# The Roles of *REV3* and *RAD57* in Double-Strand-Break-Repair-Induced Mutagenesis of *Saccharomyces cerevisiae*

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ABSTRACT

The DNA synthesis associated with recombinational repair of chromosomal double-strand breaks (DSBs) has a lower fidelity than normal replicative DNA synthesis. Here, we use an inverted-repeat substrate to monitor the fidelity of repair of a site-specific DSB. DSB induction made by the HO endonuclease stimulates recombination >5000-fold and is associated with a >1000-fold increase in mutagenesis of an adjacent gene. We demonstrate that most break-repair-induced mutations (BRIMs) are point mutations and have a higher proportion of frameshifts than do spontaneous mutations of the same substrate. Although the *REV3* translesion DNA polymerase is not required for recombination, it introduces ~75% of the BRIMs and ~90% of the base substitution mutations. Recombinational repair of the DSB is strongly dependent upon genes of the *RAD52* epistasis group; however, the residual recombinants present in *rad57* mutants are associated with a 5- to 20-fold increase in BRIMs. The spectrum of mutations in *rad57* mutants is similar to that seen in the wild-type strain and is similarly affected by *REV3*. We also find that *REV3* is required for the repair of MMS-induced lesions when recombinational repair is compromised. Our data suggest that Rad55p/Rad57p help limit the generation of substrates that require pol ζ during recombination.

DNA double-strand breaks (DSBs) are potentially lethal events that can arise spontaneously during chromosomal replication or by endogenous or exogenous DNA damage. Cellular enzymes also induce DSBs during programmed developmental pathways such as meiosis and mating-type switching in yeast or immunoglobulin (Ig) gene rearrangement in mammals. Unrepaired DSBs are lethal, and misrepaired DSBs can result in mutations with potentially harmful consequences. Occasionally, the introduction of new mutations can be beneficial to the organism. For example, somatic hypermutation of Ig genes is used to generate antibodies with increased antigen affinity (Jacobs and Bross 2001). Locus-specific DSBs have been demonstrated in cells undergoing somatic hypermutation (Sale and Neuberger 1998; Bross *et al.* 2000; Papavasiliou and Schatz 2000; Kong and Maizels 2001), suggesting a link between DSB formation and the process of hypermutation. Also, mutations that arise during adaptive mutation of *Escherichia coli* can result in the ability to overcome nonpermissive growth conditions (Torkelson *et al.* 2000; Rosenberg 2001). Although no associated DSBs have been demonstrated, their presence is suggested by the requirement for genes that are believed to function only at dsDNA ends, such as RecBC (Bull *et al.* 2000). In *Saccharomyces cerevisiae*, the mutation rate during meiosis is higher than the spontaneous rate during vegetative growth, and many of the meiotic mutations are associated with nearby crossover events (Magni and Von Borstel 1962; Magni 1964; Esposito and Bruschi 1993). In our own research we have demonstrated that the introduction of a site-specific DSB in mitotic cells directly results in increased mutation frequencies associated with the repair of the break (Strathern *et al.* 1995; Holbeck and Strathern 1997; McGill *et al.* 1998; Rattray *et al.* 2001). Our current work is focused on understanding the mechanisms by which these mutations arise.

DSBs can be repaired by homologous recombination, single-strand annealing (SSA), or nonhomologous end-joining (NHEJ; see Paques and Haber 1999 for a review). Recombinational repair utilizes a homologous sequence as a template for DNA synthesis, allowing for the restoration of chromosomal integrity between both sides of the break. The primary product of recombinational repair of a DSB is a gene conversion of the sequences surrounding the break site from the donor to the broken recipient (Figure 1). Recombinational repair is primarily an error-free process, but we previously demonstrated that it is ~100- to 3000-fold more error prone than normal replicative DNA synthesis (Strathern *et al.* 1995; Holbeck and Strathern 1997; McGill *et al.* 1998; Rattray *et al.* 2001). To further investigate the origin of break-repair-induced mutations (BRIMs), we monitored the fidelity of DSB-induced recombina-

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tional repair in haploid yeast cells by use of an inverted-repeat substrate (Figure 1A). Induction of a site-specific DSB in one copy of the inverted repeat by the HO endonuclease resulted in repair of ~99% of all events by homologous recombination resulting in gene conversions, of which 0.4% had an associated BRIM. The remaining events were gene rearrangements and deletions that were presumably repaired by NHEJ (Rattray *et al.* 2001). We present results implicating error-prone DNA polymerases in generating BRIMs and strand-exchange proteins in minimizing the production of recombination intermediates that result in the recruitment of such error-prone polymerases.

The majority of spontaneous and damage-induced mutagenesis in yeast requires the nonessential translesion DNA polymerase ζ (Lawrence and Christensen 1979; Roche *et al.* 1994), the catalytic subunit of which is encoded by the *REV3* gene (Morrison *et al.* 1989). Rev3p is most closely related to DNA polymerase δ but is lacking the 5′–3′ exonucleolytic proofreading domain (Braithwaite and Ito 1993). *In vivo*, *REV3* appears to be important for the insertion of both base substitution (BS) and frameshift (FS) mutations (Roche *et al.* 1994). *In vitro*, pol ζ is more efficient at synthesizing across damaged DNA bases and at extending abnormal primer termini than are most replicative DNA polymerases (Johnson *et al.* 2000), but does not appear to be very processive, generally extending only a few nucleotides (Nelson *et al.* 1996; Lawrence *et al.* 2000). Previously, while monitoring the reversion of mutations associated with repair of a nearby DSB, we found that *REV3* was important for reversion of a BS mutation, but not for two different FS mutations (Holbeck and Strathern 1997).

Homologous recombination requires the functions encoded by members of the *RAD52* epistasis group (see Game 2000 for a review). The *RAD52* gene product is of central importance and is required for efficient repair of DSBs. Biochemically, Rad52p promotes annealing of ssDNA oligomers (Mortensen *et al.* 1996) and enhances the strand-exchange activity of Rad51p (see below). Although the *in vivo* role of Rad52p is not entirely clear, a critical early step in DSB repair is the invasion of a homologous duplex by a 3′ end from the broken strand to initiate strand exchange and new DNA synthesis on the unbroken template. It is presumably during this new DNA synthesis that errors that result in BRIMs occur. Strand invasion and exchange are promoted by Rad51p, which has homology to the *E. coli* RecA protein (Aboussekhra *et al.* 1992; Shinohara *et al.* 1992). *In vitro*, the strand-exchange activity of Rad51p is greatly enhanced by the addition of a number of factors, including Rad52p (Benson *et al.* 1998; New *et al.* 1998; Shinohara and Ogawa 1998; Song and Sung 2000) and Rad55p/Rad57p (Sung 1997). *RAD55* and *RAD57* also encode RecA homologs (Kans and Mortimer 1991; Lovett 1994) and presumably act together as a single functional unit, as they copurify from cells as a heterodimer (Sung 1997). Furthermore, null mutations in either gene, or in the double mutant, have indistinguishable phenotypes, including a much stronger defect for recombination and repair at 20° than at 30° (Lovett and Mortimer 1987; Johnson and Symington 1995; Rattray and Symington 1995). The Rad55p/Rad57p heterodimer does not appear to have strand-exchange activity by itself, but rather appears to function by enhancing Rad51p activity (Sung 1997).

In the work presented here we have analyzed BRIMs at a molecular level, demonstrating that most BRIMs are point mutations. We have also analyzed the roles of members of the *RAD52* epistasis group and of *REV3* on the efficiency of BRIM formation and on the spectrum of BRIMs. From our data, we propose that error-prone polymerases are recruited to bypass secondary structure or base damage of the ssDNA that is produced as a recombination intermediate and that efficient strand exchange reduces BRIMs by minimizing the exposure of ssDNA.

## MATERIALS AND METHODS

**Strains and plasmids:** The strains used in this study are listed in Table 1. All of the strains are derivatives of strains GRY1650 or GRY1654, whose construction has been described previously (Rattray *et al.* 2001). *mush18/21* refers to the inverted-repeat substrate inserted near the *MAT-inc* locus on chromosome *III* (Figure 1). The *rad57-m431* and *can1-5′Δ-m362* alleles were originally isolated by UV mutagenesis of strain GRY1650 as previously described (Rattray *et al.* 2001), resulting in strains GRY1670 and GRY1668, respectively. The *can1-m362* allele is a C → A transversion at position +1272 (where +1 refers to the A of the initiating ATG) of the *CAN1* open reading frame (ORF) resulting in a stop codon. All other strains were constructed either by transplacement of the wild-type gene with a disruption allele or by genetic crosses as noted in Table 1. For the strains that were made by one-step transplacement (Rothstein 1983), cells were transformed by the LiAc transformation procedure (Ito *et al.* 1983) with the appropriate DNA fragments from the disruption plasmids as noted in Table 1. Expected phenotypes were initially tested [*i.e.*, methyl methanesulfonate (MMS) sensitivity, sporulation defects, UV-induced mutagenesis], and the presence of the disruption allele was then confirmed by Southern blot analysis (Ausubel *et al.* 1994, and see below). At least three independent transformants were analyzed, and subsequent fluctuation test analysis was done with at least two independent transformants. For the strains made by crosses, at least three haploid spores bearing the desired mutation that were also isogenic for the markers of the noted parental strain were selected. The mutations were examined for the expected phenotypes and, where possible, further confirmed by Southern blot analysis. Fluctuation analysis was done with at least two different spores of the appropriate genotype.

