Genetic Requirements for the Single-Strand Annealing Pathway of Double-Strand Break Repair in Saccharomyces cerevisiae

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ABSTRACT

HO endonuclease-induced double-strand breaks (DSBs) within a direct duplication of Escherichia coli lacZ genes are repaired either by gene conversion or by single-strand annealing (SSA), with >80% being SSA. Previously it was demonstrated that the RAD52 gene is required for DSB-induced SSA. In the present study, the effects of other genes belonging to the RAD52 epistasis group were analyzed. We show that RAD51, RAD54, RAD55, and RAD57 genes are not required for SSA irrespective of whether recombination occurred in plasmid or chromosomal DNA. In both plasmid and chromosomal constructs with homologous sequences in direct orientation, the proportion of SSA events over gene conversion was significantly elevated in the mutant strains. However, gene conversion was not affected when the two lacZ sequences were in inverted orientation. These results suggest that there is a competition between SSA and gene conversion processes that favors SSA in the absence of RAD51, RAD54, RAD55 and RAD57. Mutations in RAD50 and XRS2 genes do not prevent the completion, but markedly retard the kinetics, of DSB repair by both mechanisms in the lacZ direct repeat plasmid, a result resembling the effects of these genes during mating-type (MAT) switching.

Environmental stresses induce a wide variety of injuries to DNA, forcing cells to restore the integrity of the genome by DNA repair. Among the different forms of DNA damage, DNA double-strand breaks (DSBs) are considered as the most harmful, because unrepaired DSBs result in the loss of broken chromosomes, leading to the death of haploid cells or aneuploidy in diploids. Considerable evidence has accumulated during the past 20 years that in yeast the major cellular mechanism of DSB repair involves homologous recombination, culminating in the DSB repair model (SZOSTAK et al. 1983). In this model, a DSB or a gap is repaired by a gene conversion event involving broken ends that invade and copy DNA from an intact, homologous donor copy (Figure 1a).

Further supporting the idea that DSB repair involves homologous recombination is a striking overlap in the genetic requirements for homologous recombination and for DNA repair after treatments known to induce DSBs (e.g., ionizing radiation). Mutations in RAD51, RAD52, RAD54, RAD55, and RAD57 genes confer high levels of sensitivity to ionizing radiation; the very same mutations demonstrate defects in homologous recombination both in vegetative cells and during meiosis (reviewed in PETES et al. 1991). More recently, the RFA1 gene coding for the large subunit of the single-stranded DNA-binding protein RPA was shown to be required for both DSB-induced gene conversion and DNA repair (FIRMENICH et al. 1995).

During the last few years, it has become evident that another pathway exists for DSB repair via homologous recombination. This pathway, called single-strand annealing (SSA), relies on the presence of two homologous sequences in direct orientation flanking the site of a DSB (Figure 1b). According to current schemes of SSA, bidirectional 5' to 3' degradation of the DSB ends leads to the exposure of complementary sequences, which then anneal. Subsequent removal of nonhomologous 3'-ended tails followed by ligation completes the process. The outcome of this reaction is the creation of a deletion of one repeat along with the intervening sequence. The mechanism of SSA was first postulated to explain some features of extrachromosomal recombination in mammalian cells (LIN et al. 1984, 1990). We have shown that the SSA process operates in yeast cells as well, representing an alternative, kinetically separable and independently modulated pathway of DSB repair (FISHMAN-LOBELL et al. 1992). SSA is a truly homologous recombination process, in that it displays essentially the same homology requirements between interacting sequences as does gene conversion (SUGAWARA and HABER 1992; N. SUGAWARA, unpublished results). It is also evident that both SSA and gene conversion strongly depend on the 5' to 3' degradation of DSB ends (CAO et al. 1990; WHITE and HABER...
chromosomal gene conversion and nonhomology that are created during annealing (Figures. In agreement with this assumption, rad1 and degradation was shown to be under similar genetic control and is not expected to occur during recombination by gene conversion involving fully homologous sequences. However, the major problem with interpreting these data is that SSA is only one of a few possible mechanisms of deletion formation during direct-repeat recombination (reviewed in Klein 1995), and the fraction of events being processed through the SSA pathway is unknown. In addition, the identity of the lesion initiating a recombination event (DSB vs. single-stranded break) cannot be revealed in any system employing spontaneous recombination.

