Role of Reciprocal Exchange, One-Ended Invasion Crossover and Single-Strand Annealing on Inverted and Direct Repeat Recombination in Yeast: Different Requirements for the *RAD1*, *RAD10*, and *RAD52* Genes

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ABSTRACT

We have constructed novel DNA substrates (one inverted and three direct repeats) based on the same 0.6-kb repeat sequence to study deletions and inversions in *Saccharomyces cerevisiae*. Spontaneous deletions occur six to eight times more frequently than inversions, irrespective of the distance between the repeats. This difference can be explained by the observation that deletion events can be mediated by a recombination mechanism that can initiate within the intervening sequence of the repeats. Spontaneous and double-strand break (DSB)-induced deletions occur as *RAD52*-dependent and *RAD52*-independent events. Those deletion events initiated through a DSB in the unique intervening sequence require the Rad1/Rad10 endonuclease only if the break is distantly located from the flanking DNA repeats. We propose that deletions can occur as three types of recombination events: the conservative *RAD52*-dependent reciprocal exchange and the nonconservative events, one-ended invasion crossover, and single-strand annealing (SSA). We suggest that one-ended invasion is *RAD52* dependent, whereas SSA is *RAD52* independent. Whereas deletions, like inversions, occur through reciprocal exchange, deletions can also occur through SSA or one-ended invasion. We propose that the contribution of reciprocal exchange and one-ended invasion crossover *vs.* SSA events to overall spontaneous deletions is a feature specific for each repeat system, determined by the initiation event and the availability of the Rad52 protein. We discuss the role of the Rad1/Rad10 endonuclease on the initial steps of one-ended invasion crossover and SSA as a function of the location of the initiation event relative to the repeats. We also show that the frequency of recombination between repeats is the same independent of their location (whether on circular plasmids, linear minichromosomes, or natural chromosomes) and have similar *RAD52* dependence.

MITOTIC recombination between two homologous DNA sequences occurs irrespective of whether these DNA sequences are located on the same chromosome, on homologous chromosomes, on nonhomologous chromosomes, or on plasmids (LISKAY and STACHELEK 1983; PETES and HILL 1988; BOLLAG *et al.* 1989; BAUR *et al.* 1990; MARYON and CARROLL 1991; PETES *et al.* 1991). An important substrate for recombination is repetitive DNA, present in large amounts in eukaryotic genomes. Although gene conversion between DNA repeats can eventually cause sequence homogeneity (BALTIMORE 1981; EGEL 1981), reciprocal recombination can be a source of genome instability, as it can cause deletions, inversions, translocations, and other chromosomal aberrations. In particular, tandem DNA repeats can be a potential source of genome instability of important consequences for the cell. Genetic diseases such as Werner syndrome (FUKUCHI *et al.* 1989) or Ataxia telangectasia (MEYN 1993) and certain forms of colorectal cancer (IONOV *et al.* 1993; THIBODEAU *et al.* 1993) are associated with instability of DNA repeats, showing the importance of stabilization mechanisms of DNA repeats in eukaryotic cells.

The mechanisms leading to the instability of DNA repeats are dependent on the length of the repeat. For short DNA direct repeats and simple repetitive DNA, deletions ($\leq$10 nucleotides long) may occur due to errors in DNA replication or repair, as suggested in *Escherichia coli* (ALBERTINI *et al.* 1982; MAZIN *et al.* 1991) and yeast (GORDENIN *et al.* 1992; HENDERSON and PETES 1992; RUSKIN and FINK 1993). In yeast it has been shown that these events are independent of the recombinational repair function Rad52 (HENDERSON and PETES 1992) and that the alteration of DNA mismatch repair destabilizes repetitive DNA (STRAND *et al.* 1993). Long DNA repeats, however, have been shown to be good substrates for recombination (JACKSON and FINK 1981; KLEIN 1984). Recombination between inverted DNA repeat systems in yeast is *RAD52* dependent (AHN and LIVINGSTON 1986; WILLIS and KLEIN 1987; AGUILERA and KLEIN 1989; DORNFELD and LIVINGSTON 1992).

Recombination between long DNA direct repeats

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(several hundred base pair long) is not yet completely understood, even though it is particularly relevant for genomic stability. Two types of events can be observed in direct repeat recombination: gene conversion between both DNA repeats and deletion of one repeat unit plus the intervening sequence. Gene conversion has been shown to depend strongly on the Rad52 function (MALONE and ESPOSITO 1980; JACKSON and FINK 1981; KLEIN 1988; DORNFELD and LIVINGSTON 1992); therefore it is believed to occur through a double-strand break (DSB) repair model of recombination (SZOSTAK *et al.* 1983). In contrast, spontaneous deletions show a low dependency on the *RAD52* gene (JACKSON and FINK 1981; KLEIN 1988; DORNFELD and LIVINGSTON 1992), and the frequency at which they occur is independent of the length of homology of the repeats (YUAN and KEIL 1990). We have recently shown that null mutants of the *HPR1* gene show a specific increase in deletions between DNA repeats that is not accompanied by an increase in reciprocal exchange in either direct or inverted repeats (SANTOS-ROSA and AGUILERA 1994). These data suggest that at least a second mechanism, besides a DSB repair type of mechanism, should be responsible for spontaneous deletions between direct repeats. The use of the HO endonuclease-cut site in the study of recombination has led to the proposal that deletions between long direct repeats can also occur through the single-strand annealing (SSA) model for DSB repair (LIN *et al.* 1984), consisting on the resection of one DNA strand at both sides of the DSB and alignment of the released single DNA strands at the homologous repeat sequences. It has been observed that deletions (1) are stimulated by an HO-cut in the nonhomologous intervening sequence (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989; RUDIN *et al.* 1989), (2) appear earlier than gene conversions in contiguous DNA repeats with an HO-cut site in between the repeats (FISHMAN-LOBELL *et al.* 1992), and (3) can occur in the absence of *RAD52* if initiated by an HO-cut in the rDNA region (OZENBERGER and ROEDER 1991). However, deletions could also occur through unequal sister chromatid exchange, unequal sister chromatid gene conversion (MALONEY and FOGEL 1987; ROTHSTEIN *et al.* 1987), half crossing over (TAKAHASHI *et al.* 1992), or mispairing of the replication fork (LOVETT *et al.* 1993).

To understand the mechanisms by which spontaneous recombination (in particular, deletions) between long DNA repeats occurs, we have compared recombination in direct *vs.* inverted repeats using the same DNA sequences as repeats. We have examined how recombination is affected by (1) distance between the repeats; (2) location of the repeats (circular plasmid, linear minichromosome, or natural chromosome); (3) spontaneous *vs.* DSB-induced events; (4) location of the DSB relative to the repeats; and (5) mutations in the excision repair genes *RAD1*, *RAD10*, and the recombinational repair gene *RAD52*. We propose that the recombination events detected in our systems occur through crossing over (whether reciprocal exchange or one-ended invasion crossover) or SSA. Whereas SSA produces only deletions (nonconservative events), crossing over can produce deletions and inversions (reciprocal exchange events) or just deletions (nonconservative one-ended invasion crossovers). We discuss the factors that determine the contribution of crossing over and SSA to overall deletions and the requirement for the *RAD1*, *RAD10*, and *RAD52* gene products.

MATERIALS AND METHODS

Strains: The yeast strains used in this study are listed in Table 1. All strains are genetically related to strains A3Y3A and W303-1A. The *rad* mutations were introduced by genetic crosses.

Media and growth conditions: Standard media such as rich medium YEPD, synthetic complete medium (SC) with bases and amino acids omitted as specified, and sporulation medium were prepared according to standard procedures (SHERMAN *et al.* 1986). All yeast strains were grown at 30° with horizontal shaking for liquid cultures. Yeast strains were transformed using the lithium acetate method (ITO *et al.* 1983) modified according to SCHIESTL and GIETZ (1989).

Mutagenesis: Yeast strains transformed with plasmids pRS314-LU or pRS314-SU were grown overnight in 5 ml SC-trp to select for the presence of the plasmids. Cells in early stationary phase were resuspended in 0.1 M Tris-maleate, pH 7.8, to a density of 10^8 cells/ml and mutagenized with a final concentration of 20 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) for 15 min as described (CALDERON and CERDA-OLMEDO 1983). Mutations were allowed to segregate by culturing the mutagenized cells in liquid SC-trp for 6–8 h at 30° before plating. The viability of the cells after mutagenesis was ~30%. Mutagenized cells were plated on SC-trp plates to isolate single colonies. After 3 days at 30°, colonies from SC-trp plates were replica-plated onto SC-leu. Colonies unable to form papilla on SC-leu plates were selected as Rec^- mutant candidates.