Plasmid pAL215, used for gap repair of the *can1* mutations (Orr-Weaver *et al.* 1983), contains partial sequences of *TRP1* and *HIS3* separated by a unique restriction site. The relative orientation of the *TRP1* and *HIS3* sequences is such that gap repair of cells with the linearized substrate will result in transfer of the entire *CAN1* ORF and will reconstitute the *HIS3* gene. The plasmid backbone is pRS426 (2μ origin of replica-

**TABLE 1**

**Summary of strains**

| Strain | Genotype[a] | Source/construction | Reference |
|---|---|---|---|
| GRY1650 | *MAT***a***-inc*::*mush18/21*[b] *ade2-101 can1*Δ*::hisG his3-*Δ*200 leu2-*Δ*1 lys2*Δ*::hisG trp1*Δ*::hisG ura3-52* + pGalHO | | Rattray *et al.* (2001) |
| GRY1654 | *MAT*α*-inc*::*mush18/21 can1*Δ*::hisG his3-*Δ*200 leu2-*Δ*1 lys2*Δ*::hisG trp1*Δ*::hisG ura3-52 tyr7-1* + pGalHO | | Rattray *et al.* (2001) |
| GRY1668 | GRY1650 *mush18/21-(can1-5′*Δ*-m362)*[c] | UV mutagenesis of GRY1650 | Rattray *et al.* (2001) |
| GRY1670 | GRY1650 *rad57-m431* | UV mutagenesis of GRY1650 | Rattray *et al.* (2001) |
| GRY1673 | *MAT***a***-inc* 21 *can1*Δ*::hisG his3-*Δ*200 leu2-*Δ*1 lys2*Δ*::hisG trp1*Δ*::hisG ura3-52 tyr7-1 cyh2*[r] | | This study |
| YAR332 | GRY1654 *mush18/21-can1-5′*Δ*-m362* + pGalHO | From GRY1654 × GRY1668 | This study |
| YAR638 | GRY1650 *rev3*Δ*::LEU2* | One-step transplacement with pAM56 (A. Morrison) | This study |
| YAR640 | GRY1654 *rev3*Δ*::LEU2* | One-step transplacement with pAM56 (A. Morrison) | This study |
| YAR665 | GRY1654 *rad52::LEU2* | One-step transplacement with pSM20 (D. Schild) | This study |
| YAR666 | GRY1654 *rad57::LEU2* | One-step transplacement with pSM51 (D. Schild) | This study |
| YAR667 | GRY1654 *rad51::LEU2* | One-step transplacement with pAM28 (M. Aker) | This study |
| YAR676 | GRY1654 *rev3*Δ*::LEU2 rad57-m431* | From YAR640 × GRY1670 | This study |
| YAR678 | YAR666 *rev3*Δ*::LEU2* | From YAR638 × YAR666 | This study |
| YAR692 | YAR665 *rev3*Δ*::LEU2* | From YAR638 × YAR665 | This study |
| YAR693 | YAR667 *rev3*Δ*::LEU2* | From YAR638 × YAR667 | This study |
| YAR764 | YAR332 *rad57::LEU2* | One-step transplacement with pSM51 (D. Schild) | This study |
| YAR784 | GRY798 + YEp13-*RAD51* | | This study |
| YAR798 | GRY1654 *rad57-m431* | From GRY1670 × GRY1654 | This study |

[a] All markers are isogenic to parental strains except those noted.
[b] *mush18/21* refers to the inverted-repeat construct shown in Figure 1A.
[c] *mush18/21-(can1-5′*Δ*-m362)* refers to the inverted-repeat construct with the *can1-5′*Δ*-m362* allele.

tion, *URA3*; Sikorski and Hieter 1989). Further details on the sequence and construction of pAL215 are available upon request.

**Analysis of recombination and mutation frequencies:** Fluctuation tests were performed as described previously (Rattray *et al.* 2001). Briefly, log-phase cells were grown in glucose medium lacking uracil (to select for the HO plasmid) and aliquots were plated on the appropriate selective medium to determine the $Trp^+$, $Can^r$, and total cell titers. Cells were then washed and grown in galactose medium lacking uracil for ~18 hr, after which aliquots were again plated to determine the $Trp^+$, $Can^r$, and total cell titers. At least 15 independent colonies were analyzed for each strain. The frequency of associated BRIMs was then determined by replica plating $Trp^+$ prototrophs to determine the proportion that were also $Can^r$. Although it is more convenient to determine the rate of BRIM formation by directly selecting for *TRP1 can1* cells, we previously showed that this underestimates the actual rate (Rattray *et al.* 2001). We find that after galactose induction, ~98% of all cells are still inducible to *TRP1*, suggesting inefficient cleavage of our substrate by HO. Therefore, it is not possible to determine any loss of viability associated with DSB induction in recombination-defective mutants. Statistical significance was determined by a $\chi^2$ contingency test.

**Physical analysis of recombinants:** DNA from independent recombinants was isolated by glass bead disruption of cells (Hoffman and Winston 1987), digested with the appropriate restriction enzymes, electrophoresed in agarose, and transferred to Hybond N+ (Amersham, Buckinghamshire, UK) as previously described (Rattray *et al.* 2001). After hybridization with the appropriate $^{32}$P-labeled probes, washed blots were analyzed on a Typhoon scanner with ImageQuant 1.1 software.

**Sequencing of *TRP1 can1 HIS3* events:** DNA from independent recombinants was isolated, digested with Xba1, and cotransformed into GRY1673 cells with linearized pAL215 to gap repair the entire *CAN1* ORF (Orr-Weaver *et al.* 1983). $Ura^+$ $His^+$ transformants were selected, and DNA from these cells was transformed into *E. coli* KC8 cells (CLONTECH, Palo Alto, CA) by electroporation. Plasmid DNA from individual $His^+$ *E. coli* transformants was isolated by minipreps (QIAGEN, Chatsworth, CA). The *can1* ORF was sequenced on an ABI3700 automated sequencer using four forward and four reverse primers of *CAN1*. Sequencing was performed by the Laboratory of Molecular Technology (National Cancer Institute-FCRDC). The sequences were compiled and analyzed with Sequencher 3.1 software program (GeneCodes). A detailed list of the sequenced mutations is presented in the supplemental table at http://www.genetics.org/supplemental/. We noted two nucleotide differences in our wild-type *CAN1* sequence compared to those published in the Saccharomyces Genome Database: a T → G transversion at base pair +465, V115V (where +1 refers to the A of the initiating ATG) and an A → G transition at base pair +1600, I534V.

**Analysis of MMS sensitivity:** Cells were grown to mid-log phase in YPD, after which cells were concentrated to ~$10^9$/ml in YPD. Fifty-microliter aliquots of cells were added to 96-well microtiter plates containing an equal volume (50 μl) of prewarmed YPD with twice the indicated concentration of MMS (Figure 3). After incubating the cells for 10 min at 30°, the MMS was inactivated by adding 100 μl of 10% sodium thiosulfate. Ten-fold serial dilutions were then spotted onto YPD plates and incubated at 30° for 5 days.

## RESULTS

Several years ago our laboratory demonstrated that the fidelity of DSB repair is much lower than the fidelity of normal S-phase DNA synthesis (see Introduction), suggesting intrinsic differences in the DNA synthetic complexes, substrates, or error-correction mechanisms between the two processes. These differences have allowed us to monitor the fidelity of DSB repair as an assay for the identification and characterization of functions involved in this process, with a goal of gaining a clearer understanding of the mechanisms involved in DSB repair and its role in mutagenesis.

We have used an inverted-repeat substrate in which a site-specific DSB is introduced into one of the repeats (Figure 1A). The substrate consists of a reporter for homologous recombination (*trp1*) and a reporter for associated mutagenesis (*CAN1*). The substrate has a unique recognition sequence for the HO endonuclease (HOcs) present between the *trp1-3′Δ* allele and a full-length wild-type copy of *CAN1*. The normal HO recognition sequence at the *MAT* locus is mutated to be uncleavable by HO (*MAT-inc*). The HO endonuclease is under control of the *GAL1* promoter such that transfer of the cells from glucose to galactose induces expression of HO and promotes DSB formation at the HOcs. The DSB is repaired by homologous recombination using the duplicated sequences present in the second repeat (consisting of the *trp1-5′Δ* and *can1-5′Δ* alleles), which does not have an HO recognition sequence but does have homology to both sides of the break (Figure 1A). Recombinants are scored as tryptophan prototrophs (*TRP1*), and mutations associated with the repair event are scored as canavanine-resistant (*can1*) mutations among the *TRP1* recombinants. The construction and characterization of this substrate has been previously described (Rattray *et al.* 2001).