To gain more insight into the genetic control of the SSA pathway of DSB repair in yeast, we studied the effects of RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, and XRS2 genes on the repair of a single, HO endonuclease-induced DSB, using two directly repeated \( \text{lacZ} \) genes of \textit{Escherichia coli} as a recombination substrate (Rudin et al. 1989; Fishman-Lobell and Haber 1992; Fishman-Lobell et al. 1992; Ivanov and Haber 1995). The main advantage of this experimental system for studying the genetic control of SSA is that >80% of

\[\text{Deletion product}\]

FIGURE 1.—Two alternative pathways of DSB repair as adapted for the plasmid substrate employed in this work. (a) DSB repair by gene conversion could theoretically yield both conversions without crossing-over and with crossing-over (Szostak et al. 1983). However, repair of an HO-induced DSB occurring in direct repeats of the \( \text{lacZ} \) gene was shown to occur without the formation of the reciprocal crossover product (Rudin and Haber 1988; Fishman-Lobell et al. 1992). (b) SSA pathway repairs a DSB leading to a deletion of one of the direct repeats. In this pathway, the small reciprocal circular product is not expected to be formed. For discussion of current schemes of SSA, see Ivanov and Haber (1995).
HO-induced DSBs are repaired by this pathway, the rest being gene conversion events. In this communication, we present data on the repair of HO-induced DSBs when the lacZ duplication was carried on a low copy-number plasmid as well as when integrated into a chromosome.

MATERIALS AND METHODS

Plasmids: As a substrate to study HO-induced DSB repair, we used two plasmids (FISMAN-LOBELE, et al. 1992). Plasmid pJF6 (Figure 2a) carries two inactive copies of the E. coli lacZ gene in direct orientation, one of which is promoterless while the other is interrupted by the HO cut site. pJF5 differs from pJF6 in that the two copies of lacZ are in inverted orientation separated with the URA3 gene. (a) in the case of pJF6, galactose-induced HO cleaves an 11.4-kb PstI-fragment containing the lacZ duplication into 8.7- and 2.4-kb cut fragments. Repair of the resulting DSB by SSA leads to the appearance of an 7.0-kb PstI-fragment containing the deletion product. Repair of the DSB by gene conversion results in the transformation of a 4.4-kb HindIII-SmaI fragment into a 4.5-kb HindIII-SmaI fragment. A triple HindIII/PstI/SmaI restriction digest allows detection of both recombination products simultaneously. In this latter case, the deletion product is represented by a 5.8-kb PstI-SmaI fragment. (b) in the case of pJF5, the 7.4-kb PstI-fragment containing the HO cut site is cleaved upon galactose induction into 4.4- and 3.0-kb fragments. Repair of this DSB by using the 6.0-kb PstI-fragment as a donor can be accomplished by a gene conversion event with or without associated crossing-over. In the former case, two fragments of 7.0 and 6.5 kb are formed. In the latter case, a single 7.3-kb PstI fragment is formed.

Figure 2.—Structure of plasmids pJF6 (a) and pJF5 (b) and their recombination derivatives. The positions of relevant HindIII, PstI and SmaI sites are shown; for more detail, see FISMAN-LOBELE and HABER (1992). pJF6 contains two inactive copies of lacZ in direct orientation, one of which is promoterless while the other is interrupted by the HO cut site. pJF5 differs from pJF6 in that the two copies of lacZ are in inverted orientation separated with the URA3 gene. (a) in the case of pJF6, galactose-induced HO cleaves an 11.4-kb PstI-fragment containing the lacZ duplication into 8.7- and 2.4-kb cut fragments. Repair of the resulting DSB by SSA leads to the appearance of an 7.0-kb PstI-fragment containing the deletion product. Repair of the DSB by gene conversion results in the transformation of a 4.4-kb HindIII-SmaI fragment into a 4.5-kb HindIII-SmaI fragment. A triple HindIII/PstI/SmaI restriction digest allows detection of both recombination products simultaneously. In this latter case, the deletion product is represented by a 5.8-kb PstI-SmaI fragment. (b) in the case of pJF5, the 7.4-kb PstI-fragment containing the HO cut site is cleaved upon galactose induction into 4.4- and 3.0-kb fragments. Repair of this DSB by using the 6.0-kb PstI-fragment as a donor can be accomplished by a gene conversion event with or without associated crossing-over. In the former case, two fragments of 7.0 and 6.5 kb are formed. In the latter case, a single 7.3-kb PstI fragment is formed.