Determination of recombination frequencies: Recombination frequencies were calculated using the median method of LEA and COULSON (1948). Fluctuation test experiments were performed as described previously (AGUILERA and KLEIN 1989), using six independent colonies for each strain studied. Yeast strains were grown on YEPD plates (those carrying the repeat systems on the chromosome) or on SC-trp (those carrying the repeat system on plasmid pRS314-derivatives or minichromosome pTEL-LU). After 3 days independent colonies were picked, resuspended in water, and plated on SC-trp, SC-leu, or YEPD to determine the number of Trp^+, Leu^+, or viable colonies, respectively. The median frequency of recombination for each strain was calculated per viable cell number (determined on SC-trp or YEPD).

The frequency of recombination of DNA repeats located on plasmids, immediately after being introduced into the cell by transformation, was calculated by isolating single transformants on SC-trp. The proportion of Leu^+ or Leu^p (Leu^+ papillating phenotype) and Ura^- transformant colonies was determined by subsequent replica plating onto SC-leu and SC-ura, respectively. The method used was independent of whether the plasmid carrying the repeat (pRS314 derivative) was uncut or cut before transformation.

Plasmid constructions: *pRS314-IL3:* The 1.4-kb *Cla*I–*Sal*I

TABLE 1

Strains

Strain	Genotype	Source
A3Y3A	*MATα leu2-k::URA3-ADE2::leu2-k his3Δ200 ade2 ura3*	AGUILERA and KLEIN (1989)
W303-1A	*MATa leu2-3, 112 trp1 can1-100 ura3-1 ade2-1 his3-11, 15*	R. ROTHSTEIN
W303-1B	*MATα leu2-3, 112 trp1 can1-100 ura3-1 ade2-1 his3-11, 15*	R. ROTHSTEIN
AWI-1B	*MATα leu2-3, 112 trp1 can1-100 ura3 ade2 his3*	This study
315-9C	*MATa leu2Δ68 trp1 ura3-52 ade2-101 his3Δ200*	H. KLEIN
356-11A	*MATa leu2-3, 112 trp1 ura3-52 rad1-1*	H. KLEIN
AWF-2D	*MATα leu2-3, 112 trp1 ura3-52 rad1-1*	This study
FX315-2D	*MATα ura3-52 trp1 leu2Δ68 his3Δ200 rad 52-1*	This study
FX315-4D	*MATa ura3-52 trp1 leu2Δ68 his3Δ200 rad52-1*	This study
FX315-5B	*MATα ura3-52 trp1 leu2Δ68 his3Δ200 rad52-1*	This study
VW-YH5	*MATα his3Δ200 lys2-801 ade2-101 ura3-52 rad10Δ::URA3*	YGSC[a]
UWA-4A	*MATa leu2-3, 112 his3 ade2 trp1 rad10Δ::URA3*	This study
UWA-3C	*MATa leu2-3, 112 his3 ade2 trp1 rad10Δ::URA3*	This study
W303-LU	*MATa leu2-3, 112 trp1 can1-100 ura3-1 ade2-1 HIS3::LU*	This study
W303-L	*MATa leu2-3, 112 trp1 can1-100 ura3-1 ade2-1 HIS3::L*	This study
W303-SU	*MATa leu2-3, 112 trp1 can1-100 ura3-1 ade2-1 HIS3::SU*	This study
FXLU-1A	*MATa leu2Δ68 ura3 trp1 ade2 HIS3::LU*	This study
FLUX-10B	*MATa leu2Δ68 ura3 trp1 HIS3::LU*	This study
FXLU-2B	*MATα leu2-3, 112 ura3 trp1 HIS3::LU rad52-1*	This study
FXLU-13C	*MATα leu2-3, 112 ura3 trp1 HIS3::LU rad52-1*	This study
FXSU-3B	*MATa leu2Δ68 ura3 trp1 ade2-1 HIS3::SU*	This study
FXSU-10C	*MATα leu2-3, 112 ura3 trp1 ade2-1 HIS3::SU*	This study
FXSU-4C	*MATa leu2Δ68 ura3 trp1 ade2-1 HIS3::SU rad52-1*	This study
FXSU-11A	*MATa leu2Δ68 ura3 trp1 ade2-1 HIS3::SU rad52-1*	This study
FXL-6A	*MATα leu2-3, 112 ura3 trp1 HIS3::L*	This study
FXL-6D	*MATa leu2Δ68 ura3 trp1 ade2-1 HIS3::L*	This study
FXL-1C	*MATα leu2-3, 112 ura3 trp1 ade2-1 HIS3::L rad52-1*	This study
FXL-5B	*MATα leu2-3, 112 ura3 trp1 ade2-1 HIS3::L rad52-1*	This study

[a] Yeast Genetics Stock Center (Berkeley, CA).

fragment from the *LEU2* region was inserted at the *Cla*I–*Xho*I site of the 4.8-kb yeast centromeric vector pRS314, based on pBLUESCRIPT (KS+) and *TRP1*, *CEN6*, and *ARSH4* yeast DNA sequences (SIKORSKI and HIETER 1989) to create plasmid pRS314–1L3 (6.2 kb).

pRS314-L: The unique *Cla*I site of pRS314–1L3 was eliminated by *Cla*I digestion, Klenow reaction, and ligation, creating a new *Nru*I site in the resulting plasmid, pRS314–1L3N. The 1.9-kb *Eco*RV–*Bgl*II fragment from the *LEU2* region was inserted into the *Sma*I–*Bam*HI site of this new plasmid to create plasmid pRS314-L (8.1 kb).

pRS314-LU: A 2.5-kb YIp5 *Cla*I–*Sma*I fragment, containing pBR322 sequences and the complete *URA3* gene from yeast, was inserted at the *Cla*I–*Sma*I site of pRS314–1L3 to create plasmid pRS314–1LU2. The 1.9-kb *Eco*RV–*Bgl*II fragment of the *LEU2* region was inserted into the pRS314–1LU2 opened at *Sma*I and the *Bam*HI site of the KS+ polylinker (partial digestion required) to create plasmid pRS314-LU (10.6 kb).

pRS314-SU: The 1.9-kb *Eco*RV–*Bgl*II fragment of the *LEU2* region was inserted into the pRS314–1LU2 opened at *Sma*I and the *Bam*HI site of the internal *tet* gene (partial digestion required) to create plasmid pRS314-SU (8.14 kb).

pTEL-LU: A 2.55-kb *Bam*HI–*Xba*I fragment from YCpA13 (AGUILERA and KLEIN 1990) was inserted at the *Bam*HI–*Xba*I site of the centromeric vector pRS316, which is similar to pRS314 but carries the *HIS3* gene instead of *TRP1* (SIKORSKI and HIETER 1989), to create plasmid pTEL1–2. A 2.5-kb *Bam*HI–*Hin*dIII fragment containing telomeric sequences was inserted at the *Bam*HI–*Hin*dIII site of pTEL1–2 to form plasmid pTEL2–1. A 2.6-kb *Xho*I–*Sal*I fragment of pTEL2–1 was inserted at the unique *Xho*I site of pRS314-LU to make pTEL-LU (13.2 kb).

pRS314-LY: A *Bgl*II linker (dCAGATCTG) was inserted at the unique *Nru*I site pRS314–1L3N to form pRS314–1L3B. The 1.9-kb *Eco*RV–*Bgl*II fragment from the *LEU2* region was inserted at the *Sma*I–*Bam*HI site of this new plasmid to create plasmid pRS314-LB. The YIp5 vector opened at *Bam*HI was inserted at the *Bgl*II site of pRS314-LB to form plasmid pRS314-LY (13.6 kb).

pRS303-L, pRS303-LU, and pRS303-SU: These plasmids were constructed by an *in vivo* cloning strategy previously reported (PRADO and AGUILERA 1994). A transformant of strain W303–1A with plasmid pRS314-L was retransformed with plasmid pRS303 (SIKORSKI and HIETER 1989), based on pBLUESCRIPT-KS and the yeast *HIS3* gene, opened at the *Xho*I and *Xba*I sites of the pKS polylinker. A dimeric cointegrate that arose through recombination between both plasmids was isolated from the new transformants, cut at the *Sca*I site to release the two new monomers, and religated. After *E. coli* transformation, plasmid pRS303-L containing the complete L system (see Figure 1) at the KS polylinker of pRS303 was isolated. To obtain plasmids pRS303-LU and pRS303-SU we used the same strategy. Transformants of the W303–1A strain with plasmids pRS314-LU and pRS314-SU were retransformed with plasmid pRS303 opened at the *Xho*I and *Xba*I sites of the KS polylinker and identical steps were followed to create pRS303-LU and pRS303-SU (PRADO and AGUILERA 1994).

Construction of direct repeat systems in the chromo-

some: Plasmids pRS303-L, pRS303-LU, and pRS303-SU were linearized by cutting at the *BglII* sites in the *HIS3* gene. Each linear plasmid was used to transform the strain W303–1A independently. Stable integrative transformants were isolated on SC-his and tested for the presence of each of the systems independently (L, LU, and SU) by the ability to form papilla on SC-leu media. Southern analysis of the genomic DNA of these transformants was performed to confirm that the plasmids were integrated in single copy in chromosome *XV* at the *HIS3* locus. By subsequent genetic crosses, strains containing each of the systems L, LU, and SU on chromosome *XV* were created, with the only difference that the chromosomal LU system has an inactive *URA3* gene.