**Most BRIMs are point mutations:** The introduction of an HO-induced DSB into the inverted-repeat substrate results in a large (>5000-fold) increase in *TRP1* recombinants, representing ~99% of all HO-induced events (Table 2, wild type, spontaneous *vs.* induced). Analysis of the DSB-induced *TRP1* recombinants indicates that most (99.6%) are repaired in an efficient and error-free manner. However, the frequency of BRIMs (*TRP1 can1*; $4 \times 10^{-3}$; Table 2) is ~3000-fold higher than the spontaneous frequency of mutation to *can1* ($1.3 \times 10^{-6}$). Introduction of a DSB also results in an ~150-fold increase in total *can1* events (not selected for *TRP1*), representing ~1% of all HO-induced events. Further analysis of the DSB-induced *can1* events indicates that most (>95%) of these events are also *trp1 his3* and represent rearrangements and/or deletions of the substrate (data not shown). These events are presumably the result of NHEJ and have been termed break-repair-induced rearrangements (BRIRs) to distinguish them from events repaired by homologous recombination.

From a Southern blot analysis of independent *TRP1 can1 HIS3* DSB repair events we find that most (96%) are gene conversions, of which 24% are associated with an inversion of the substrate (Table 3, wild type). The

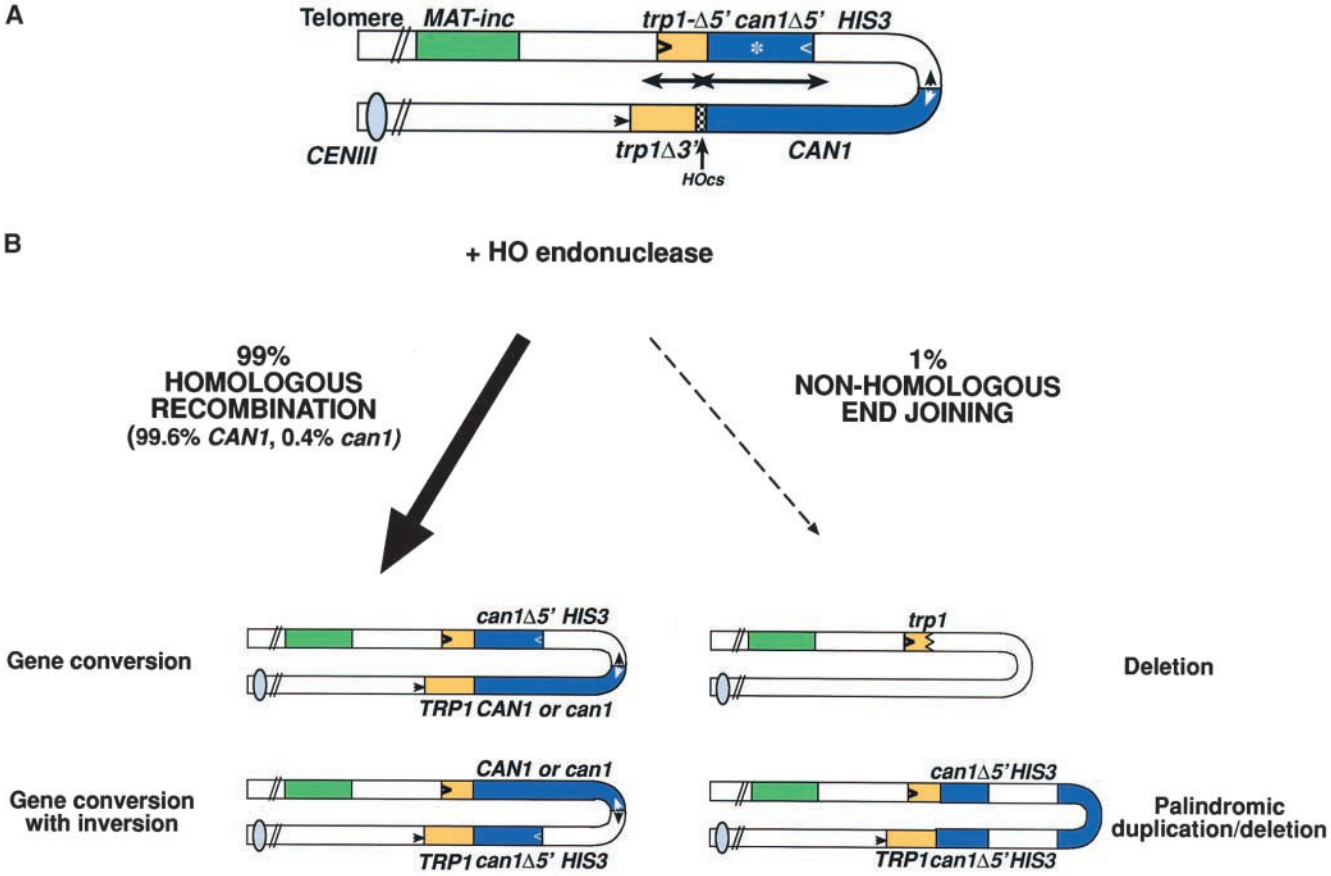


FIGURE 1.—Inverted-repeat substrate and products of DSB repair. (A) Substrate for fidelity of DSB repair. The substrate consists of inverted repeats of sequences from the *TRP1* (yellow) and *CAN1* (blue) genes. One repeat (A, bottom) is composed of a portion of *TRP1* including its promoter but lacking the 3′ end of the gene (*trp1-3′*Δ). Adjacent to the *trp1-3′*Δ allele is a 30-bp recognition sequence for the HO endonuclease (HOcs, stippled) and a full-length copy of the wild-type *CAN1* sequence including the promoter (*CAN1*). The second repeat (top) consists of a second truncated copy of the *TRP1* gene lacking the promoter but containing the 3′ end of the gene (*trp1-5′*Δ). Adjacent to the *trp1-5′*Δ allele is a portion of the *CAN1* gene lacking the promoter and 5′ end of the ORF (*can1-5′*Δ). The inverted repeats are separated by a full-length wild-type copy of the *HIS3* gene, including its promoter. The entire substrate is located near the *MAT* locus (green). The normal HO recognition sequence at MAT has been mutated to be uncleaveable by the HO endonuclease (*MAT-inc*). The double-headed arrows in the center of the structure indicate the extent of homology between the repeats, which consists of 374 bp of *TRP1* sequence and 1300 bp of *CAN1* sequence. Small solid arrows indicate the promoters, and open triangles represent the orientation of the gene for sequences lacking a promoter. The asterisk in the *can1-5′*Δ repeat indicates the approximate location of the *can1-m362* allele present only in strains GRY1668, YAR332, and YAR764. (B) Products of HO-induced DSBs. The majority (99%) of the DSBs are repaired by homologous recombination to yield *TRP1* gene conversion events, a subset of which are associated with an inversion of the substrate (lower left and Table 3). About 1% of the DSBs are repaired by a mechanism that leads to rearrangements of the substrate that are presumably repaired by nonhomologous end-joining. These rearrangements include many different types of events, of which only two examples are shown. Top right: a deletion. Bottom right: a palindromic event due to a duplication/deletion as previously described (RATTRAY *et al.* 2001).

remaining three events are associated with a rearrangement of the substrate. Among the gene conversions, the DNA fragments have no obvious alterations in the expected mobility, suggesting that the mutations are primarily small alterations such as point mutations. For comparison, we also include data from the analysis of 26 independent *TRP1 CAN1 HIS3* gene conversion events. Of the 26 gene conversions, 2 (8%) are associated with inversions (Table 3). Although BRIMs appear to have a larger proportion of inversions, the number of events examined is too small to be of statistical significance ($P > 0.05$).

