# Removal of One Nonhomologous DNA End During Gene Conversion by a *RAD1*- and *MSH2*-Independent Pathway

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ABSTRACT

Repair of a double-strand break (DSB) by homologous recombination depends on the invasion of a 3′-ended strand into an intact template sequence to initiate new DNA synthesis. When the end of the invading DNA is not homologous to the donor, the nonhomologous sequences must be removed before new synthesis can begin. In *Saccharomyces cerevisiae*, the removal of these ends depends on both the nucleotide excision repair endonuclease Rad1p/Rad10p and the mismatch repair proteins Msh2p/Msh3p. In *rad1* or *msh2* mutants, when both ends of the DSB have nonhomologous ends, repair is reduced ~90-fold compared to a plasmid with perfect ends; however, with only one nonhomologous end, repair is reduced on average only 5-fold. These results suggest that yeast has an alternative, but less efficient, way to remove a nonhomologous tail from the second end participating in gene conversion. When the removal of one nonhomologous end is impaired in *rad1* and *msh2* mutants, there is also a 1-hr delay in the appearance of crossover products of gene conversion, compared to noncrossovers. We interpret these results in terms of the formation and resolution of alternative intermediates of a synthesis-dependent strand annealing mechanism.

IN *Saccharomyces cerevisiae*, the site-specific HO endonuclease can be used to generate a double-strand break (DSB) that initiates DNA repair by gene conversion (reviewed in Haber 1995; Osman and Subramani 1998). When the *HO* gene is expressed under the control of a galactose-inducible promoter (Jensen and Herskowitz 1984), DSBs can be introduced synchronously into a large population of cells and it is then possible to monitor different steps in the recombinational repair of the DSB by Southern blots or polymerase chain reaction (PCR; Connolly *et al.* 1988; Raveh *et al.* 1989; Rudin *et al.* 1989; White and Haber 1990; Fishman-Lobell and Haber 1992). With these techniques it has been possible to demonstrate that the DSB is processed by 5′–3′ resection of the DNA ends to produce 3′-ended tails that invade a homologous donor sequence to initiate new DNA synthesis (White and Haber 1990) and to analyze the fate of DNA molecules in various mutant yeast strains that prevent the completion of recombination (Fishman-Lobell and Haber 1992; Sugawara *et al.* 1995, 1997; Umezu *et al.* 1998).

In many experiments, we have used centromeric plasmids carrying two copies of the *Escherichia coli lacZ* sequence in opposite orientation, one of which contains an inserted HO recognition site and surrounding sequences of 117 bp (Rudin *et al.* 1989; Fishman-Lobell and Haber 1992; Sugawara *et al.* 1997). After cleavage of this site by HO, the two ends of the DSB are not homologous to the donor and must be removed before repair can be initiated. The removal of these ends requires the Rad1 and Rad10 proteins (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995; Prado and Aguilera 1995), which were shown to act as a single-stranded "flap" endonuclease in a fashion similar to their role in nucleotide excision repair (Sung *et al.* 1993; Tomkinson *et al.* 1993; Bardwell *et al.* 1994; Tomkinson *et al.* 1994). Other nucleotide excision repair genes are not required (Ivanov and Haber 1995). The removal of the 3′ nonhomologous ends also requires two members of the mismatch repair family—the mutS homologues Msh2p and Msh3p—but not the mutL homologues, Pms1p and Mlh1p (Saparbaev *et al.* 1996; Kirkpatrick and Petes 1997; Sugawara *et al.* 1997). In addition, this process requires a 3′–5′ helicase, Srs2p (Pâques and Haber 1997).

In the double-strand break repair model of Szostak *et al.* (1983), one would imagine that both nonhomologous ends would likely be removed in the same way, allowing the two invading ends to prime new DNA synthesis from the two strands of the donor template. However, in the past several years a substantial body of evidence that supports alternative models of DNA repair, called synthesis-dependent strand annealing (SDSA), has accumulated (Nasmyth 1982; Hastings 1988; Thaler and Stahl 1988; McGill *et al.* 1989; Pâques and Wegnez 1993; Nassif *et al.* 1994; Ferguson and