DNA manipulation: Plasmid DNA was isolated from *E. coli* using CsCl gradient centrifugation as described (CLEWELL and HELINSKI 1970). Small-scale plasmid DNA preparations were made according to BOLIVAR and BACKMAN (1979). Yeast genomic DNA was prepared from 5 ml YEPD cultures as previously reported (SHERMAN *et al.* 1986) and was used for Southern blot analysis (SOUTHERN 1975). Plasmid yeast DNA was prepared according to HOFFMAN and WINSTON (1987) and used directly to transform *E. coli* as described (COHEN *et al.* 1972).

Digoxigenine-dUTP-labeled DNA probes were prepared as described (FEINBERG and VOLGELSTEIN 1984). Hybridization was performed in 50% formamide 5 × SSC, 0.01% *N*-laurosylsarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer Mannheim) at 42° for 18 h. Detection of Digoxigenine-labeled DNA was performed following Boehringer Mannheim recommendations.

Linear DNA fragments were recovered directly from agarose gels and used in DNA labeling experiments or in ligation reactions with T4 DNA ligase overnight at 14°.

RESULTS

Spontaneous recombination in direct *vs.* inverted repeats located on autonomous plasmids: To understand the mechanisms leading to deletion formation between direct repeats, we have devised several DNA repeat systems that use the same repeat unit in direct and inverted orientation and separated by intervening sequences of different sizes. We have constructed three direct repeat systems, L, LU, and LY, based on the same 600-bp repeat (an internal fragment of the *LEU2* coding region) that was separated by 31 bp (L), 2.51 kb (LU), or 5.57 kb (LY) (Figure 1). The three direct repeat systems (L, LU, and LY) differ not only in the length of the intervening sequence but also in the source of this sequence. The L system contained no pBR322 sequences in the intervening region, the LU system contained just a short 1.4-kb fragment of pBR322 plus the yeast *URA3* gene, and the LY system contained a complete pBR322 sequence plus the *URA3* gene (see Figure 1). To study inversions, we constructed an inverted repeat system, the SU system, based on the same 600-bp repeat sequence that was separated by 1.66 kb. The four systems were constructed in the pRS314 plasmid (Figure 2). In all four systems, recombination between the two 600-bp nonfunctional *LEU2* internal fragments generates a complete and functional *LEU2* gene, allowing recombinants to be scored as Leu$^+$ colonies.

Our approach was based on two premises. First, inversions between repeats correspond to intramolecular reciprocal exchange events, whereas deletions could either result from an intramolecular reciprocal exchange or from another type of event, such as unequal sister chromatid exchange (SCE), unequal gene conversion, nonconservative recombination, and so on. If spontaneous deletions correspond only to intramolecular reciprocal exchanges, the frequency of Leu$^+$ recombinants for direct repeat systems LU and LY and inverted repeat system SU should be similar. Second, the pairing of two 600 bp direct repeats along their entire lengths may have sterical limitations for the L system where the repeats were separated by just 31 bp, as compared with the 2.51- or 5.57-kb repeat separation for the LU or LY systems (four and nine times the size of the repeat, respectively). Table 2 shows that the frequency of Leu$^+$ is similar for the L, LU, and LY direct repeat systems and considerably higher (six- to eightfold) than for the SU inverted repeat system. These results suggest that reciprocal exchange by itself cannot explain all deletion events in the direct repeat systems and that no steric constraint should differentially affect the direct repeat recombination systems.

To better understand the mechanisms leading to Leu$^+$ recombinants in the four systems studied, we have determined the effect of mutations in the genes *RAD52* and *RAD1*, previously shown to be involved in repeat recombination (KLEIN 1988; SCHIESTL and PRAKASH 1988). We used the *rad1–1* and *rad52–1* alleles for convenience and because the effect on recombination is as strong or stronger than that of the disrupted alleles (JACKSON and FINK 1981; KLEIN 1988; SCHIESTL and PRAKASH 1988; AGUILERA and KLEIN 1989). Table 2 shows that whereas the frequency of spontaneous Leu$^+$ recombination events in *rad1* cells is similar to wild-type cells, it is strongly reduced (50- to 200-fold) in *rad52* strains in all systems studied. Yet the frequency of deletions in the three systems studied is significant (10^{-5}) in *rad52–1* strains, and it is three to eight times higher than the frequency of inversions. Our results contrast with the low dependency on Rad52 observed for deletions between chromosomal direct repeats in previous studies (JACKSON and FINK 1981; AGUILERA and KLEIN 1988; KLEIN 1988). We have confirmed that deletion formation depends slightly on *RAD52* in two other related systems. These systems were the *leu2–101::pBR322-URA3::leu2–102*, based on the 400 bp *Eco*RI *LEU2* fragment as the repeat (CHAN and BOTSTEIN 1993), and the *leu2–112::pBR322-URA3::leu2-k*, based on the 2.16-kb *Sal*I–*Xho*I *LEU2* fragment as the repeat (KLEIN 1988). In both cases the intervening sequence was 5.54 kb long. We observed that the frequency of deletions in *rad52–1* strains was 3 and 10 times lower than in wild-type strains (data not shown).

We have determined by Southern analysis that in

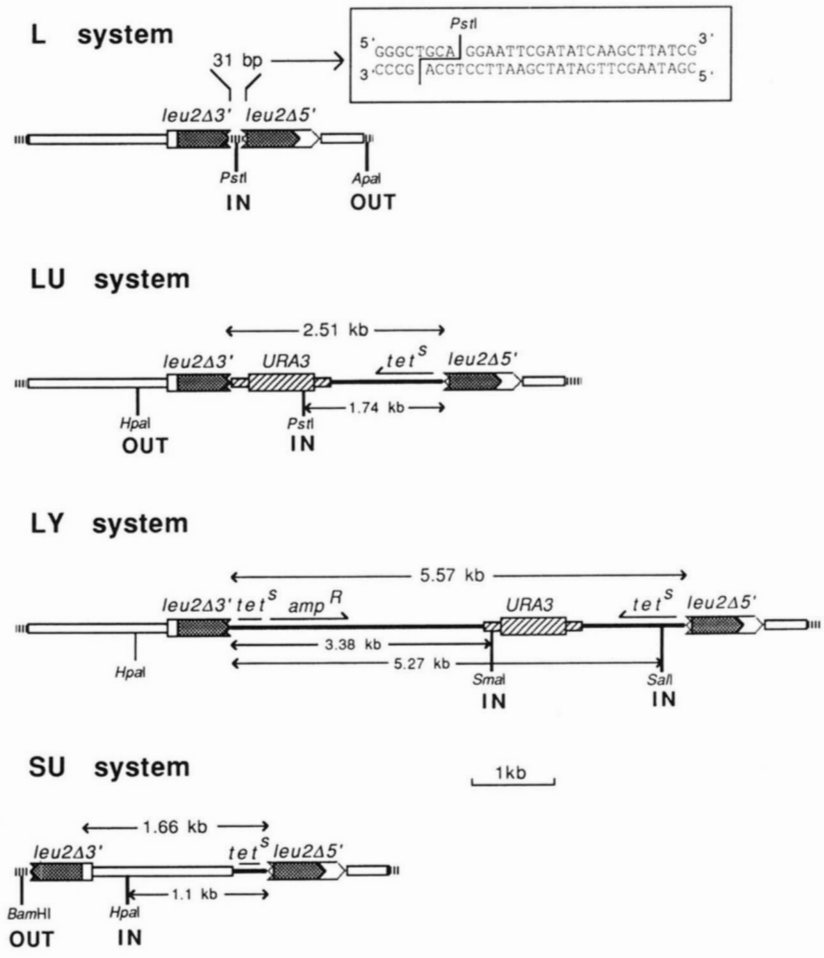

FIGURE 1.—DNA repeat systems constructed for this study. Wide boxes represent the *LEU2* or *URA3* coding regions. They are immediately flanked by thinner boxes that represent DNA sequences from the respective yeast chromosomal regions. The 0.6-kb internal *LEU2* fragment used as the repeat is shown as a filled area inside each open box. Bacterial sequences are shown as a continuous dark line, and sequences from the KS+ polylinker are shown as dashed lines. Arrows indicate the direction of transcription. IN and OUT indicate the DSB sites used to cut the plasmids before transformation. The restriction enzyme used is specified for each case. The figure is approximately drawn to scale.

wild-type strains, Leu$^+$ events obtained with the LU or the SU system show the pattern of a deletion or an inversion in 15/15 Leu$^+$ recombinants examined for each case (data not shown). The genetic analysis of the LU Leu$^+$ events revealed that ~60% were also Ura$^+$. This result was confirmed by Southern analysis for both the LU and the SU Leu$^+$ events. Similar results were also obtained for the LU system in *rad52–1* strains. These data indicated that the Leu$^+$ recombinants contained two plasmids, one carrying the original intact system and the other carrying the recombinant system. The presence of two plasmids is presumably the result of mitotic missegregation of the *CEN* plasmids (MURRAY and SZOSTAK 1983). However, THOMAS and ROTHSTEIN (1989) have suggested that deletions between chromosomal DNA repeats are associated with the occurrence of disomy, an interpretation that could be consistent with our results, although we have also observed this association with inversions.