To further elucidate the nature of the BRIMs, we sequenced the entire *can1* ORF from 42 independent DSB-induced *TRP1 can1 HIS3* events from our wild-type strain. As a basis for comparison, we have also sequenced the entire *can1* ORF from 26 spontaneous (non-DSB-induced) *can1* mutations from the same strain. We find that the majority (85%) of the spontaneous mutations are base substitutions (Table 4). The remaining 4 mutations are single nucleotide deletions or insertions resulting in frameshifts. Therefore, among the spontaneous mutations is a FS:BS ratio of ~0.2. Most of the mutations (69%) are in the unduplicated region of the

**TABLE 2**

**Frequency of recombination, mutation, and BRIM formation in the *mush18/21* inverted-repeat substrate**

| Strain | Relevant genotype | Spontaneous ($\times 10^6$)[a] ($\pm$SD)[b] | | HO-induced ($\times 10^6$)[c] ($\pm$SD)[b] | | Phenotype of HO-induced *TRP1 HIS3* cells[d] (*N*)[e] | |
|---|---|---|---|---|---|---|---|
| | | *TRP1* | *can1* | *TRP1* | *can1* | % *CAN1* | % *can1* |
| GRY1654 | Wild type | 4.0 (3.1) | 1.3 (0.5) | 21,000 (11,000) | 200 (96) | 99.6 (1,500) | 0.4 |
| YAR640 | *rev3*Δ | 1.7 (1.5) | 0.4 (0.2) | 26,000 (13,000) | 3.5 (0.8) | 99.9 (4,183) | 0.1 |
| YAR798 | *rad57-m431* | 3.0 (0.7) | 7.5 (4.4) | 410 (170) | 47 (18) | 97.5 (962) | 2.5 |
| YAR676 | *rad57-m431 rev3*Δ | 2.9 (1.2) | 1.4 (1.7) | 170 (95) | 70 (65) | 95.7 (376) | 4.3 |
| YAR784 | *rad57-m431* + YEp13-*RAD51* | 3.3 (0.9) | 7.5 (5.6) | 2,500 (400) | 120 (45) | 99.3 (698) | 0.7 |
| YAR666 | *rad57*Δ | 2.4 (1.3) | 16 (12) | 180 (210) | 140 (60) | 91.5 (749) | 8.5 |
| YAR666 | *rad57*Δ (20°) | 2.0 (1.0) | 32 (24) | 30 (30) | 35 (25) | 100 (300) | <0.3 |
| YAR667 | *rad51*Δ | 0.4 (0.04) | 16 (13) | 1.6 (0.4) | 10 (4.8) | 99.2 (368) | 0.8 |
| YAR665 | *rad52*Δ | <0.01 | 10 (13) | 0.4 (0.5) | 7.7 (2.0) | 100 (457) | <0.2 |

[a] Average of median frequencies (see MATERIALS AND METHODS).
[b] Standard deviation.
[c] Results after induction of a DSB by inducing expression of the HO endonuclease by transferring cells to galactose.
[d] Determined by replica plating *TRP1 HIS3* events from fluctuation analysis (see MATERIALS AND METHODS).
[e] Total number analyzed.

*CAN1* gene, which comprises only 38% of the ORF (Figure 2, spontaneous).

A summary of the sequence analysis of the 42 BRIMs from DSB-induced *TRP1 can1 HIS3* events is shown in Figure 2 and Table 4 (wild type). The mutations were composed of 17 BS, 27 FS, and a TTT → AAAA substitution (see supplemental table at http://www.genetics.org/supplemental/ for details). We note that the majority of the FS mutations occur in mononucleotide repeats ≥3. We find significantly more FS among BRIMs than among spontaneous mutations, resulting in a FS:BS ratio of 1.4 for the BRIMs as compared with 0.2 for the spontaneous mutations (Table 4, $P < 0.05$). Although there is no apparent gradient in mutational events from the site of the HO break, located 200 bp from the 3′ end of the *CAN1* ORF, a larger proportion (54%) of the mutations is located in the duplicated region of *CAN1* (closer to the HO break site) than that found for spontaneous mutations (Figure 2).

***REV3* is required for most BRIMs:** In previous experiments, we showed that *REV3* was important for DSB-induced reversion of a BS mutation, but not of two different FS mutations (HOLBECK and STRATHERN 1997). Our results were surprising given the importance of *REV3* for the introduction of both BS and FS mutations during spontaneous or UV-induced mutagenesis (ROCHE *et al.* 1995; LAWRENCE *et al.* 2000). To test the generality of the distinction between the origin of BS and FS errors, we examined the effect of a *rev3*Δ mutation on the mutation spectra of BRIMs in the inverted-repeat assay,

**TABLE 3**

**Physical analysis of DSB-induced *TRP1 HIS3* recombinants**

| Strain | Relevant genotype | *CAN1* phenotype | Gene conversion | | Other[b] |
|---|---|---|---|---|---|
| | | | Total GC[a] | % inversions | |
| GRY1654 | Wild type | *CAN1* | 26 | 8 | 1 |
| | | *can1* | 41 | 24 | 3 |
| YAR640 | *rev3*Δ | *CAN1* | 22 | 0 | 1 |
| | | *can1* | 40 | 13 | 1 |
| YAR798 | *rad57-m431* | *CAN1* | 9 | 0 | 2 |
| | | *can1* | 28 | 18 | 3 |
| YAR676 | *rad57-m431 rev3*Δ | *can1* | 15 | 44 | 0 |
| YAR666 | *rad57*Δ | *CAN1* | 9 | 11 | 2 |
| | | *can1* | 30 | 17 | 1 |

[a] Total number of gene conversions among events analyzed.
[b] Unpredicted rearrangement of the substrate.

**TABLE 4**

**Summary of mutational spectra at *CAN1***

| | | Frameshift | | Base substitution | | Complex[c] | |
|---|---|---|---|---|---|---|---|
| | | Frequency (%) | Type[a] | Frequency (%) | Type[b] | Frequency (%) | FS:BS[d] |
| Spontaneous | Native locus[e] | 11/40 (28) | 8:3 | 26/40 (65) | 11:13 | 3/40 (7) | 0.4 |
| | Inverted repeat | 4/26 (15) | 4:0 | 22/26 (85) | 8:14 | 0/26 | 0.2 |
| BRIMs | Genotype[f] | | | | | | |
| | Wild type | 24/42 (57) | 17:7 | 17/42 (41) | 6:11 | 1/42 (2) | 1.4 |
| | *rev3*Δ | 25/29 (86) | 18:7 | 4/29 (14) | 3:1 | 0/29 | 6.3 |
| | *rad57-m431* | 11/22 (50) | 9:2 | 8/22 (36) | 6:2 | 3/22 (14) | 1.4 |
| | *rad57-m431 rev3*Δ | 16/21 (76) | 14:2 | 3/21 (14) | 1:2 | 2/21 (10) | 5.3 |

See supplemental table at http://www.genetics.org/supplemental/ for detailed list of mutations.
[a] Contractions:expansions.
[b] Transitions:transversions.
[c] See text for details.
[d] Frameshift:base substitution ratio.
[e] Data from Tishkoff *et al.* (1997) and Tran *et al.* (2001).
[f] Only relevant genotype is shown.

which uses the forward mutation reporter *CAN1* and is therefore a target for many different types of mutations. As described below, our experiments reveal a major pathway for FS errors that is independent of *REV3*.

The rate of recombination and mutation in a *rev3*Δ strain is shown in Table 2 (*rev3*Δ). As expected, we find that *rev3*Δ strains are reduced about threefold for spontaneous mutation to *can1* (Table 2). The introduction of a *rev3*Δ mutation does not affect the rate of recombination to *TRP1*, which is consistent with our previous finding that *REV3* is not important for homologous recombination *per se*. We were surprised to find a large reduction in *can1* events (not selected for *TRP1*) after HO induction (Table 2, $P < 0.01$) since the majority of DSB-induced *can1* events in wild-type cells were also *trp1 his3* (>95%) and presumably require NHEJ. These data suggest a previously unrecognized role for *REV3* in BRIRs. These events require further characterization and will be presented elsewhere.

We observe that only 0.1% of the *TRP1* recombinants from a *rev3*Δ strain have an associated mutation in *can1*, representing a 4-fold reduction in BRIMs as compared to the wild-type strain (Table 2; $P < 0.01$). It is notable that the frequency of BRIMs in the *rev3*Δ strain ($2.6 \times 10^{-5}$) is 65-fold higher than the spontaneous *can1* mutation frequency (Table 2), indicating that BRIMs are still induced in a *rev3*Δ strain ($P < 0.01$).

The Southern blot data from 41 independent *TRP1 can1 HIS3* BRIM events from a *rev3*Δ strain are shown in Table 3. Most (93%) of the BRIMs are gene conversions, of which 13% are associated with an inversion. We sequenced the *can1* ORF from 29 independent *TRP1 can1 HIS3* BRIM events from a *rev3*Δ strain and found that ~86% of the mutations are FS (Table 4, Figure 2) and only 14% are BS. Significantly more FS mutations are among the *rev3*Δ BRIMs than among wild-type BRIMs, resulting in a change in the FS:BS ratio from 1.4 in wild-type to 6.3 in *rev3*Δ strains ($P < 0.05$). If we take into consideration the overall frequency of BRIMs for the wild-type ($8.4 \times 10^{-5}$) and *rev3*Δ strains ($2.6 \times 10^{-5}$), as well as the proportion of frameshifts and base substitutions in each strain, we find a greater reduction in BS (about ninefold) than in FS (about twofold) mutations. These data indicate that another error-prone polymerase(s) is likely to be involved in introducing BRIM FS mutations (see discussion).