STRAINS AND MEDIA: The strains of Saccharomyces cerevisiae used in this study are listed in Table 1. All of these strains are MATa- isogenic derivatives of the previously described strain JKM40 (IVANOV and HABER 1995); the MATa- mutation prevents HO cutting. These strains also carry the HO endonuclease gene under control of the inducible GAL promoter, the whole construction being integrated into chromosomal ADE3 locus (SANDELL and ZARIAN 1993). For galactose-induction experiments, strains E1534 through E1556 were transformed with plasmids pJF6 or pJF5. Strain E1558 and its derivatives bear the lacZ direct repeat integrated into the ura3-52 locus on chromosome V (RUDIN et al. 1989; see also Figure 6a) made by integrative transformation of E1534 with plasmid pNR28. The rad mutations were introduced into E1534 and E1558 strains by the one-step gene disruption/replacement method (ROTHSTEIN 1983) using the plasmids listed above. For details of strain constructions, see the corresponding references. All the strain constructions were verified by Southern blot analysis. The lithium acetate method (ITO et al. 1988) was used for yeast transformation. YEPD medium and synthetic complete media lacking leucine (SC-Leu) or uracil (SC-Ura) were described previously (SHERMAN et al. 1986). YEPGly and YEPGal were 1% yeast
TABLE 1
Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>E1534</td>
<td>MATα-1ne ade1 ade3::GAL::HO leu2-3, 112 trp1 ura3-52 RAD1</td>
</tr>
<tr>
<td>E1548</td>
<td>E1534, but rad50::hisG::URA3::hisG</td>
</tr>
<tr>
<td>E1549</td>
<td>E1534, but rad51::URA3</td>
</tr>
<tr>
<td>E1550</td>
<td>E1534, but rad52::LEU2</td>
</tr>
<tr>
<td>E1551</td>
<td>E1534, but rad53::LEU2</td>
</tr>
<tr>
<td>E1552</td>
<td>E1534, but rad55::LEU2</td>
</tr>
<tr>
<td>E1553</td>
<td>E1534, but rad56::LEU2</td>
</tr>
<tr>
<td>E1554</td>
<td>E1534, but direct lacZ duplication</td>
</tr>
<tr>
<td></td>
<td>integrated at ura3-52</td>
</tr>
<tr>
<td>E1555</td>
<td>E1558, but rad51::URA3</td>
</tr>
<tr>
<td>E1556</td>
<td>E1558, but rad52::LEU2</td>
</tr>
<tr>
<td>E1557</td>
<td>E1558, but rad54::URA3</td>
</tr>
<tr>
<td>E1558</td>
<td>E1558, but rad56::LEU2</td>
</tr>
</tbody>
</table>

**extract-2%** Bacto Peptone media supplemented with 3% (v/v) glycerin or 2% (w/v) galactose, respectively. Synthetic complete medium containing X-Gal was prepared as described (RUBY et al. 1983). YEPD medium containing 0.015% (v/v) methyl methane sulfonate (MMS) was used to follow the Rad phenotype. Cultures were incubated at 30°C, unless otherwise indicated.

**Induction of DSBs**: Strains to be tested were streaked to single colonies on SC-Leu/Ura (for strains bearing plasmids) or YEPD (for strains bearing chromosomal integration of lacZ) plates. Liquid precultures (5 ml) in the same medium were inoculated from single colonies, grown to midlog phase, and transferred to 50 ml of YEPGly for 8–10 hr. These cultures were used to inoculate 0.5 liter cultures in YEPGly followed by the growth overnight to a cell density of 10^8 cells per ml. At the time 0, an aliquot of cells was removed, while the rest of the culture was harvested and suspended in the same volume of warmed YEPGly, and incubation was continued for 30 min. The cells were harvested again and suspended in an equal volume of warmed YEPD, an aliquot of cells was removed, and incubation resumed with samples of the cells being withdrawn at the time points indicated. Appropriate dilutions of cells taken before induction (0 hr) and after induction (0.5 hr) were spread on YEPD plates, grown to colonies, and replica plated on SC-Leu/Ura plates to measure loss of plasmid DNA. We note, however, that the amount of plasmid DNA as evidenced by the appearance of cut fragments. Disappearance rapidly in the deletion product accumulated. The formation of the deletion product was severely decreased in a rad52 strain (Figure 3c); however, deletions were formed in rad51 (Figure 3b), rad54 (Figure 3d), and rad55 and rad57 (data not shown) mutant strains. A densitometric analysis (Figure 4) of the Southern blot data of Figure 3 shows that the kinetics of appearance and the amount of the deletion product formed were quite similar in the wild-type and all the rad strains, except rad52 (Figure 4a and Table 2). In Figure 4b, we also analyzed the kinetics of formation and disappearance of cut fragments that reflect the kinetics of processing of the DSB ends. The levels of DSB induction and the rate of their subsequent degradation were essentially similar for wild-type and the rad strains. We conclude that RAD51, RAD54, RAD55, and RAD57 genes are not required for DSB repair by the SSA mechanism on plasmid DNA. We note, however, that the efficiency of cutting seen in the rad52 strain (≤30%) does not correspond quantitatively to the high level of plasmid loss (≥90%). It could be that the amount of cut fragments seen in the rad52 strain is underestimated due to more extensive 5’ to 3’ degradation of one strand as observed previously in rad52 mutant cells (WHITE and HABER 1990; SUGAWARA and HABER 1992).