Our results suggest that in addition to reciprocal exchange events leading to deletions and inversions, there are two other types of nonconservative recombination events leading to spontaneous deletions: one *RAD52* independent and another *RAD52* dependent. We believe that deletions, but not inversions, can initiate at nonhomologous DNA intervening regions, as shown below. This would explain why deletions are more frequent than inversions (Table 2) and why the *RAD52* dependency of deletions varies between different systems (JACKSON and FINK 1981; WILLIS and KLEIN 1987; AGUILERA and KLEIN 1989; DORNFELD and LIVINGSTON 1992). The contribution of each recombination mechanism to the overall deletions depends on the particular structural or sequence features of each DNA repeat system that presumably influence the initiation event, as will be discussed later.

Effect of DSBs at unique DNA sequences on deletions and inversions between repeats: To determine a possible role of the initiation event in determining the differences of spontaneous recombination observed in direct *vs.* inverted repeats, we characterized the recombination events initiated by a DSB located at different sites in our four repeat systems. We transformed wild-type strains with the pRS314-derivative plasmids carrying the direct repeat systems L, LU, and LY and the inverted repeat system SU. For transformation, each of the plasmids was used either uncut or cut once in unique DNA sequences. This cut (DSB) was located

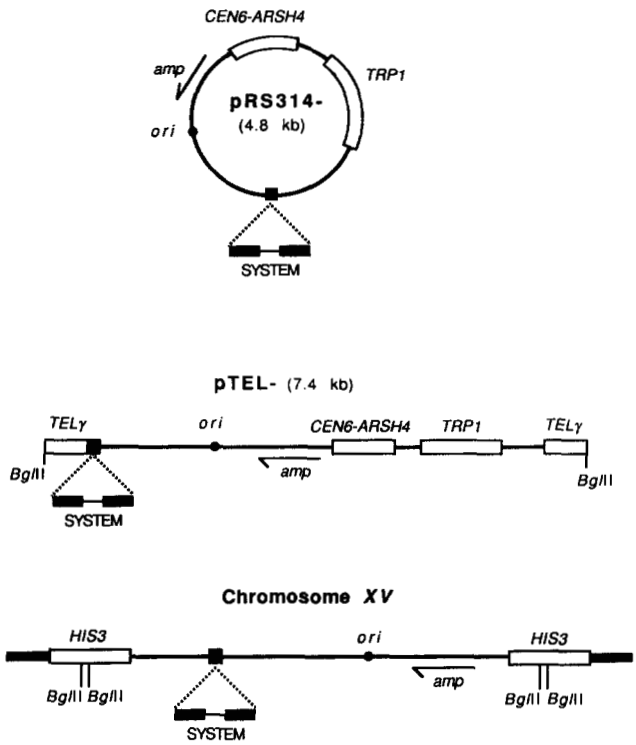

FIGURE 2.—DNA molecules where the DNA repeat systems from Figure 1 were located. The YAC vector pTEL3 is shown linearized after being cut with *Bgl*II. The location of the repeat systems are indicated. In parentheses is indicated the size of the vectors without the system.

either in the intervening sequence of the repeats (IN) or immediately outside the repeat system (OUT) (see Figure 1).

Table 3 shows that the frequency of Leu$^+$ recombinants for the uncut circular plasmid was two orders of magnitude higher immediately after transformation than when the plasmid had been stably propagated within the cell for generations (compare Tables 2 and 3). The frequency of deletions in the L, LU, and LY system was 8–10% right after transformation, about 100-fold higher than the value obtained for the system stably propagated within the cell ($8.9–11.7 \times 10^{-4}$). A similar difference was found for the frequency of inversions in the SU inverted repeat system (0.3% *vs.* 1.5×10^{-4}). This difference suggests that the DNA entering the cell at transformation is highly recombinogenic, presumably because it is free of proteins and a fraction of it is inevitably nicked as a consequence of the methods used for its isolation. However, the DNA stably propagated within the cell is intact and in a chromatin structure. This conclusion is supported by the large variation in the percentage of Leu$^+$ events obtained after transformation with uncut plasmids (between 1 and 20% for the LU system, depending on whether the DNA was obtained by minipreps, CsCl gradient centrifugation, or purified from agarose gels). Similar conclusions have been obtained recently by LARIONOV *et al.* (1994).

When the three direct repeats were cut at unique DNA sequences within the intervening region of the repeat systems (IN), we observed that the frequency of transformation was similar to that for uncut circular plasmids; however, >90% of the transformants underwent a deletion resulting in a Leu$^+$ event (Table 3). The induction of deletions by a DSB is also observed by comparing the number of Leu$^+$ recombinants/μg DNA of plasmids cut at the IN sites and uncut circular plasmids (Table 3). This result was independent of the distance between the DSB and the repeats. For the four cases analyzed, the DSB was located at 27 bp (L-*Pst*I), 1.74 kb (LU-*Pst*I), 3.38 kb (LY-*Sma*I), and 5.27 (LY-*Sal*I) from the furthest repeat unit and the frequencies of transformation and Leu$^+$ recombinants were similar (see Figure 1). The scored Leu$^+$ events were deletions because over 96% of all Leu$^+$ events in the LU and

TABLE 2

Frequency of Leu+ recombinants ($\times 10^6$) in the L, LU, and LY direct repeat systems and the SU inverted repeat system when located in the centromeric plasmid pRS314

	Direct repeats[b]			Inverted repeat[b]
Genotype[a]	L	LU	LY	SU
Wild-type	890 (×1)	1,170 (×1)	1,100 (×1)	150 (×1)
rad52-1	15 (×0.02)	20 (×0.02)	6 (×0.005)	2 (×0.01)
rad1-1	660 (×0.7)	1,190 (×1)	ND[c]	150 (×1)

[a] Strains used were as follows: wild type, 315-9C, W303-1A, W303-1B, and AWI-1B; *rad52-1*, FX315-2D, FX315-4D, and FX315-5B; *rad1-1*, 356-11A and AWF-2D.

[b] All fluctuation tests were performed on six independent colonies for each strain used as described in MATERIALS AND METHODS. When more than one strain were used the given value corresponds to the strain with the median value among those studied for each genotype. The variation for these median values for each genotype was <16%. Numbers in parentheses indicate the times increase over the wild-type value for each repeat system studied, considered as 1.

[c] Not determined.

TABLE 3

Frequency of Leu+ recombinants arising during transformation of wild-type strains with uncut and cut (DSB) pRS314 derivative plasmids carrying the repeat systems L, LU, LY, and SU

Cut site[a]	System	Transformants/μgDNA[b]	Leu+:Leu^{P}[c]	Leu+/μg DNA
Uncut	L	72,245	67:810 (8)	5,519
	LU	48,420	153:1403 (10)	4,761
	LY	11,165	4:40 (9)	1,015
	SU	76,080	8:2527 (0.3)	240
IN	L (*Pst*I)	43,040	494:40 (92)	39,816
	LU (*Pst*I)	19,200	546:86 (86)	16,587
	LY (*Sma*I)	41,220	798:13 (98)	40,559
	LY (*Sal*I)	18,815	471:18 (96)	18,122
	SU (*Hpa*I)	6,260	13:230 (5)	335
OUT	L (*Apa*I)	2,860	5:163 (3)	85
	LU (*Hpa*I)	1,734	19:144 (12)	202
	SU (*Bam*HI)	6,525	13:345 (4)	237

[a] Strains used were AWI-1B and W303-1A. IN indicates a cut in the intervening sequence located between the repeats; OUT indicates a cut outside of the repeats and the intervening region. The restriction enzyme used to cut the plasmid before transformation is indicated in parentheses. The cut sites are as indicated in Figure 1.

[b] The values correspond to the average of three transformation experiments with 200–300 ng DNA each. The standard deviation was between 15 and 25%.

[c] Data correspond to three to five experiments, with standard deviations of 20%. The number in parentheses indicates the percentage of Leu+ recombinants among total Trp+ transformants selected. Leu^{P} means papillators on SC-leu.

LY systems were Ura− (600Ura−:23Ura+ for LU and 1039Ura−:0Ura+ for LY).

Plasmids cut outside of the direct repeat systems (OUT) showed frequencies of transformation > 10 times lower than plasmids cut inside the intervening region (IN) and frequencies of deletions similar to uncut plasmids (Table 3). Plasmids cut either at the IN or OUT sites of the inverted repeat system showed frequencies of transformation and recombination similar to plasmids cut at the OUT sites of direct repeats (3–12%).