***RAD57* promotes fidelity of DSB repair:** In a screen for mutants with altered fidelity of mitotic DSB repair we identified a candidate that showed a greatly reduced ability to promote recombinational repair of the DSBs, but also demonstrated elevated levels of BRIMs among the recombinants. We previously presented a preliminary characterization of this mutant, identifying it as an allele of *RAD57* (*rad57-m431*; Rattray *et al.* 2001). A fluctuation analysis of the recombination and mutator phenotypes of this mutant is shown in Table 2 (*rad57-m431*). Prior to galactose induction, strains with this allele show a moderate (5-fold) spontaneous mutator phenotype. Introduction of a DSB induces recombination by only ~140-fold in a *rad57-m431* strain (as compared to ~5000-fold in the wild-type strain). These data agree with previous results of others showing that *RAD57* is important for DSB repair (Johnson and Symington 1995; Sugawara *et al.* 1995). In *rad57-m431* cells the frequency of associated BRIMs determined by phenotypic analysis of the *TRP1* recombinants is increased to 2.4% (Table 2, $P < 0.01$). These data suggest that *RAD57* plays an important role in mutation avoidance during DSB repair.

From the Southern blot analysis of 31 independent *TRP1 can1 HIS3* BRIM events from a *rad57-m431* strain (Table 3), we find that most (90%) are gene conversion

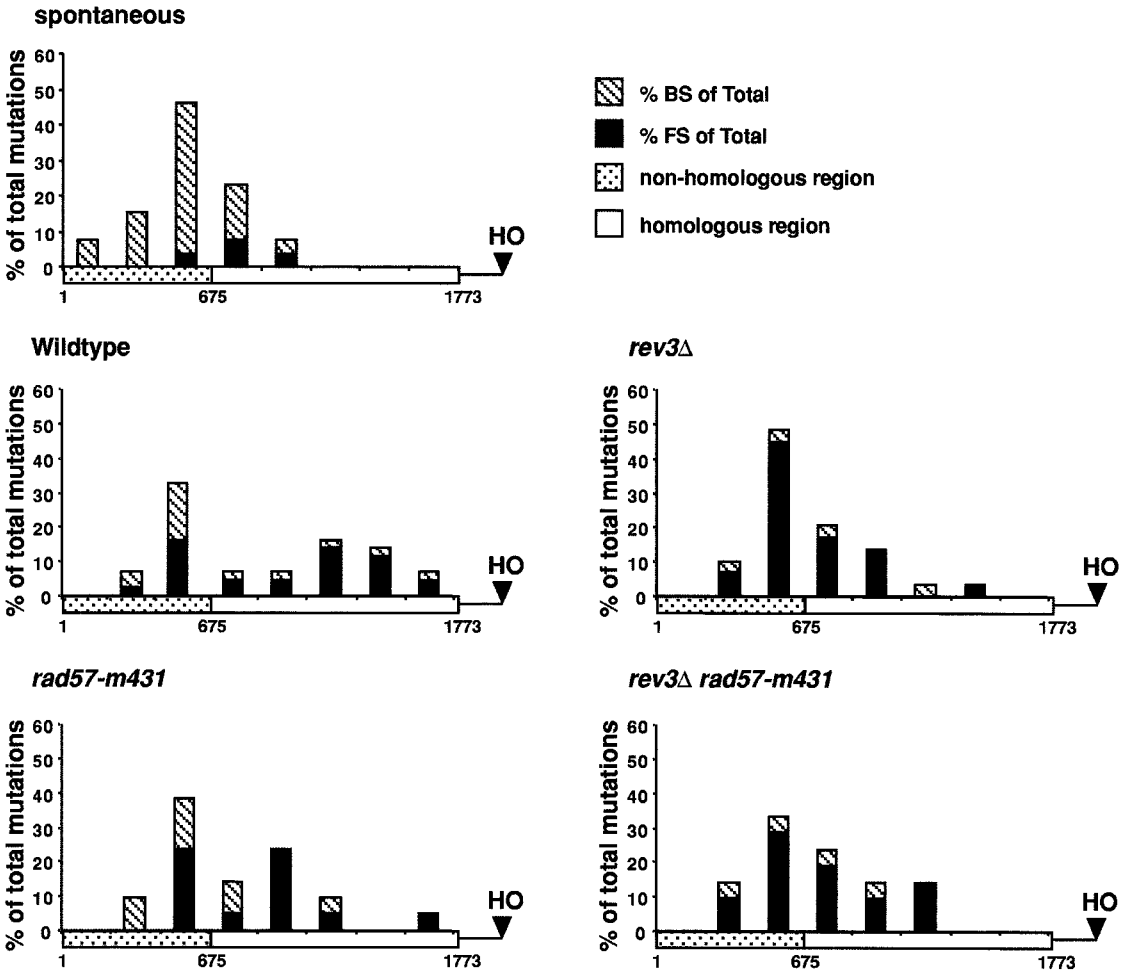


FIGURE 2.—Distribution of BRIMs from wild-type, *rev3*Δ, *rad57-m431*, and *rad57-m431 rev3*Δ strains. The *x*-axis represents the entire *CAN1* coding sequence from left to right (where 1 refers to the A of the initiating ATG). The open region (from +676 to +1773) represents sequences also present in the *can1-5′*Δ allele, whereas the stippled region (from +1 to +675) represents sequences present only in the full-length copy of *CAN1*. The HO recognition sequence is 200 bp beyond the 3′ end of the ORF. The mutations have been pooled into groups of 225 bp, and the location and proportion of each type of mutation is represented by the bars, where the *y*-axis represents the percentage of all mutations sequenced. Striped bars represent the percentage of BS mutations, and solid bars represent the percentage of FS mutations. Spontaneous refers to spontaneous mutations isolated in the absence of HO induction from strains GRY1650 and GRY1654 (data are pooled). All other graphs refer to DSB-induced BRIMs. Only relevant genotypes are indicated. Wild type, strains GRY1650 and GRY1654; *rev3*Δ, strains YAR638 and YAR640; *rad57-m431*, strain YAR647; *rad57-m431 rev3*Δ, strain YAR676. All sequenced mutations are listed in the supplemental table at http://www.genetics.org/supplemental/.

events, of which 18% are associated with an inversion. A summary of the sequence analysis of 22 BRIMs from the *rad57-m431* strain is shown in Figure 2 and Table 4. As for the wild-type strain, we found that most of the BRIMs are point mutations. Among the 22 events sequenced, 8 were single BS, 1 was a GG → TA substitution, and the remaining 13 events were FS (Table 4). The overall distribution of mutations is similar to that seen in wild-type strains (Figure 2) and has a similar FS:BS ratio (1.4, Table 4). These data suggest that *RAD57* probably does not affect the mechanism(s) by which the BRIMs arise, but rather appears to influence the frequency of their occurrence.

Since the *rad57-m431* mutant was isolated in a screen for mutants with altered fidelity, it was unclear whether the reduced fidelity was specific to this allele or was a general attribute of *rad57* mutants. Therefore, we examined a strain with a *rad57* null allele (*rad57*Δ) in our assay and found that it was very similar to the *rad57-m431* mutation at 30° (Tables 2 and 3). Null mutations in *rad57* are known to have a more severe phenotype for recombination and repair at lower temperatures (JOHNSON and SYMINGTON 1995; RATTRAY and SYMINGTON 1995). We found that reducing the temperature of incubation and DSB induction reduced the efficiency of repair of the null mutant, but not of the *rad57-m431* allele (Tables 2 and 3). The slightly greater severity and cold sensitivity of the null mutation suggests that the *rad57-m431* protein is still present and possibly provides some stability to the recombination complex.

Several studies have shown that the recombination and repair phenotypes of *rad57* and *rad55* mutants for recombination and repair are indistinguishable (Lovett and Mortimer 1987; Hays *et al.* 1995; Johnson and Symington 1995; Rattray and Symington 1995). An analysis of a *rad55*Δ strain in our assay indicates that it has a very similar phenotype to a *rad57*Δ strain (data not shown).

Overexpression of Rad51p has been shown to substantially suppress the recombination and repair defects of *rad57* (and *rad55*) mutants (Hays *et al.* 1995; Johnson and Symington 1995), suggesting that a major role of Rad57p (and Rad55p) is to promote the strand-exchange activity of Rad51p. To determine if overexpression of Rad51p could also suppress BRIMs, we introduced a high-copy plasmid expressing Rad51p under its own promoter into strains with the inverted-repeat substrate. Introduction of the vector alone (YEp13) into either strain or of YEp13-*RAD51* into a wild-type strain did not affect the frequency of recombination to *TRP1* or the frequency of associated BRIMs (data not shown). However, when YEp13-*RAD51* was introduced into a *rad57-m431* strain we found that although it did not appear to suppress the spontaneous mutator phenotype of *rad57-m431*, it was able to partially suppress the defect in promoting DSB-induced recombination to *TRP1* (Table 2). Overexpression of Rad51p resulted in an increase in *TRP1* recombinants by about sixfold and in a reduction in the fraction of BRIMs among the *TRP1* recombinants from 2.4% in the *rad57-m431* strain to 0.7% when YEp13-*RAD51* is present (Table 2; $P < 0.05$). These data are consistent with a defect in strand exchange leading to elevated levels of BRIMs.