**RESULTS**

Analysis of DSB repair by the SSA mechanism on a plasmid substrate: We first analyzed the ability of mutants belonging to the epistasis group RAD52 to repair a single, HO endonuclease-induced DSB on plasmid DNA. This DSB must be repaired before the onset of the next round of DNA replication; otherwise, the plasmid would be lost. Thus the level of plasmid loss/retention in galactose-induced cells can be used as a measure of the efficiency of DNA repair. Data presented in Table 2 show that rad51, rad54, rad55, and rad57 mutant strains displayed essentially the wild-type level of plasmid retention. Moreover, after induction >80% of all the cells became LacZ+, demonstrating successful completion of the repair event and arguing against the possibility that the high levels of plasmid retention in mutant cells were due to the inefficient cleavage of plasmid DNA. Additionally, in agreement with our previous results (FISHMAN-LOBELL et al. 1992), a pronounced plasmid loss was seen in a rad52 strain implying that HO induction was high in this particular genetic background.

The kinetics of DSB repair of the plasmid substrate were also analyzed by Southern blot hybridization using DNA samples prepared from HO-induced cells. In a wild-type strain (Figure 3a), expression of the HO endonuclease led to the cleavage of plasmid DNA as evidenced by the appearance of cut fragments. These disappeared rapidly in the deletion product accumulated. The formation of the deletion product was severely decreased in a rad52 strain (Figure 3c); however, deletions were formed in rad51 (Figure 3b), rad54 (Figure 3d), and rad55 and rad57 (data not shown) mutant strains. A densitometric analysis (Figure 4) of the Southern blot data of Figure 3 shows that the kinetics of appearance and the amount of the deletion product formed were quite similar in the wild-type and all the rad strains, except rad52 (Figure 4a and Table 2). In Figure 4b, we also analyzed the kinetics of formation and disappearance of cut fragments that reflect the kinetics of processing of the DSB ends. The levels of DSB induction and the rate of their subsequent degradation were essentially similar for wild-type and the rad strains. We conclude that RAD51, RAD54, RAD55, and RAD57 genes are not required for DSB repair by the SSA mechanism on plasmid DNA. We note, however, that the efficiency of cutting seen in the rad52 strain (≤30%) does not correspond quantitatively to the high level of plasmid loss (≥90%). It could be that the amount of cut fragments seen in the rad52 strain is underestimated due to more extensive 5’ to 3’ degradation of one strand as observed previously in rad52 mutant cells (WHITE and HABER 1990; SUGAWARA and HABER 1992).
It is also possible that in the rad52 strain, expression of HO is not completely shut off after transferring the cells into glucose-containing medium resulting in additional cutting of plasmid DNA. In any case, successful recombination to produce Lac<sup>+</sup> recombinants was nearly completely blocked in rad52 mutant strains.

In plasmid pJF6, only ~80% of HO-induced DSBs are repaired by SSA with the rest being repaired by gene conversion (Fishman-Lobell et al. 1992). We decided to test whether the distribution of deletion vs. gene conversion events is different in the rad strains. By using a triple HindIII/PstI/SmaI restriction digest of DNA prepared from galactose-induced cells, it is possible to follow the formation of both recombination products simultaneously. The results are presented in Figure 5. A densitometric analysis of these data showed that in the wild-type strain (Figure 5a) the whole recombination product (deletion plus gene conversion) represented almost 60% of the total lacZ-hybridizing signal, of which 83% were deletion and 17% were gene conversion events (Table 2). As expected, no visible recombination products were seen in the rad52 strain (Figure 5c). In the rad51, rad54, rad55, and rad57 strains (Figure 5, b and d4, respectively), although the total recombination product was formed at even higher than wild-type level (60~70% of the total lacZ-hybridizing signal), almost no gene conversion product was formed. More precisely, according to densitometric analysis, the efficiency of gene conversion was 30~80 times lower in rad51, rad54, rad55, and rad57 strains compared with the wild type (Table 2).

