the strand exchange protein Rad51 (Sung 1997b). Interestingly, this inhibition can be overcome by addition of the Rad52 protein (Sung 1997a; New *et al.* 1998; Shinohara and Ogawa 1998).

On the basis of our molecular results and these *in vitro* experiments, we suggest that in the absence of Rad52, single-strand annealing occurs inefficiently because RP-A inhibits the access of a pairing or annealing protein (complex) to the ssDNA. In strains containing the *rfa1-D228Y* mutation, the level of the RP-A complex is reduced and/or its interaction with DNA is destabilized, permitting pairing between homologous sequences. This view is consistent with the idea that the role of Rad52 in homologous pairing is to alter the binding of RP-A to allow a pairing or strand exchange protein (complex) to access the ssDNA.

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