***RAD51* and *RAD52* are required for DSB-induced recombinational repair of the inverted-repeat substrate:** To determine whether increased BRIMs are associated with other mutants of the *RAD52* epistasis group, we examined the phenotype of null alleles of *rad51* and *rad52* in the inverted-repeat assay. We found that *rad51*Δ or *rad52*Δ mutant strains show a ≥10-fold reduction in spontaneous recombination to *TRP1* (Table 2; *rad51*Δ, *rad52*Δ) and a ≥10-fold increase in spontaneous mutation to *can1*. A spontaneous mutator phenotype for *rad51* and *rad52* mutants has been reported previously (Morrison and Hastings 1979; Kunz *et al.* 1989). Induction of a DSB results in only a 4-fold increase in *TRP1* recombinants in a *rad51*Δ strain and in a slightly higher proportion of BRIMs among the *TRP1* recombinants (0.8%). In summary, we find that while *RAD51* and *RAD52* are essential for recombinational repair of induced DSBs in the inverted-repeat substrate, the rare recombinants show little or no evidence of elevated BRIMs.

***REV3* affects the spectrum of mutations in *rad57-m431* mutants:** As noted above, the spectrum of BRIMs is similar in *rad57-m431* and wild-type strains, suggesting that *RAD57* may be affecting the frequency but not the mechanism by which the mutations arise. To determine whether *REV3* is required for BRIMs in *rad57* mutants, we introduced a *rev3*Δ mutation into a *rad57-m431* strain. The data from the fluctuation analysis of the double-mutant strain are shown in Table 2 (*rad57-m431 rev3*Δ). As expected, the *rad57-m431* recombination defect was epistatic to *rev3*Δ for the overall efficiency of DSB-induced recombination (Table 2). Indeed, there is a small reduction in the DSB-induced *TRP1* recombinants in the double mutant as compared to that in the *rad57* single mutant. However, the proportion of BRIMs is not reduced by loss of *REV3*. Although the difference between the proportion of BRIMs in the double mutant and in the *rad57-m431* mutant is not statistically significant, these data suggest that the role of *RAD57* in mutation avoidance supercedes the role of *REV3* in error-prone DNA repair.

A summary of the Southern blot analysis of 26 independent *TRP1 can1 HIS3* events from a *rad57-m431 rev3*Δ strain indicates that they are all repaired by gene conversions, of which 42% are associated with an inversion (Table 3). From a sequence analysis of the *can1* ORF of 21 independent BRIMs from a *rad57-m431 rev3*Δ strain (Table 4, Figure 2), we find that the majority of the mutations are FS. Only four of the events are BS mutations, resulting in a FS:BS ratio of ~5.3. Therefore, as in the wild-type strain, loss of *rev3* results in a higher proportion of FS mutations. These data indicate that although *REV3* does not influence the overall frequency of BRIMs in *rad57* mutants, it still introduces mutations when present and that another function(s) that primarily introduces FS mutations must be able to substitute for *REV3* in its absence. Loss of *rev3* results in a reduction in the overall recombination frequency and the residual events derive from a pathway that is more error prone to making FS mutations. These data are consistent with the view that many of the recombination events in the *rad57* strain require an error-prone polymerase for their completion.

***REV3* is important for repair of MMS DNA damage when recombinational repair is impaired:** We noted that *rad57-m431 rev3*Δ double mutants grew more slowly and were more sensitive to MMS than were the single mutants. To determine if the synergistic sensitivity to MMS was a characteristic of other genes defective in recombinational repair, we constructed *rev3*Δ *rad51*Δ, *rev3*Δ *rad52*Δ, and *rev3*Δ *rad57*Δ double-mutant strains. An example of the MMS sensitivity is shown in Figure 3, where the MMS hypersensitivity of the double-mutant strains is readily apparent. These results were similar to those of the *rad57-m431 rev3*Δ double-mutant strain (not shown). In all cases, the double mutants also grew more slowly than the single mutants. The primary damage induced by MMS is alkylation of guanine residues (Pegg 1984), which may require either recombinational repair or pol ζ for lesion bypass. Therefore, whereas other error-prone DNA polymerases may be able to substitute

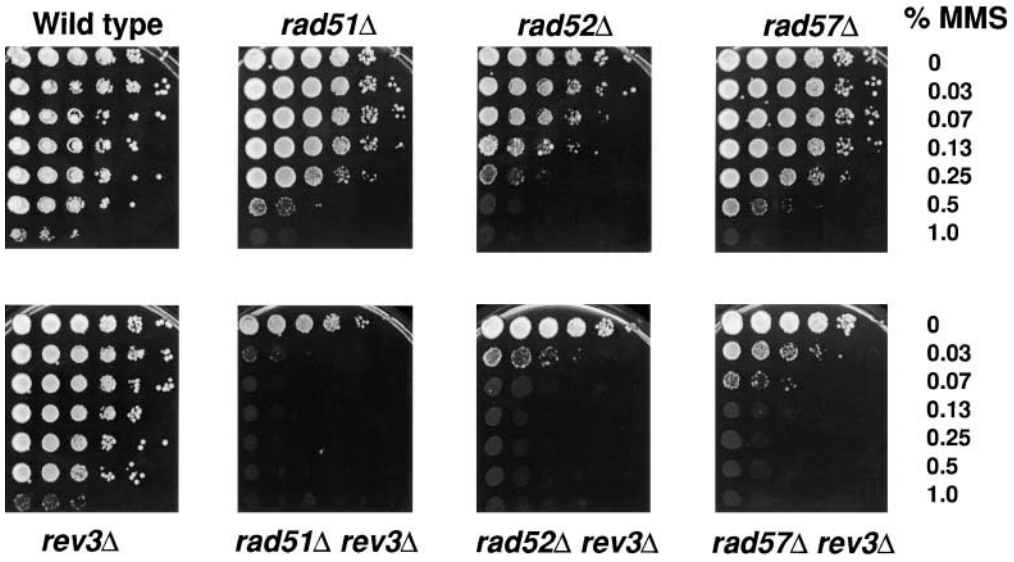


FIGURE 3.—MMS sensitivity assay. Logarithmically growing cells were incubated with the indicated concentration of MMS for 10 min after which the MMS was inactivated (see MATERIALS AND METHODS). Ten-fold serial dilutions of cells were spotted onto YPD and incubated for 5 days. Only relevant genotypes are indicated. Wild type, GRY-1654; *rev3*Δ, YAR640; *rad51*Δ, YAR667; *rad51*Δ-*rev3*Δ, YAR-693; *rad52*Δ, YAR665; *rad52*Δ *rev3*Δ, YAR692; *rad57*Δ, YAR-666; *rad57*Δ *rev3*Δ, YAR678.

for *REV3* during DSB repair, they may not be able to substitute for MMS-induced lesions. Alternatively, perhaps the increased ssDNA arising from defects in recombinational repair results in increased DNA damage that requires *REV3* (see DISCUSSION).

**DSB-induced conversion tracts in wild-type and recombination-impaired cells:** One possible explanation for the increase in BRIMs among the residual recombinants in *rad57* mutants is that these events are associated with longer gene conversion tracts. Longer gene conversion tracts might be expected to result in increased BRIMs because of the greater extent of new DNA synthesis or the greater extent of ssDNA that is used as a template. To test this hypothesis, we utilized a mutation in the *can1-5′*Δ allele of the inverted repeat, *can1-m362*, which we isolated in our laboratory (see MATERIALS AND METHODS). The mutation is ~700 bp from the HOcs and 600 bp from the end of the homologous region (asterisk, Figure 1A). Using this substrate, we determined the proportion of DSB-induced *TRP1* events that import the *can1-m362* allele into the full-length *CAN1* gene by a crossover to the right of the mutation and/or a gene conversion, thus providing some information on the tract length of the recombination event (Table 5).

In the wild-type ($Rad^+$) strain, the proportion of HO-induced *TRP1 can1* events is ~16% (Table 5), representing a 40-fold increase over the substrate without the *can1-m362* allele (Table 2); therefore the majority (>97%) of the events in this substrate are due to coconversion rather than to BRIM formation. Although only ~16% of the *TRP1* recombinants have tracts that are longer than 700 bp, it is notable that ~81% of the BRIMs are located beyond this position.

In a *rad57*Δ strain we find a marked increase in the proportion of *can1* events from 8% with the original substrate to 37% with the *can1-m362* substrate (compare Tables 2 and 5). Therefore, about twice as many of the events are associated with longer tracts in a *rad57*Δ mutant ($P < 0.01$), consistent with the view that the elevated BRIM frequency in *rad57* mutants is related to increased tract length.