In a parallel series of experiments, we analyzed the structure of recombinant plasmids from individual LacZ<sup>+</sup> colonies grown on YEPD plates after induction. Each colony contained a single lacZ-hybridizing band, either the 7-kb band corresponding to the deletion product or the ~11-kb band corresponding to the gene conversion event (see Figures 2 and 3). The results of this analysis were in general agreement with the time-course experiments. Among 38 LacZ<sup>+</sup> clones derived from the wild-type strain, 31 (82%) were deletions and seven (18%) were gene convertants. For 39 lacZ<sup>+</sup> clones derived from the rad57 mutant, these figures were 38 (97%) and one (3%), respectively. All 20 LacZ<sup>+</sup> clones analyzed for the rad51 mutant were deletions. Thus the proportion of gene convertants was greatly affected by rad51, rad54, rad55, and rad57 mutations within direct repeats of lacZ genes.

This result was rather unexpected because previously we showed that rad51, rad54, rad55, rad57 mutants are proficient in HO-induced gene conversion between two MAT genes held on a plasmid (Sugawara et al. 1995). To test whether the absence of gene conversion in these rad mutants was an intrinsic property of lacZ-containing plasmids, we used plasmid pJF5 in which the two lacZ genes are in inverted rather than in direct configuration. As shown in Figure 2b, an HO-induced DSB is repaired in this system exclusively by gene conversion. In any case, successful recombination to produce Lac<sup>+</sup> recombinants was nearly completely blocked in rad52 mutant strains.

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induced cells containing p[F5]. The results of this analysis are shown in Figure 6. In the wild-type strain (Figure 6a), bands characteristic of the crossover product started to appear as early as 30–60 min after induction of a DSB. In rad51 (Figure 6b), rad54 (Figure 6d) and rad55 and rad57 (data not shown) strains, the formation of recombination product was indistinguishable from that of wild type both in kinetics and in the final amount. Recombination was still absent in the rad52 strain (Figure 6c).

All of the above-mentioned experiments were carried out at 30°. However, it was shown that in rad55 and rad57 mutants defects in DNA repair and recombination are exaggerated by incubation at a lower temperature (Lovett and Mortimer 1987; Hays et al. 1995; Rajagopal and Symington 1995). As the same rad55 and rad57 mutant strains show complete deficiency in MAT switching when tested at 30° (Sugawara et al. 1995), we felt it unlikely that the proficiency of the rad55 and rad57 mutants in lacZ recombination was due to the higher temperature. However, to rule out this possibility completely, we carried out HO induction on plates containing galactose at 23° or 30° and then plasmid loss was estimated. No increase in loss of either p[F6 or p[F5 at 23° was seen in the rad55 and rad57 mutants, nor in the wild-type, rad51 or rad54 mutants (data not shown).

In summary, RAD51, RAD54, RAD55, and RAD57 genes are not required for DSB repair by the SSA mechanism when duplicated lacZ genes are in direct orientation. The very same genes are not required for HO-induced gene conversion between lacZ sequences in inverted orientation. However, in the corresponding mutants, gene conversion is severely reduced when the recombining lacZ sequences are in direct orientation and SSA is a competing process.

Analysis of DSB repair by the SSA mechanism on a chromosome: Previously, we have shown that rad51, rad54, rad55, and rad57 mutants are able to perform MAT switching on a plasmid, but not on the chromosome (Sugawara et al. 1995). Thus we decided to test whether these genes are required for DSB repair by the SSA mechanism when recombining sequences constitute a part of a chromosome. A direct duplication of lacZ genes used before was integrated into chromosome V as described in MATERIALS AND METHODS. Repair of HO-induced DSBs was analyzed for a wild-type strain

Fig. 3.—Southern blot analysis of the kinetics of deletion formation in wild-type and mutant strains. Expression of the HO endonuclease was initiated by the addition of galactose at time 0. At the time points indicated, samples of cells were taken, and DNA was prepared and digested with PstI. The DNA fragments were separated on neutral 0.8% agarose gels and probed with a lacZ-specific probe as described in MATERIALS AND METHODS. Positions of the 11.1-kb duplication fragment (df), 8.7- and 2.4-kb cut fragments (cf), and the 7.0-kb deletion product (del) are indicated. The other product of recombination, a gene conversion event, cannot be seen on these Southern blots due to a small difference in sizes of the gene conversion and the parental bands. A DNA band at the position of 3.4 kb seen on some of the blots is probably due to nonspecific hybridization because it is seen in noninduced cells. (a) wild type; (b) rad51Δ; (c) rad52Δ; (d) rad54Δ; (e) rad50Δ; (f) xrs2Δ.
and three rad mutants: rad51, rad52, and rad54. In these strains, the chromosomal MATα-inc locus is not cleaved by HO endonuclease so that the only intact HO cut site in the genome is located in one of the duplicated lacZ genes.