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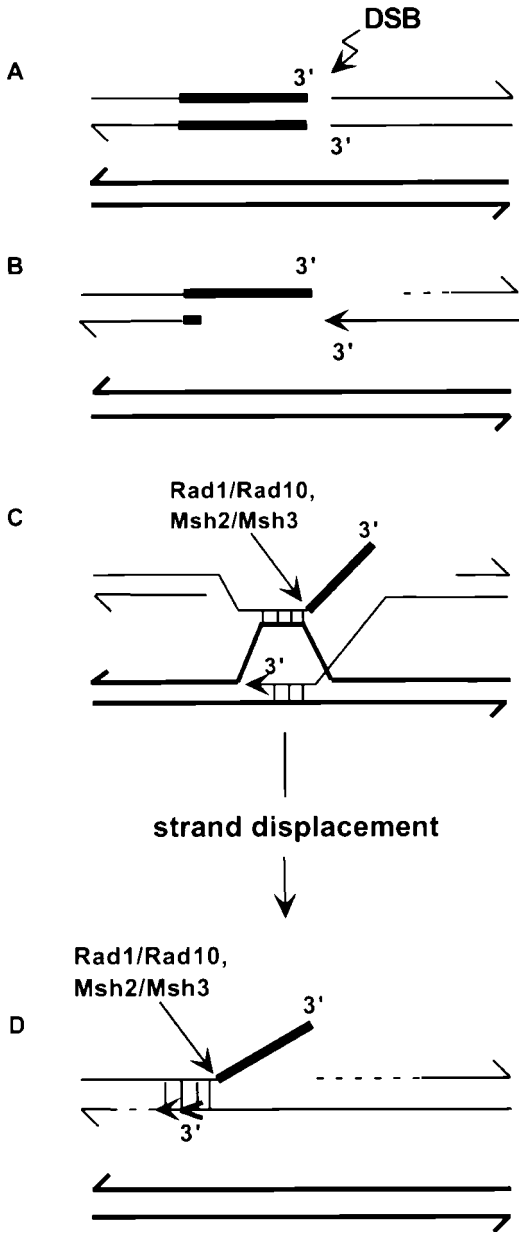


Figure 1.—Removal of a nonhomologous tail by Rad1/Rad10 endonuclease, assisted by Msh2/Msh3 mismatch repair proteins. Following formation of a DSB (A), the ends are resected by one or more 5′–3′ exonucleases (B). Once strand invasion occurs from an end that has perfect homology, the nonhomologous tail (depicted as a thick line) could be removed after annealing of this DNA end with either a D-loop created by strand invasion (C) or when the newly synthesized strand is itself displaced and anneals with the second end (D). For simplicity, the repair pathway described above is shown only as generating gene conversions. Alternatively, the Holliday junction-containing intermediate depicted in C could be resolved to generate a gene conversion with an associated crossover (see Figure 8).

Holloman 1996; Pâques and Haber 1997). Especially in the more recent conceptions, new DNA synthesis is initiated by invasion of only one end and the newly synthesized DNA is unwound from the template to pair with the second end, which then primes synthesis of the second strand, thus placing nearly all of both newly synthesized DNA strands into the recipient (Figure 1).

To examine the requirements of removing the nonhomologous tail, we created a set of centromeric plasmids with two inverted copies of *lacZ*, one of which contains an HO endonuclease cleavage site (Rudin *et al.* 1989; Sugawara *et al.* 1997). These new plasmids (Figure 2) were modified by deletions and additions so that the DSB would contain only one nonhomologous end. Our data support the idea that recombination is initiated efficiently by the perfectly homologous end and that the processing of the second end to excise the nonhomologous DNA tail usually involves Rad1p/Rad10p and Msh2p/Msh3p. In the absence of these proteins, however, there is a second, less efficient back-up system that can remove the tail and allow the delayed completion of recombination. In the absence of efficient nonhomologous tail removal, there is a 1-hr difference in the appearance of gene conversions with and without crossovers. Moreover, although *rad1* and *msh2* had no effect on the proportion of crossing over associated with gene conversion when the plasmid had two homologous ends (Sugawara *et al.* 1997), these mutants exhibited a reduced level of exchange in some cases where the DSB had one nonhomologous end.

## MATERIALS AND METHODS

**Plasmid construction:** The five different plasmids analyzed in this study were derived from Ted, a centromeric plasmid marked by the *URA3* gene provided by W. Kramer. These plasmids carry two copies of the *lacZ* gene from *E. coli*. These genes are in inverted orientation and one of the copies contains an HO endonuclease cut site (Figure 2). The second copy of *lacZ*, used as a template for repair, lacks an HO cut site, carries only half of the HO cut site, or carries a mutated "inc" cut site (Weiffenbach and Haber 1981), which renders it uncleavable. Thus, repair involves loss of the original HO cut site and no recutting occurs. pFP122 and pFP120 have been previously described in Pâques and Haber (1997) and in Sugawara *et al.* (1997).