## DISCUSSION

Here we have addressed the role of functions that are important for recombinational repair and the role of the translesion DNA polymerase ζ in the generation of BRIMs during recombinational repair of DNA DSBs. Our major findings can be summarized as follows:

1. More FS mutations are associated with recombinational repair of DNA DSBs than with spontaneous mutations, suggesting that spontaneous mutations and BRIMs differ in either their genesis or their repair.
2. *REV3* is required for introducing ~75% of BRIMs, including both FS and BS mutations.
3. The preponderance of FS mutations in the absence of *REV3* provides evidence for another *REV3*-independent mechanism that primarily generates FS mutations.
4. The absence of *rad57* results in decreased recombination, as expected, but is associated with an increase in BRIMs associated with the residual repair, indicating that *RAD57* and *RAD55* play an important role in mutation avoidance.
5. In wild-type cells, ~16% of the DSB-induced *TRP1* recombinants are associated with gene conversion tract lengths >700 bp, whereas this proportion is 37% in *rad57* mutants, providing evidence that *rad57* mutants result in longer gene conversion tracts.
6. Mutants defective in both recombinational repair

**TABLE 5**

**Frequency of recombination and cosegregation of *TRP1* and *can1-m362***

| Strain | Relevant genotype | Spontaneous | | | | HO induced | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Frequency ($\times 10^6$)[a] | | Phenotype of *TRP1*[b] | | Frequency ($\times 10^6$) | | Phenotype of *TRP1* | |
| | | *TRP1* | *can1* | % *CAN1* | % *can1* | *TRP1* | *can1* | % *CAN1* | % *can1* |
| YAR332 | Wild type | 3.8 | 16 | 78 (200) | 22 | 12,000 | 1,600 | 84 (400) | 16 |
| YAR764 | *rad57*Δ | 1.5 | 33 | 66 (186) | 34 | 330 | 150 | 63 (199) | 37 |
| YAR764 | *rad57*Δ (20°) | 1.4 | 27 | 84 (192) | 16 | 13 | 24 | 73 (150) | 27 |

[a] Median frequency (see MATERIALS AND METHODS).
[b] Determined by replica plating *TRP1 HIS3* events (see MATERIALS AND METHODS).

and *rev3* show a synergistic sensitivity to MMS as compared with the single mutants, suggesting that *rev3* and recombinational repair define different pathways for DNA damage tolerance.

Sequence analysis of spontaneous mutations of our substrate shows that most are single BS mutations and have a FS:BS ratio of ~0.2 (Table 4, inverted repeat). Spontaneous mutations from the native *CAN1* locus have also been sequenced (TISHKOFF *et al.* 1997; TRAN *et al.* 2001) and have a FS:BS ratio of ~0.4 (Table 4, native locus). At other loci where spontaneous mutation spectra have been examined, the FS:BS ratio is ~0.1–0.2 (see KUNZ *et al.* 1998). Therefore, it appears that, in general, most spontaneous mutations are single base substitutions.

The introduction of a DSB in our substrate is primarily repaired by recombination with a homologous sequence lacking the break site. Most of the induced DSBs are repaired by gene conversion to *TRP1* and retain a functional *CAN1* gene. However, among cells that repaired the DSB by recombination, the frequency of BRIMs is $\sim 4 \times 10^{-3}$, a >3000-fold increase over the spontaneous mutation frequency of our substrate ($\sim 1 \times 10^{-6}$). In the simplest view, we assume that the DNA is degraded primarily from the 5′ termini at the site of the break, leaving 3′ tails, which can then invade a homologous duplex and prime *de novo* DNA synthesis (see SZOSTAK *et al.* 1983 and PAQUES and HABER 1999 for a description of the DSB repair model). Presumably, during this new DNA synthesis errors are generated. Indeed, most of the BRIMs analyzed from our wild-type strain are repaired by gene conversion and have no obvious unpredicted rearrangements in the substrate. Sequence analysis of BRIMs demonstrates that they are primarily point mutations with a significantly larger proportion of FS mutations than is found among spontaneous mutations of the same substrate. Frameshifts are generally believed to be introduced by template-primer slippage events during polymerization and removed by mismatch repair (MMR; see PFEIFER 2000 for recent reviews; BROOMFIELD *et al.* 2001). From a sequence analysis of spontaneous mutations at the native *CAN1* locus it was shown that cells deficient in MMR result in a ~7-fold increase in the FS:BS ratio (TRAN *et al.* 2001). One possibility is that the increased FS:BS ratio seen among BRIMs is due to inefficient MMR. For example, the DNA repair complex may be less likely than a normal replication complex to recruit MMR functions. Interestingly, there is evidence for reduced mismatch repair during the process of adaptive mutation in *E. coli* (LONGERICH *et al.* 1995).

Previous results from our laboratory indicated a role for *REV3*, encoding the catalytic subunit of yeast DNA polymerase ζ (MORRISON *et al.* 1989) in the reversion of BS but not of FS mutations (HOLBECK and STRATHERN 1997). This result was surprising, since studies have shown that *REV3* is required for both FS and BS spontaneous and UV-induced mutations (see LAWRENCE *et al.* 2000; LAWRENCE and MAHER 2001 for reviews). Here we have analyzed BRIMs in a forward mutation assay, which is not limited to any specific class of mutation. We again find that *REV3* does not affect the overall efficiency of recombination to *TRP1*, confirming our previous results indicating that *REV3* is unlikely to be a major DSB-repair-associated polymerase (HOLBECK and STRATHERN 1997). However, *REV3* is required for generating ~75% of the BRIMs. Among BRIMs, we find a significant change in the FS:BS ratio from 1.4 in wild type to 6.3 in *rev3* ($P < 0.05$) and calculate that ~50% of the FS and ~90% of the BS are *REV3* dependent. Therefore, *REV3* is required for the introduction of both FS and BS in our substrate, although it has a greater effect on BS mutations. In contrast, *rev3* mutants have little effect on the spontaneous or damage-induced FS:BS ratio at *sup4-o* (ROCHE *et al.* 1994; KUNZ *et al.* 1998); the different requirements for *REV3* in the generation of FS mutations in our assay as compared with spontaneous or damage-induced mutations reflect differences in the DNA template, the type of DNA damage, or the enzymes involved in repair synthesis.

Clearly pol ζ is not the only DNA polymerase generating BRIMs, since BRIMs are still induced 65-fold in a

*rev3*Δ strain. Also, the preponderance of −1 FS mutations among BRIMs from the wild-type strain and the small effect of *rev3* on this particular class of mutations suggests the involvement of another DNA polymerase(s) with a tendency to introduce FS mutations. In this context it is interesting that a mutation in DNA polymerase δ that specifically reduces −1 FS mutations from short mononucleotide repeats has recently been described (Hadjimarcou *et al.* 2001). Perhaps the *REV3*-independent FS BRIMs reflect a role of pol δ in recombination.

Although pol ζ is not a very processive enzyme *in vitro*, it does appear to be quite error prone (Nelson *et al.* 1996; Lawrence *et al.* 2000). *In vivo*, there is evidence that it can insert multiple closely associated mutations (Harfe and Jinks-Robertson 2000). We see no evidence for multiple closely associated mutations among the sequenced BRIMs. Of the 42 events sequenced from the wild-type strain, only 2 events had more than a single nucleotide change. One was a TTT → AAAA substitution, and the other had two single base mutations located >1 kb from each other (one of the mutations did not result in an amino acid change and has not been included in the data set presented in the supplemental table at http://www.genetics.org/supplemental/). Given that loss of *rev3* does not affect the overall rate of DSB-induced recombination to *TRP1*, and given the paucity of events with multiple closely spaced mutations, it is likely that pol ζ is recruited to synthesize only short stretches of DNA and that other polymerase(s) are responsible for the majority of DNA synthesis associated with DSB repair.

Although the distribution data were not reported for the native locus, P. Tran and R. M. Liskay kindly provided us with this information from their data set (Tran *et al.* 2001), where they found that 55% of the mutations were located in the region of *CAN1* that is duplicated in the inverted-repeat substrate (which comprises 62% of the ORF). In the inverted-repeat substrate, we found that only 31% of the spontaneous mutations are located in this region. We speculate that the presence of homology in our substrate may allow for correction of spontaneous DNA damage by homologous recombination, thus "erasing" mutations that might arise in the duplicated region.

Two features of the distribution of the BRIM *can1* mutations reveal aspects of their origin. First, there is no gradient of mutations from the site of the break (3′ end of the gene; see Figure 2). Many (45%) of the BRIMs are located in the unduplicated region, suggesting that exonucleolytic degradation often extends beyond the homologous region requiring new DNA synthesis. Second, as monitored by coconversion of the *can1-m362* allele, only 16% of the HO-induced *TRP1* recombinants have gene conversion tracts >700 bp. In contrast, 81% of the BRIMs are located >700 bp from the HO site. Therefore, it appears that BRIMs are more prevalent among events associated with longer gene conversion tracts.