We first analyzed the effects of a short induction when the cultures were incubated in YEPGal medium for 1 hr. The percentage of LacZ' cells after induction in the strains carrying the integration construction was significantly lower (45–64%) than in strains carrying lacZ genes on a plasmid (Table 2). We attributed the lower appearance of LacZ' cells to an inefficient cutting of HO cut site in this particular position and to the formation of competing deletions between flanking _ura3-52_ and _URA3_ sequences (Rudin et al. 1989). Thus, we employed HO induction by growing the strains on YEPgal plates. Table 3 summarizes the results of these experiments. Survival of wild-type cells was not affected, but the survival of rad52 cells decreased ~200-fold. There was some drop in viability of rad51 and rad54 mutant strains, which is probably due to the cutting at silent _HM_ loci under conditions of continuous expression of HO (Connolly et al. 1988). However, the levels of LacZ' were very high and equal for the wild-type, rad51 and rad54 strains demonstrating successful completion of DSB repair in all three strains.

The kinetics of DSB repair were monitored at the DNA level. Data presented in Figure 7B and Table 3 show that among three rad mutants tested, only the rad52 mutant displayed a dramatic reduction in deletion formation. The rad51 and rad54 mutants were not only proficient in deletion formation, but in fact demonstrated even higher than wild-type levels of the recombination product.

The two other fragments represent parental bands. The cut fragments created by HO cleavage of the 4.4-kb HindIII-Smal fragment cannot be seen on this blot.

FIGURE 5.—Southern blot analysis of the formation of deletion and gene conversion products of HO-induced recombination in wild-type and mutant strains. Samples of cells were taken 6 hr after expression of HO. DNA was prepared and digested with HindIII/PstI/Smal. The DNA fragments were separated on neutral 0.5% agarose gels and probed with a lacZ-specific probe. Positions of the 5.8-kb deletion product are indicated. Also indicated are positions of the 4.3-kb gene conversion product seen in wild type (a), rad50Δ (g), and _xrs2Δ_ (h) strains; this product cannot be seen in rad51Δ (b), rad52Δ (c), rad53Δ (d), rad55Δ (e), and rad57Δ (f) strains. The two other fragments represent parental bands. The cut fragments created by HO cleavage of the 4.4-kb HindIII-Smal fragment cannot be seen on this blot.
We then wanted to know whether the distribution of recombination products (deletions vs. gene conversion) resembled that for plasmid substrate. Analysis of the structure of individual LacZ' colonies showed that 37 of 37 clones derived from rad51 strain and 40 of 40 clones derived from rad54 strain were deletions; no gene convertants were found among these two sets of LacZ' colonies. Among 40 LacZ' clones derived from the wild-type strain, five (13%) were gene convertants and 33 (87%) were deletions, which is close to the distribution seen for the plasmid substrate. We conclude from these data that as for the plasmid pJF6 recombination substrate, RAD51 and RAD54 genes are not required to carry out SSA on a chromosome, but in the corresponding mutants, HO-induced gene conversion between directly repeated lacZ genes is severely repressed or being prevailed by SSA.

**Mutations in RAD50 and XRS2 genes decrease the efficiency of SSA on a plasmid:** Although RAD50 and XRS2 genes belong to the RAD52 epistasis group, these two genes have some properties making them very different from other members of this group [for detailed discussion of this point, see Ivanov et al. (1994)]. Although rad50Δ and xrs2Δ deletion alleles do not prevent the completion of MAT switching, the kinetics of this process in mutant cells are markedly retarded. Molecular analysis of this phenomenon demonstrated that the slower kinetics of MAT switching in rad50Δ and xrs2Δ mutants is at least partly due to the decreased 5' to 3' degradation of the ends of HO-induced DSBs (Sugawara and Haber 1992; Ivanov et al. 1994). As the SSA process is critically dependent on 5' to 3' degradation of DSB ends (Figure 1b), we analyzed the effects of rad50Δ and xrs2Δ mutations on the efficiency and kinetics of DSB repair in the pJF6 plasmid system.