These results clearly suggest that a DSB located in a unique DNA region flanked by direct repeats is repaired very efficiently via induced deletions (the number of transformants was similar for cut and uncut plasmid and >90% of the transformants carried a deletion of the repeat system). In contrast, a DSB at a unique region not flanked by direct repeats (IN in inverted repeats and OUT in either inverted or direct repeats) is not repaired efficiently and does not induce intramolecular recombination between repeats. This result can explain why spontaneous deletions are six to eight times more frequent than inversions. Inversions can only be mediated by reciprocal exchange, which only initiate in the homologous DNA repeat, whereas deletions can be mediated by an additional mechanism and this can also initiate within the intervening sequence.

DSB-induced deletions require the *RAD1*, *RAD10*, and *RAD52* genes: To understand the mechanisms responsible for deletions, we decided to determine the effect of mutations in the *RAD1*, *RAD10*, and *RAD52* genes on DSB-induced deletions. Our rationale was that the differential effect of each mutation on recombination (KLEIN 1988; SCHIESTL and PRAKASH 1988, 1990; THOMAS and ROTHSTEIN 1989) could help to define different recombination mechanisms in our repeat systems. We decided to include a *rad10* strain in this set of experiments because *RAD10* has been shown to have similar effects on recombination as *RAD1* (SCHIESTL and PRAKASH 1990) and because the Rad1 and Rad10 proteins have been shown to form a complex with endonucleolytic activity (BARDWELL *et al.* 1992; TOMKINSON *et al.* 1993). The results on transformation efficiency and recombination frequencies for plasmids carrying direct repeat systems either uncut or cut at the respective IN sites in *rad1-1*, *rad10Δ*, and *rad52-1* mutants are shown in Table 4.

When we used uncut circular plasmids, we observed that transformation frequencies were reduced in *rad1* (2- to 4-fold), *rad10* (2- to 4-fold), and *rad52* strains (4- to 10-fold) as compared with the wild-type values. However, the frequency of Leu^{+} recombinants decreased ~20- to 40-fold below the wild-type values in *rad1* and *rad10* cells and over 100-fold in *rad52* cells (compare Tables 3 and 4). This reduction was similar for all plasmids used. This result suggests that *RAD1* and *RAD10* may also be required for the formation of deletions in our repeats, as observed for other repeats (KLEIN 1988; SCHIESTL and PRAKASH 1990), even though this effect was not observed when the repeats were stably propagated in the cell (Table 2).

When we used the plasmid cut at the IN sites, we observed a reduction in the frequency of Trp^+ transformants that was dependent on the distance between the DSB and the furthest repeat unit in *rad1* and *rad10* strains (Table 4; Figure 3A). The frequency of transformation for pRS314-L cut at 8 and 27 bases from the 3′-end of each homologous repeat (see Figure 1) was similar in wild-type, *rad1*, and *rad10* cells. However, the transformation levels of *rad1* and *rad10* cells decreased strongly as the distance between the DSB and the furthest repeat increased, reaching a 25-fold reduction at a distance of 3.38 kb (LY-*Sal*I). For *rad52–1* strains there is also a strong reduction in the frequency of transformation (20- to 90-fold below the wild-type values) with small differences due to the distance betwen the DSB and the repeats (Table 4; Figure 3A). Part of this reduction was already observed with uncut plasmids in *rad52* strains, and the frequency of transformation of *rad52* strains with cut plasmids is reduced three- to fivefold compared with uncut plasmids.

Table 4 shows that in *rad1* and *rad10* mutants >90% of the transformants with cut plasmids contained a deletion if the DSB produced a fragment of 8 and 27 bases of nonhomologous DNA at the 3′ end of each flanking DNA repeat [L(*Pst*I)]. This result is consistent with the transformation frequencies in these strains, suggesting that *rad1* and *rad10* mutants have wild-type capacity to repair via deletion a DSB flanked by very proximal repeats. However, in both *rad1* and *rad10* strains the frequency of deletions decreased to 26–60% of wild-type frequency when the DSB was at 0.77 and 1.74 kb [LU(*Pst*I)], 3.38, and 2.19 kb [LY(*Sma*I)] and 5.27 and 0.3 kb [LY(*Sal*I)] from the flanking repeats (Table 4; Figure 3B). This is consistent with the decrease in the frequency of transformation reported below and indicates that both the *RAD1* and the *RAD10* genes are required for the repair, via deletions, of DSBs flanked by distant repeats. We confirmed by Southern analysis that 16/16 Leu^+ recombinants of the LU system tested from *rad1* strains carried a deletion as expected (data not shown).

In *rad52* mutants, however, ~84–92% of the transformants carried a deletion if the DSB was approximately symmetric relative to the flanking repeats [L(*Pst*I), 8 and 27 bp; LU(*Pst*I), 0.76 and 1.74 kb; and LY(*Sma*I), 3.38 and 2.19 kb]. However, this value decreased to 26% if the DSB was very asymmetrically located [LY(*Sal*I), 0.3 and 5.27 kb]. Southern analysis of 16 Leu^+ recombinants of the LU system confirmed that all carried a deletion as expected (data not shown). These results suggest that DSB-induced deletions occur as *RAD52*-dependent and *RAD52*-independent events and that *RAD52*-dependent events are presumably more predominant as the DSB is more asymmetrically located between the repeats.

The plotting of the Leu^+ recombinants as a function

TABLE 4

Frequency of Leu+ recombinants arising during transformation of *rad1*, *rad10*, and *rad52* strains with uncut and cut (DSB) pRS314 derivative plasmids carrying the repeat systems L, LU, and LY

Cut site[b]	System	*rad1-1*[a]			*rad10Δ*[a]			*rad52-1*[a]		
		Transformants/ μg DNA[c]	Leu+:Leu^p[d]	Leu+/μg DNA	Transformants/ μg DNA[c]	Leu+:Leu^p[d]	Leu+/μg DNA	Transformants/ μg DNA[c]	Leu+:Leu^p[d]	Leu+/μg DNA
Uncut	L	19,640	7:1322 (0.5)	98	18,760	3:582 (0.5)	94	10,040	0:902 (<0.1)	<10
	LU	16,920	1:562 (0.2)	677	21,160	1:528 (0.2)	42	5,160	0:131 (<0.5)	<26
	LY	7,744	3:121 (2)	155	7,520	1:42 (2)	451	13,648	0:622 (<0.2)	<7
IN	L (*Pst*I)	56,315	1237:78 (94)	52,936	20,960	255:8 (97)	20,331	3,680	320:54 (85)	3,128
	LU (*Pst*I)	16,120	181:238 (43)	6,931	4,720	208:298 (41)	1,935	3,720	196:38 (84)	3,125
	LY (*Sma*I)	4,768	56:156 (26)	1,240	1,748	100:99 (50)	874	1,460	329:27 (90)	1,314
	LY(*Sal*I)	2,016	92:123 (43)	867	616	129:83 (61)	370	688	63:178 (26)	179

[a] Strains used were as follows: *rad1-1*, AWF-2D and 356-11A, *rad10Δ*, UWA-4A and UWA-3C; *rad52-1*; FX315-4D and FX315-5B.

[b] IN indicates a cut in the intervening sequence located between the repeats. The cut sites (shown in parentheses) are as indicated in Figure 1.

[c] The values correspond to the average of three transformation experiments. The standard deviation oscillated between 15 and 25%.

[d] Data correspond to three to five experiments, with standard deviations of 20%. The number in parentheses indicate the percentage of Leu+ recombinants among total Trp+ transformants obtained.