We report here that *rad57* mutants have a reduced but detectable level of repair of DSBs to yield recombinants that are physically identical to recombinants from a wild-type strain, except for an increased level of associated BRIMs. Furthermore, the spectrum of BRIMs is indistinguishable from that seen in wild-type cells (FS:BS ratio of ~1.4, Table 4), and loss of *rev3* results in a similar shift in the distribution of FS and BS mutations (FS:BS ratio of 6.3 for the wild-type strain and 5.3 for the *rad57* strain, Table 4). Taken together, these observations suggest that the mechanism(s) by which BRIMs arise in both wild-type and *rad57* strains is likely to be similar.

We found that mutants defective in recombinational repair have a synergistic sensitivity to MMS (Figure 3). A synergistic sensitivity to oxidative agents has also been noted for *rad52 rev3* double-mutant strains (Swanson *et al.* 1999). Although the contribution of *REV3* to tolerance of DNA-damaging agents is minor (Nelson *et al.* 1996), our observation indicates that it must provide an important pathway for the repair of some types of DNA damage when recombinational repair is compromised. We suggest that repair intermediates generated in the absence of efficient recombination require *REV3* for the bypass of these lesions.

What might this enhanced frequency of BRIMs in *rad57*-deficient cells tell us about the origins of these mutations? One possibility is that BRIMs arise from a subset of events that are repaired via a mechanism such as break-induced replication (BIR) followed by SSA (see Kang and Symington 2000; Malagón and Aguilera 2001 for a description of this model). Indeed, both BIR and SSA appear to be enhanced in *rad57* strains (Ivanov *et al.* 1996; Signon *et al.* 2001). Although both BIR and SSA have been shown to occur in the absence of *RAD51* (Malkova *et al.* 1996; Kang and Symington 2000), the extreme dependence of our substrate on *RAD51* argues against such a mechanism operating in our assay. Also, we found that only 0.7% of the events in *rad51* mutants have an associated BRIM. Furthermore, a BIR + SSA mechanism is expected to result in an equal ratio of inversions, whereas we see an excess of events without inversions (Table 3). However, it is possible that in our substrate Rad51p (and associated factors) are necessary for the initial invasion step (Sugawara *et al.* 1995; Malagón and Aguilera 2001) and that some aspect of our substrate (*i.e.*, length of DNA being duplicated) leads to preferential resolution as a gene conversion without inversion by SSA after BIR. One major difference between BIR and other models of DSB repair is that whereas two-ended DSB repair events presumably require only leading-strand DNA synthesis, BIR is expected to require a full replication fork, which may provide a more (or less) likely substrate for the recruitment of factors such as *REV3* (see below).

Another possibility is that BRIMs are associated with more extensive regions of ssDNA. This is suggested by the finding that many of the BRIMs are located outside of the duplicated region. We found that *rad57* mutants have longer gene conversion tracts and an increased proportion of BRIMs. The defect in *rad57* strains may reflect a delay in the formation or extent of the Rad51p filament, its stability, or its ability to find a homologous partner as suggested by studies that indicate that Rad57p (together with Rad55p) enhance the strand-exchange activity of Rad51p (Sung 1997). Indeed, increased ssDNA has been seen upon physical monitoring of HO-induced breaks at the *MAT* locus in *rad57* mutants (Sugawara *et al.* 1995). In this scenario, Rad51p may provide a protective environment for the DNA or simply hasten the repair, via recombination, of the broken DNA back to duplex. Our finding that overexpression of *RAD51* reduces the frequency of BRIMs is consistent with such a view.

We consider that a model for BRIM formation that is more consistent with ssDNA as a precursor to BRIM formation is the synthesis-dependent strand-annealing (SDSA) model (see Paques and Haber 1999 for a review). As shown in Figure 4, we suggest that after invasion of the homologous duplex by one end of the broken DNA, the replication fork migrates forward by leading-strand DNA synthesis displacing the newly synthesized DNA. The displaced strand may "capture" the other end of the broken DNA by annealing. Because of the inverted-repeat structure of our substrate, slower or less efficient repair would lead to longer regions of ssDNA exposure, particularly in the nonduplicated region of the *CAN1* gene. One feature of the SDSA model is that it involves conservative DNA replication and predicts that the errors will be found on the recipient (or broken) DNA molecule. In previous studies using a heteroallelic substrate, we found that the majority of BRIMs were located on the recipient (Strathern *et al.* 1995; McGill *et al.* 1998), providing strong evidence for a conservative mode of DNA synthesis during BRIM formation.

Is ssDNA more likely to be a target for base damage, and thus requires *trans*-lesion DNA polymerases to bypass the lesions, or is copying a ssDNA template more mutagenic because it is a poorer template perhaps because it introduces pause sites due to its secondary structure? We cannot distinguish between these models; however, a number of studies indicate that ssDNA is more susceptible to mutagenesis than is dsDNA. For example, it has been shown that cytosine is deaminated >100-fold more frequently in ssDNA than in dsDNA (Federico *et al.* 1990). Also, increased transcription of a gene has been shown to increase its mutability (Herman and Dworkin 1971; Datta and Jinks-Robertson 1995; Beletskii and Bhagwat 1996; Wright *et al.* 1999). We

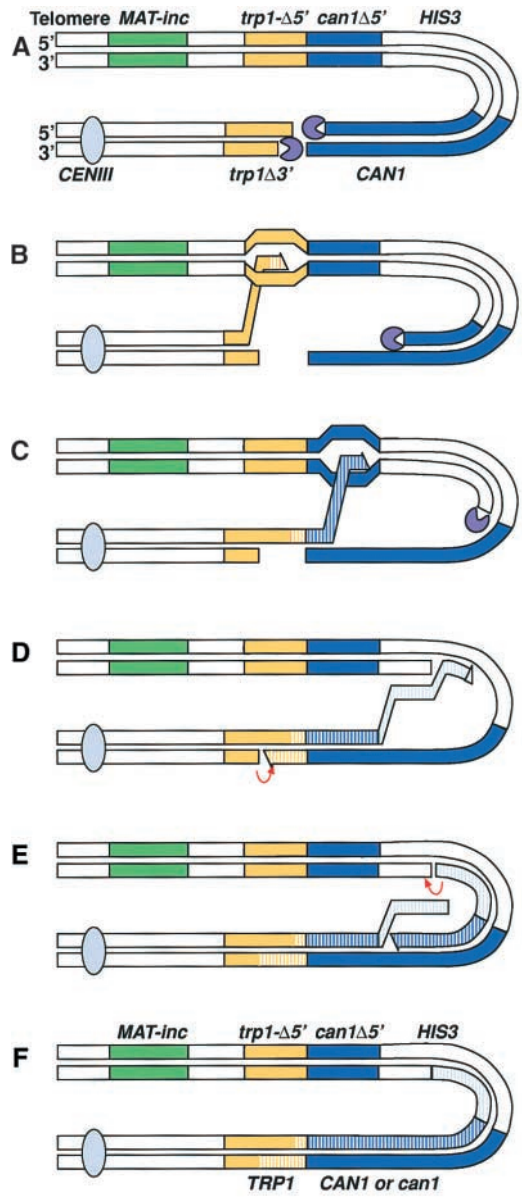


Figure 4.—SDSA model for BRIM formation. (A) Induction of the HO endonuclease results in a DSB that is processed by 5′ → 3′ exonuclease(s) (purple) to reveal 3′ ssDNA ends. (B) Invasion of the homologous sequence, presumably requiring the functions of the *RAD52* epistasis group and trimming of the nonhomologous sequence at the end by flap endonuclease(s), reveals a 3′ hydroxyl that is used to prime new DNA synthesis using the unbroken strand as a template. (C) As DNA synthesis proceeds, the newly synthesized DNA strand (striped) is displaced and can anneal with homologous sequences from the other side of the break, allowing the initiation of DNA synthesis on the second strand. (D) The newly synthesized displaced strand can anneal only to the other copy of the repeat over the duplicated region. Once the 5′ → 3′ exonuclease has proceeded as far as the replication bubble, the new DNA synthesis must proceed on a ssDNA template. (E and F) After removal of nonhomologous sequences, ligation allows resolution of the molecule, leading to a gene conversion. Resolution prior to extention beyond the duplicated region could also lead to an inversion (not shown).

postulate that the elevated mutation rate seen in meiosis and treatments that cause stalled replication forks are related to BRIMs in that they reflect the sensitivity of ssDNA to damage.

We previously suggested that error-prone DNA polymerases might have roles in somatic hypermutation of Ig genes (Holbeck and Strathern 1997). Support for that proposal has recently been provided by a study in which a reduction in the expression of the *REV3* homolog was correlated with reduced somatic hypermutation levels (Zan *et al.* 2001). The recent demonstration of DSBs in the genes undergoing somatic hypermutation (Sale and Neuberger 1998; Bross *et al.* 2000; Papavasiliou and Schatz 2000; Kong and Maizels 2001) suggests that pol ζ is recruited to that region in much the same way that it is recruited to the repair of DSBs in yeast resulting in BRIMs.

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