**TABLE 3**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Survival*</th>
<th>Fraction of LacZ' cells (%)</th>
<th>Relative efficiency of deletion formation*</th>
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<tr>
<td>Wild type</td>
<td>1.1</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td><em>rad51Δ</em></td>
<td>0.67</td>
<td>97</td>
<td>1.43</td>
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<td><em>rad52Δ</em></td>
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<td>ND</td>
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<tr>
<td><em>rad54Δ</em></td>
<td>0.70</td>
<td>96</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* Survival was determined as number of colonies grown on YEPGal plates divided by the number of colonies grown on YEPD plates.

* Fraction of colonies turned blue on X-Gal plates among all the colonies grown on YEPGal plates. Approximately 200 colonies were analyzed for each strain. ND, not determined.

Densitometric analysis of Southern blot data shown in Figure 7B was performed as described in Table 2. The efficiency of deletion formation for the wild-type strain was 40.4%. Presented are data averaged from two independent determinations for each strain.
with the wild-type and proficient rad mutants indicating successful completion of recombination events. One explanation for this apparent discrepancy could be that rad50 and xrs2 mutants do not prevent but markedly retard recombinational repair of HO-induced DSBs between direct repeats of lacZ genes, as was shown to be the case for MAT switching (Ivanov et al. 1994). If so, a significant fraction of DSB-containing plasmids would remain unrepaired at the next round of DNA replication leading to increased plasmid loss. At the same time among recovered plasmids, completion of recombination should have the high proportion of LacZ' cells seen in wild-type cells.

Analysis of the repair events at the DNA level supports the observation that the efficiency of DSB repair within the lacZ duplication was substantially reduced in rad50 (Figure 8a) and xrs2 (Figure 8b) mutants. By 6 hr after induction, only 40–45% of the wild-type level of deletion production was seen in the mutant strains (Figure 8a). Characteristically, the amount of persistent cut fragment was dramatically increased in mutant cells (Figure 8b). Disappearance of the cut fragments reflects the degradation of DSB ends. Thus accumulation of this intermediate in mutant cells supports the idea that the extent of 5' to 3' degradation of DSBs is decreased in rad50 and xrs2 cells.

We then wanted to know whether both deletion and gene conversion formation were affected to the same extent by rad50 and xrs2 mutations. Data presented in Figure 5, g and h, respectively, and in Table 2 show that the efficiency of gene conversion was decreased two- to threefold in rad50 and xrs2 strains compared with wild type, according to a densitometric analysis of Southern blot data. To exclude the possibility that there was a bias in survival of LacZ' colonies bearing a deletion or a gene conversion plasmid derivative, we analyzed individual LacZ' clones derived from the xrs2 strain. For 20 clones analyzed, two (10%) were gene convertants and 18 (90%) were deletions, which is close to the results derived from time-course experiments. We conclude that rad50A and xrs2A deletion mutations decreased HO-induced recombination in directly repeated lacZ genes, both deletion and gene conversion events being affected approximately to the same extent.

**DISCUSSION**

Genes belonging to the epistasis group RAD52 share many common effects on DNA repair and recombina-
Despite the finding that the Rad51 protein has a strand-exchange activity (Sung 1994), our data suggest that Rad51 may play a different role in vivo. One would expect more general defects in recombination in rad51 mutants if this strand-exchange activity were the major cellular one.

The main result of this work is that RAD51, RAD54, RAD55, and RAD57 genes are also not required for HO-induced DSB repair by the SSA mechanism. SSA, either on a plasmid substrate (Figures 3 and 4 and Table 2); or on a chromosome (Figure 7 and Table 3), is not affected by null mutations in these genes. It is important to note that SSA is greatly repressed, both on a plasmid and on a chromosome, by a rad52 mutation demonstrating again the difference between RAD52 and other genes of the RAD52 epistasis group with respect to HO-induced recombination.