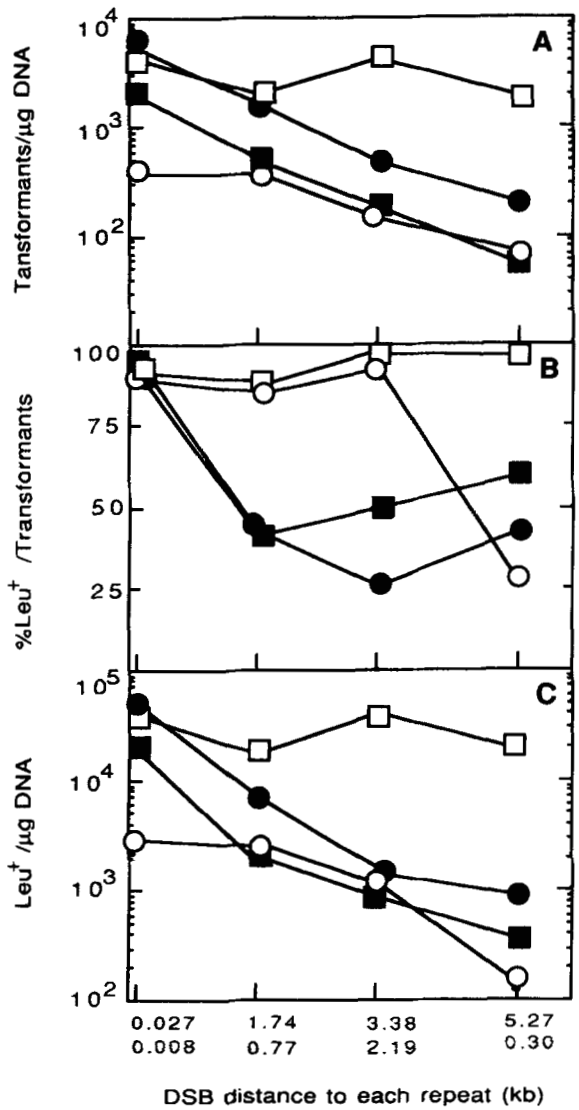

FIGURE 3.—Plotting of transformants/μg DNA (A), %Leu$^+$ deletions/total transformants (B), and Leu$^+$ deletions/μg DNA (C) as a function of the distance of the DSB to the furthest flanking repeat in wild-type (□), *rad1-1* (●), *rad10*Δ (■), and *rad52-1* (○) strains transformed with pRS314-derivative plasmids containing the L, LU, and LY systems cut at the IN sites (see Figure 1). The horizontal axis shows the distance from the DSB to the furthest (top) and the nearest repeat (bottom).

of transformants obtained (Figure 3B) or micrograms of DNA used for transformation (Figure 3C) clearly shows that the repair of a DSB flanked by distant direct repeats depends on the *RAD1* and *RAD10* genes. When the proximal flanking repeat is just eight bases from the DSB, the Rad1 and Rad10 functions are not required to repair DSBs via deletions. It is remarkable that the Rad1, Rad10, and Rad52 functions are equally important for the repair of a DSB located at 0.3 and 5.27 kb from the repeats [LY(*SalI*)].

Spontaneous recombination between DNA repeats located on minichromosomes and chromosomes: Because previous studies on spontaneous deletions between repeats have used direct repeat systems located on chromosomes (JACKSON and FINK 1981; KLEIN and PETES 1981; AGUILERA and KLEIN 1988; KLEIN 1988; THOMAS and ROTHSTEIN 1989), we decided to investigate whether the different location of our DNA repeat systems in the yeast genome could alter the recombination characteristics of the DNA repeats. We reasoned that the type of mechanism acting on repeats and responsible for deletions or inversions could depend on whether the repeats were located on a circular *vs.* a linear molecule, because of possible differences in supercoiling, or whether the repeats were located on a plasmid *vs.* a chromosome, because of possible differences in chromatin structure.

We constructed plasmid pTEL-LU by adding telomere sequences to plasmid pRS314-LU (see MATERIALS AND METHODS). This plasmid was used for yeast transformation as a closed circular DNA molecule and as a linear minichromosome (Figure 2). The latter resulted from the restriction digestion of plasmid pTEL-LU with *Bgl*II before yeast transformation. The linearity of the minichromosome inside the yeast cell was confirmed by Southern analysis (data not shown). Table 5 shows that there is no difference in the frequency of Leu$^+$ events for the circular *vs.* the linear structure. Also the events observed in both the linear and the circular structures showed a strong dependency on the Rad52 function.

The two L and LU direct repeat systems and the SU inverted repeat system were inserted at the *HIS3* locus on chromosome *XV*. To do this, we used linearized pRS303-L, pRS303-LU, and pRS303-SU to transform W303-1A cells (see MATERIALS AND METHODS and Figure 2). For each system we selected one transformant carrying the system integrated at the *HIS3* locus, as confirmed by genetic and Southern analysis (data not shown). Table 5 shows that there were no important differences in the frequency of Leu$^+$ recombinants for the three systems when located on a chromosome than when located on a plasmid (see Table 2). In all cases Leu$^+$ deletion or inversion events showed the same dependency on the Rad52 function, as observed for the same systems located on plasmids pRS314-L, pRS314-LU and pRS314-SU. Therefore, we can conclude that the recombination characteristics of our repeat systems are the same, independent of whether the repeats are located on circular plasmids, linear artificial minichromosomes, or natural chromosomes.

A search for deletion- and inversion-minus mutants identified five new alleles of the *RAD52* gene: To investigate whether different recombination pathways are responsible for spontaneous deletions and inversions, we decided to search for Rec$^-$ mutants for the systems LU and SU independently, in the hope of isolating muta-

TABLE 5

Frequency of Leu+ recombinants ($\times 10^6$) in the systems LU, L, and/or SU located on a minichromosome or on chromosome *XV*

	pTEL (circle)[b]	pTEL (linear)[b]	Chromosome *XV*[b]		
Genotype[a]	LU	LU	LU	L	SU
Wild-type	940 (×1)	470 (×1)	630 (×1)	230 (×1)	90 (×1)
rad52-1	4 (×0.004)	0.5 (×0.001)	10 (×0.015)	8 (×0.03)	2 (×0.02)

[a] Strains used for plasmids pTEL-LU were as follows: wild-type; 315-9C and W303-1A; *rad52-1;* FX315-5B. Strains used for the systems L, LU, and LY integrated at chromosome *XV* were as follows: wild-type; W303-L, FXL-6A, and FXL-6D (for system L); W303-LU, FXLU-1A, and FXLU-10B (system LU); and W303-SU, FXSU-3B, and FXSU-10C (system SU); *rad52-1,* FXL-1C and FXL-5B (system L), FXLU-2B and FXLU-13C (system LU), and FXSU-4C and FXSU-11A (system SU).

[b] All fluctuation tests were performed on six independent colonies for each strain used as described in MATERIALS AND METHODS. When more than one strain were used the given value corresponds to the strain with the median value among those studied for each genotype. The variation for these median values for each genotype was <12%. Numbers in parentheses indicate the fold number increase over the wild-type value for each repeat system studied.

tions that might specifically affect one of the repeat systems. We used the strain 315–9C transformed either with pRS314-LU or pRS314-SU. These transformants were propagated on SC-trp to select for the presence of the plasmid in the cell and mutagenized with nitrosoguanidine according to MATERIALS AND METHODS. From 11,787 mutagenized colonies from transformant 315–9C[SU], we selected three candidates that were unable to papillate on SC-leu, and from 9,277 mutagenized colonies from transformant 315–9C[LU], we selected seven candidates. The 10 candidates were cured of the plasmid and retransformed with the original pRS314-derivative plasmids to exclude the possibility that the repeat systems had been altered after mutagenesis. The selected strains were also subjected to fluctuation tests and genetic analysis to determine the frequency of Leu^+ recombinants and whether the mutations were in single genes. After these studies only five mutants (two with the inverted repeat system SU and three with the direct repeat system LU) fulfilled the requirements for single gene mutations that decreased the frequency of recombination ≥10-fold below the wild-type levels. We found that the five mutants were sensitive to 0.02% MMS in solid YEPD medium, and subsequent crosses with *rad* mutants of the *RAD52* epistasis group (FRIEDBERG 1988) revealed that all five were allelic to *RAD52*. This mutagenesis study suggests that there are very few genes besides *RAD52* that by a single mutation drastically reduce spontaneous deletions or inversions in our repeat systems (Table 6). This confirms that contrary to previous reported direct repeats, *RAD52* is essential to the formation of spontaneous deletions between our repeats.

DISCUSSION

We have constructed new DNA substrates (three DNA direct repeats and one inverted repeat) for the specific study of the formation of deletions and inversions in *Saccharomyces cerevisiae*. All substrates were based on the same 0.6-kb DNA repeat sequence separated by different distances (31 bp, 2.51 kb, and 5.57 kb for the direct repeats and 1.66 kb for the inverted repeats). The results on spontaneous and DSB-induced recombination in these substrates suggest three conclusions: (1) there are three type of recombination events leading to deletions: *RAD52*-dependent conservative events (reciprocal exchange), *RAD52*-dependent nonconservative events (presumably one-ended invasion), and *RAD52*-independent nonconservative events (presumably SSA); (2) the Rad1/Rad10 endonuclease is required for SSA and one-ended invasion crossover; and (3) the importance of each type of recombination event in the formation of overall deletions may depend on the Rad52 protein and on the site of the initiation event relative to the repeats. These conclusions are discussed below.

Three types of intramolecular recombination events are responsible for deletions between repeats: We have observed that spontaneous deletions occur at frequencies six-to eightfold higher than inversions between the same 0.6-kb repeats, independent of the distance between the direct repeats (31 bp, 2.51 kb, and 5.57 kb). Because only complete reciprocal exchanges can lead to inversions between repeats, we believe that the six-to eightfold predominance of deletions over inversions indicates that there are additional recombination events leading to deletions. Our data are consistent with those reported by JINKS-ROBERTSON *et al.* (1993), who for chromosomal *ura3* repeats longer than 200 bp found that Ura^+ recombinants arose by deletion at a frequency 3–11 times higher than by inversions. DORNFELD and LIVINGSTON (1992) also studied spontaneous recombination between two *his3* heteroalleles in direct

TABLE 6

Frequency of Leu+ deletions and inversions ($\times 10^6$) of the LU and SU repeat systems, respectively, in five new Rec-*rad52* strains

Genotype[a]	LU[b]	SU[b]
Wild-type	430 (×1)	61 (×1)
rad52-11	12 (×0.03)	2 (×0.03)
rad52-12	ND	4 (×0.07)
rad52-13	11 (×0.03)	ND[c]
rad52-14	30 (×0.07)	ND
rad52-15	69 (×0.16)	ND

[a] The wild-type strain used was the parental 315-9C from which the mutants were isolated.

[b] All fluctuation tests were performed on six independent colonies from each mutant transformed with either the LU or SU system, as described in MATERIALS AND METHODS. The median frequency value is given for each case. Numbers in parentheses indicate the relative frequency with respect to the wild-type value for each repeat system studied.

[c] Not determined.

and inverted orientation. However, their data also include gene conversion events and are not directly comparable with ours.

From our comparative study with DSB-induced recombination, we propose that deletions are six to eight times more frequent than inversions because they can initiate in the homologous repeats and in the unique intervening sequence flanked by the direct repeats. In contrast, inversions cannot initiate in nonhomologous DNA sequences (Table 3). We have observed that an *in vitro* DSB flanked by direct repeats is very efficiently repaired, independent of the distance of the DSB to either one of the repeats (Table 3). The repair of these DSBs occurs via induced deletions (over 95% of transformants have deleted the repeats; Table 3). A DSB at a unique DNA sequence not flanked by direct repeats (either direct repeats cut at OUT or inverted repeat cut at IN or OUT) is not efficiently repaired and does not induce either deletions or inversions (Table 3). These results suggest that a DSB at unique DNA sequences is processed very efficiently in *S. cerevisiae* by a recombinational repair mechanism that completes a successful recombination event only when flanked by direct repeats. This means that many spontaneous deletions, but not inversions, could be initiated in the nonhomologous intervening sequence.

Both deletions and inversions in our repeat systems decrease significantly (50- to 200-fold) in *rad52* strains. This is particularly relevant, because a two order of magnitude decrease in gene conversion (JACKSON and FINK 1981; KLEIN 1988; AGUILERA and KLEIN 1989) and reciprocal exchange events scored as inversions (WILLIS and KLEIN 1987; AGUILERA and KLEIN 1989) had been observed in *rad52-1* strains for different heteroallelic repeat systems tested. However, only a 0- to 10-fold decrease in deletions had been reported for different direct repeat systems (JACKSON and FINK 1981; KLEIN 1988; RONNE and ROTHSTEIN 1988; AGUILERA and KLEIN 1989; THOMAS and ROTHSTEIN 1989; DORNFELD and LIVINGSTON 1992; SANTOS-ROSA and AGUILERA 1994). These previously reported data clearly suggest that there is a *RAD52*-independent recombination mechanism responsible for deletions. This *RAD52*-independent mechanism is, indeed, detected in our three direct repeat systems ($0.6-2 \times 10^{-5}$ deletions in *rad52-1* strains). However, and contrary to previously reported results, deletions in our repeats occur predominantly by an additional *RAD52*-dependent mechanism that specifically yields deletions and no viable inversions and is ~50 times more efficient than the *RAD52*-independent mechanism. These results are corroborated by our mutant search that yielded alleles of the *RAD52* gene as either deletion- or inversion-minus mutants. Similar conclusions are obtained from the recombination results of each of the repeat systems right after transformation with uncut circular plasmids (Tables 3 and 4). We believe that in our repeat systems the *RAD52*-dependent nonconservative recombination mechanism of deletions is, for unknown structural reasons, favored over the *RAD52*-independent one.

From our results on DSB-induced recombination, we propose that there are two nonconservative recombination mechanisms for deletion formation. The first one is SSA as proposed by LIN *et al.* (1984) in mammals. It was previously shown that an HO-cut at unique DNA sequences flanked by direct repeats induces deletions betweeen the repeats (RUDIN and HABER 1988; NICKOLOFF *et al.* 1989) that can occur in *rad52* strains (OZENBERGER and ROEDER 1991; FISHMAN-LOBELL *et al.* 1992; SUGAWARA and HABER 1992; MEZARD and NICOLAS 1994). It has been suggested that in yeast SSA can lead to deletions between repeats (OZENBERGER and ROEDER 1991; FISHMAN-LOBELL *et al.* 1992). In our repeats, deletions in *rad52-1* strains are also induced by a DSB flanked by direct repeats (up to 85% of total transformants) (Table 4; Figure 3). Thus, our results are consistent with the idea that DSBs flanked by direct repeats are repaired via deletions by SSA.

We propose that a second nonconservative mechanism of spontaneous deletion formation is one-ended invasion crossover. We have observed that there is a decrease in the frequency of transformation in *rad52-1* strains (not observed for uncut plasmids) that indicates that the DSB-induced deletions occur more frequently by a *RAD52*-dependent mechanism than by a *RAD52*-independent one (Figure 3). One of these mechanism would be SSA and the other one-ended invasion crossing over, which presumably occurs according to the DSB repair model of recombination (SZOSTAK *et al.* 1983; BELMAAZA and CHARTRAND 1994). A crossing over induced by a DSB flanked by repeats occurs by

one-ended invasion, which actually may not repair the break, but eliminates it as part of one of the products of the resulting recombination event. The product of this recombination event (one-ended invasion crossover) is equivalent to the product of a half-reciprocal exchange or half-crossover event, as suggested for *E. coli* (TAKAHASHI *et al.* 1992), even though mechanistically it may occur via full crossing over. Figure 4 illustrates this mechanism and Figure 5 shows how deletions are the only viable recombination products produced through one-ended invasion.

Although we cannot show which of the two nonconservative mechanisms, SSA or one-ended invasion, correspond to the observed *RAD52*-dependent or *RAD52*-independent deletion events, we believe that SSA is the *RAD52*-independent mechanism and one-ended invasion the *RAD52*-dependent one. This is consistent with the original interpretation that SSA explained the *RAD52*-independent events observed in the rDNA region (OZENBERGER and ROEDER 1991) and the deletions events observed by FISHMAN-LOBELL and HABER (1992). The nonconservative *RAD52*-dependent events that these authors eventually found are perfectly explained by one-ended invasion. Consistent with this idea, we have found that our spontaneous *RAD52*-dependent deletion events are independent of the distance between the repeats, whereas the HO-induced SSA deletion events proposed by FISHMAN-LOBELL and HABER (1992) depend on the distance between the repeats. Our hypothesis is also consistent with the idea that the initial steps of the propossed one-ended invasion mechanism correspond to the same initial steps of the *RAD52*-dependent DSB repair model of recombination, which in contrast to SSA requires strand exchange. We believe that the *RAD52*-dependent mechanism of deletion formation is neither intrachromatid reciprocal exchange nor unequal sister-chromatid gene conversion (MALONEY and FOGEL 1987; ROTHSTEIN *et al.* 1987); otherwise similar frequencies of inversions and deletions should have been obtained. We also discard unequal sister-chromatid exchange, because its product would be an unstable dicentric cointegrate and our molecular analysis revealed that all deletions correspond to intramolecular events.

Therefore, we believe there are three types of recombination events responsible for spontaneous and DSB-induced deletions: reciprocal exchange, one-ended invasion, and SSA. Reciprocal exchange (crossover) leads to either deletions or inversions if the event is initiated at the repeats, following a DSB repair recombination mechanism (SZOSTAK *et al.* 1983). However, one-ended invasion and SSA only yields deletions between repeats, independently of whether the initiation event occurred at the homologous repeats or at the unique DNA sequences flanked by the repeats. Therefore, the predominance of spontaneous deletions over