The absence of requirements for RAD51, RAD54, RAD55, and RAD57 genes in the SSA pathway is complicated, however, by the negative effects of corresponding mutations on the gene conversion process in the same direct-duplication system. For the chromosomal substrate these effects could be explained by the requirements for these RAD genes to promote recombination in the context of chromatin (Sugawara et al. 1995). At the same time, it is difficult to understand why gene conversion is repressed on plasmid DNA (Figure 5 and Table 2) given the essentially normal levels of gene conversion between lacZ sequences in inverted orientation. We explain this apparent contradiction in the following way. As was shown before (Fishman-Lobell et al. 1992), in the pJF6 plasmid system, deletion and gene conversion events represent two alternative, competing and independently modulated outcomes of repair of an HO-induced DSB. Factors decreasing the efficiency of deletion formation (for example, increasing the distance between two recombining sequences) increase the fraction of gene conversion; conversely, factors preventing gene conversion increase the proportion of deletions (Fishman-Lobell et al. 1992). We propose that in rad51, rad54, rad55, and rad57 mutant cells, the gene conversion process on a plasmid has a modest defect. This defect cannot be seen when the recombining sequences are in inverted orientation (as in the case of plasmid pF5) because gene conversion is the only way to repair an HO-induced DSB. However, in the direct repeat configuration, this defect is apparent, due to the presence of an alternative, highly efficient SSA pathway that competes with gene conversion. In agreement with this view are results obtained with plasmid pNR43 that contains a 4.4-kb insert between the direct repeats leading to a higher proportion of gene conversion in wild-type cells (Fishman-Lobell et al. 1992). In a rad51 mutant cells, this also leads to the appearance of some gene conversion product, albeit with slower kinetics and at a lower level compared with wild type (J. Fishman-Lobell, unpublished results).

Alternatively, one can imagine that gene conversion in rad51, rad54, rad55, and rad57 cells is unaffected but instead the efficiency of the SSA process is increased.

**Figure 8.** Densitometric analysis of Southern blot data on the formation of deletions in rad50Δ (Figure 3e) and xrs2Δ (Figure 3f) strains. The analysis was carried out as described in the legend to Figure 4. The data for the wild-type strain are the same as in Figure 4. ■, wild type; □, rad50Δ; ◊, xrs2Δ. The data for the rad50Δ and xrs2Δ strains are from four independent experiments for each strain.

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due, for example, to higher levels of single-stranded DNA formation as seen in the case of MAT switching (Sugawara et al. 1995). This idea, however, does not find support when analyzing the kinetics of DSB repair on plasmid DNA. Indeed, if in these rad cells DSB ends were degraded and processed into the next recombinational intermediate faster than in wild-type strains, this would cause an apparent decrease in the amount of cut fragments on Southern blots (Figure 3). However, a densitometric analysis of the Southern blot data (Figure 4) does not suggest that the stability of DSB ends is changed in these mutants.

In any case, our results show that in rad51, rad54, rad55, and rad57 mutants DNA formation proceeds almost exclusively via the SSA mechanism. This could serve as a basis to explain increased deletion formation seen in these mutants during spontaneous mitotic recombination involving directly repeated sequences (Fan and Klein 1994; McDonald and Rothstein 1994; Aguilera 1995; Lieffritz et al. 1995).

In this communication, we report that null mutations in RAD50 and XRS2 genes affect the efficiency of SSA leading to increased plasmid loss. Our results confirm our previous observations that rad50Δ and xrs2Δ mutations decrease the extent of 5' to 3' degradation of the ends of HO-induced DSBs (Sugawara and Haber 1992; Ivanov et al. 1994). The observed decrease in the efficiency of deletion formation, however, is only two- to threefold (Table 2). This could explain why during spontaneous mitotic direct-repeat recombination, rad50 mutants show little or no effect (McDonald and Rothstein 1994).

The effects of rad50 and xrs2 mutations on MAT switching suggest that the rate of 5' to 3' degradation of DSB ends is a rate-limiting factor for this specialized gene conversion event (Ivanov et al. 1994). In lacZ direct-duplication substrates, rad50 and xrs2 mutations decrease deletion and gene conversion events to approximately the same extent (Figure 5 and Table 2). This argues that degradation of HO-induced DSB ends is a rate-limiting factor for gene conversion not only during MAT switching but in other recombination systems as well.

So far, only a handful of genes are known to significantly affect the SSA process. These include the RAD52 (Fishman-Lobell et al. 1992; Sugawara and Haber 1992), RAD1, and RAD10 (Fishman-Lobell and Haber 1999; Ivanov and Haber 1995) genes. Recently, a mutation in the CDC1 gene was described that most likely affects the SSA pathway of HO-induced intrachromosomal recombination (Halbrook and Hoekstra 1994). At the same time, many genes known to be essential for "classical" homologous recombination were shown here not to be required for SSA. This means that the genetic requirements of SSA are different from those of gene conversion. Thus additional genes controlling SSA remain to be discovered. To this point, we have recently undertaken a genetic screen to isolate mutants defective in SSA. Among the mutations isolated, some are neither UV or MMS sensitive, making them different from mutations in the above-mentioned genes (Sato, A. Malkova and J. E. Haber, unpublished results). We hope that characterization of these mutations will allow us to identify new genes responsible for SSA in yeast and to use them as probes to detect their human counterparts.

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