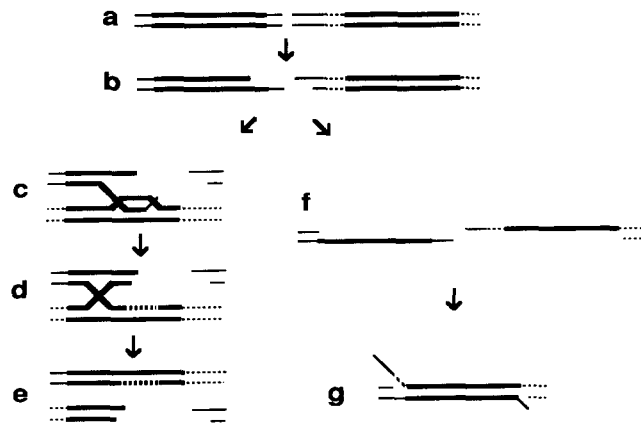

FIGURE 4.—Diagram of the mechanisms of strand-invasion crossover and single-strand annealing initiated by a DSB at the nonhomologous intervening region. (a) DSB at the unique DNA sequence. (b) 5′ to 3′ single-strand exonucleolytic degradation. If the initiation occurrs very asymmetrically between the DNA repeats, strand invasion crossover (steps c–e) will be favored. (c) The 3′ end invades the homologous repeat copy. The heterologous DNA sequence at the 3′ end of the invading DNA should be removed by the Rad1/Rad10 endonuclease. (d) D-loop nicking, DNA synthesis, and formation of a Holliday junction. (e) Resolution of the Holliday junction showing only the product of the crossing over. If the initiation event took place more or less symmetrically between both DNA repeats, SSA (steps f–g) will be favored. (f) Both DNA repeats become single stranded by the action of a 5′ to 3′ single stranded exonuclease. (g) Annealing of both homologous single strand sequences. In contrast to strand-invasion crossover, the Rad1/Rad10 endonuclease would be required to remove both heterologous DNA sequences at each 3′ end. Homologous DNA strands are shown as thick lines that represent newly synthesized DNA when discontinuous. Heterologous DNA strands are shown as either continuous or discontinuous thin lines.

inversions is explained by the nonconservative SSA and one-ended invasion crossover events. Reciprocal exchange and one-ended invasion crossover could represent two different outcomes of the same recombination mechanism, presumably a DSB repair mechanism. One-ended invasion would occur when the DSB is in a nonhomologous region, because only the side of the break that is processed toward the homologous region can recombine with the homologous-repeat partner (see Figure 4).

Finally, we have demonstrated that the particular recombinational behavior of our DNA repeats is intrinsic to the system and not caused by their location on circular plasmids, because the same results were obtained on a linear minichromosome and on chromosome *XV* (Table 5). Whether this conclusion can be extended for any type of DNA repeats or chromosomal location requires further investigation, because recently KAYTOR and LIVINGSTON (1994) have shown that the frequency of recombination between chromosomal *his3* inverted repeats was 100-fold lower than that seen for the repeats on *CEN* vectors.

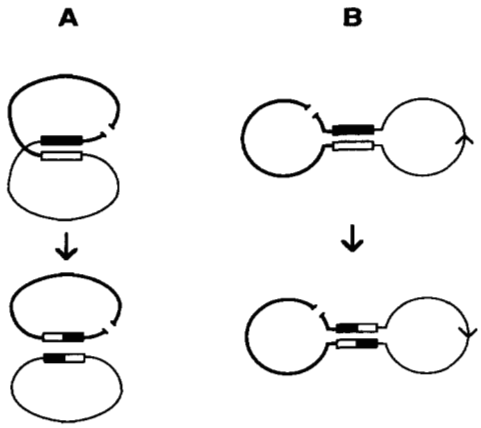

FIGURE 5.—Potential recombination products of a strand-invasion crossover event initiated by a DSB at unique nonhomologous DNA sequences in the DNA repeat systems used in this study. If the formed Holliday junction resolves as noncrossover, then a unique nonrecoverable DNA molecule carrying a DSB would be formed. If it resolves as a crossover, the result would depend on the orientation of the repeats. (A) A crossover between direct repeats induced by a DSB at unique DNA sequences will produce two recombinant molecules, one of which will retain the unrepaired DSB. A recombinant product will be recovered in our systems only if the DSB was at an IN site, because the DSB would be lost with the recombinant product formed by the deleted intervening sequence. When the DSB was at the OUT site, the theoretically viable recombination product would not be recoverable in our direct repeats, because it will not carry an *ARS* sequence. (B) A crossover between inverted repeats produces one nonrecoverable recombinant molecule containing the unrepaired DSB.

The Rad1/Rad10 endonuclease is required for one-ended invasion and SSA recombination initiated at nonhomologous DNA: We have observed that the level of spontaneous deletions and inversions is similar for wild-type and *rad1* mutants. However, total deletions are considerably reduced in direct repeat systems immediately after transformation in *rad1* and *rad10* mutants. It is possible that the partially nicked state of DNA after entering the cell during transformation induces deletions in a different way. Our results, however, are in general agreement with published data showing no or little effect of single *rad1* (KLEIN 1988; RONNE and ROTHSTEIN 1988; SCHIESTL and PRAKASH 1988; THOMAS and ROTHSTEIN 1989) and *rad10* mutations (SCHIESTL and PRAKASH 1988) on spontaneous repeat recombination.

The repair of DSBs flanked by very proximal direct repeats (8 bp from the closest DNA repeat) does not require either the *RAD1* or *RAD10* gene products (Table 4). However, when the DSB is separated from the repeats by more than several hundred base pairs, both *RAD1* and *RAD10* genes are required. The longer the distance between the DSB and the furthest repeat (1.74, 3.38, or 5.27 kb), the lower is the efficiency of transformation, which reaches levels as low as those found for *rad52* strains when the DSB is at 5.27 kb from the repeat. The frequency of deletions among transformants is clearly lower if the DSB is more than several hundred base pairs from the repeats (26–60%) than if it is just 8 and 27 bases from the 3′-end of each repeat (94–97%). These data clearly indicate that *RAD1* and *RAD10* are required for the processing of a DSB at nonhomologous sequences distant from the flanking repeats but not for DSBs flanked by very proximal repeats.

Recently, FISHMAN-LOBELL and HABER (1992) have suggested that the *RAD1* gene is required to remove nonhomologous single-stranded DNA from the 3′ ends of recombining DNA. They propose that this *RAD1* activity would be involved in SSA. Our data are consistent with their conclusion and extend it to the *RAD10* gene. This is consistent with the finding that the Rad1 and Rad10 proteins form a complex *in vitro* (BAILLY *et al.* 1992; BARDWELL *et al.* 1992) that has single-strand DNA endonucleolytic activity (TOMKINSON *et al.*1993) and annealing-promoting activity between homologous single-stranded DNA (SUNG *et al.* 1992). However, the strong requirement for *RAD1* and *RAD10* genes in the repair of a DSB located 0.3 and 5.27 kb from the flanking repeats, for which more *RAD52*-dependent deletions occur, suggests that the Rad1/Rad10 complex also participates in one-ended invasion crossover. Therefore, a DSB at a unique nonhomologous DNA sequence located more than eight bases from the 3′-end of the repeats may need the Rad1/Rad10 endonuclease activity to remove the nonhomologous sequences. If this activity were not required to eliminate eight bases of nonhomologous DNA sequence at one 3′-end of the repeat, one-ended invasion initiated by such a 3′-end could lead to a successful deletion in *rad1* or *rad10* strains, whereas SSA would be less favored because it would also require the removal of the 27 bases of the other 3′-end to complete a successful deletion event.

Importance of reciprocal exchange, one-ended invasion, and SSA in the formation of spontaneous deletions between repeats: What factors determine the importance of one-ended invasion crossover *vs.* SSA in the formation of overall spontaneous deletions? From our parallel study on spontaneous and DSB-induced recombination, we conclude that there are at least two important factors in determining the contribution of one mechanism *vs.* the other. These factors can explain the different *RAD52* dependence in of our systems relative to previously reported repeats.

One factor could be the Rad52 protein itself, as suggested previously (MEZARD and NICOLAS 1994). It has been shown that single-strand exonucleolytic degradation is more extensive in *rad52* strains (WHITE and HABER 1990; SUGAWARA and HABER 1992), indicating that the Rad52 protein prevents extensive single-strand exo-

nucleolytic degradation. Also, according to MEZARD and NICOLAS (1994), a Rad52-dependent protein complex (where the RecA homologous Rad51 protein could participate) could catalize the pairing of homologous DNA repeats required for one-ended invasion. If Rad52 is not present in the cell or does not have easy access to the region of initiation, then the initial steps required for one-ended invasion will not take place and SSA will be favored.

We propose that a second and very important factor that can determine the degree of contribution of a particular recombination mechanism to overall deletions is the location of the initiation event. For the spontaneous deletions that do not occur via reciprocal exchange (those leading to the six to eight times more deletions than inversions), one-ended invasion would be more important than SSA when the initiation event is located more asymmetrically in the unique region between the flanking repeats [compare results of LY(*Sma*I) and LY-(*Sal*I) from Table 4 and Figure 3]. This could explain the observation that the initiation event (presumably a DSB) was equally processed at both sides by single-strand exonucleolytic degradation (FISHMAN-LOBELL and HABER 1992). The closer in time both repeats became single-stranded (more symmetrically located DSB), the better substrates they would be for a nonconservative *RAD52*-independent mechanism, presumably SSA; however, if one repeat became single-stranded much earlier (asymmetrically located DSB), it would serve as a substrate for a nonconservative *RAD52*-dependent mechanism, presumably one-ended invasion. This would explain the importance of reciprocal exchange, one-ended invasion, and SSA or the participation of the Rad1/Rad10 endonuclease in overall spontaneous deletions as a feature specific for each DNA direct repeat system. It remains to be determined what the factors are that influence the location of the initiation event.

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