For both pFP130 and pFP131, one copy of *lacZ* was inserted into the polylinker of Ted. This copy carries a 40-bp synthetic HO cut site (Pâques and Haber 1997) that replaces a 235-bp *Eco*RV-*Bcl*I fragment. In pFP130 the second copy of *lacZ* was modified by replacing the *Sac*I-*Eco*RV fragment with a 20-bp sequence (AACAGTATAATTTTATAAAC). In pFP131 the second copy of *lacZ* was modified by replacing the *Bcl*I-*Cla*I fragment with a 24-bp sequence (GATCAGTTTCAGCTTTCCGCAACA). On both pFP130 and pFP131, the fragments inserted in the second copy of *lacZ* recreated half of the cut site so that the nonhomologous sequence was present only on one side of the break.

pMC9 was constructed by PCR-amplifying 301 bp of λ DNA from position 2963 to 3264 and inserting it into the *Cla*I site of pFP131. This increased the amount of nonhomologous sequence flanking one side of the HO cut site without altering the amount of homology shared between the two copies of *lacZ*.

In all the plasmids above, repair involves loss of the HO cut site and any additional nonhomologous sequences flank-

**TABLE 1**

**Yeast strains**

| Strain | Genotype | Source |
|---|---|---|
| R167 | Δ*ho HML*α Δ*mat::LEU2 hmr-3*Δ *mal2 leu2-3,112 ura3-52 trp1 thr4* | Rudin and Haber (1988) |
| tNS1368 | Δ*ho HML*α Δ*mat::leu2::hisG hmr-3*Δ *mal2 leu2-3,112 ura3-52 trp1 thr4 ade3::GAL-HO* | Sugawara *et al.* (1997) |
| JKM146 | Δ*ho* Δ*hml::ADE1 MAT***a***-inc* Δ*hmr::ADE1 ade1 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL-HO* | Moore and Haber (1996) |
| YFP103 | JKM146 Δ*rad1::LEU2* | Sugawara *et al.* (1997) |
| YFP255 | JKM146 Δ*msh2::LEU2* | Sugawara *et al.* (1997) |
| tMC43 | tNS1368 (pFP120) | This study |
| tMC44 | tNS1368 (pFP122) | Sugawara *et al.* (1997) |
| tMC47 | tNS1368 (pFP130) | This study |
| tMC48 | tNS1368 (pFP131) | This study |
| tMC90 | tNS1368 (pMC9) | This study |
| YFP112 | YFP103 (pFP120) | Pâques and Haber (1997) |
| YFP116 | YFP103 (pFP122) | Sugawara *et al.* (1997) |
| YFP124 | YFP103 (pFP130) | This study |
| YFP126 | YFP103 (pFP131) | This study |
| tMC96 | YFP103 (pMC9) | This study |
| YFP265 | YFP255 (pFP120) | Pâques and Haber (1997) |
| YFP261 | YFP255 (pFP122) | Sugawara *et al.* (1997) |
| YFP375 | YFP255 (pFP130) | This study |
| YFP376 | YFP255 (pFP131) | This study |
| tMC93 | YFP255 (pMC9) | This study |

ing the break. Through restriction-endonuclease digestions the products of repair are clearly separable in size from one another, from the parental *lacZ*-containing fragments, and from products resulting from nonhomologous end-joining.

**Strains:** Strains used in this study are listed in Table 1. They were all isogenic derivatives of either R167 (Rudin and Haber 1988) or JKM146 (Moore and Haber 1996). The two original strains are not isogenic. As a control, HO-induced recombination of the same plasmid (pJF5; Fishman-Lobell and Haber 1992) was assayed in both strains. The efficiency and kinetics of repair as well as the final levels of gene conversion associated with crossovers were the same for both strains (data not shown).

Strain tNS1368 was constructed in our laboratory by integrating the HO endonuclease under the galactose promoter at *ADE3*, in strain R167. This strain is deleted for *MAT* and *HMR*; therefore no mating type switching occurs upon induction of the HO endonuclease.

Strains deleted for *rad1* (YFP103) or *msh2* (YFP255) were constructed as described in Sugawara *et al.* (1997). They are deleted for *HML* and *HMR* and carry the *MAT***a**-inc mutation that is not cleaved by HO (Weiffenbach and Haber 1981). Mating type switching does not occur in these strains upon induction of the HO endonuclease. The *GAL::HO* fusion is also integrated at the *ADE3* locus (Sandell and Zakian 1993).

All other strains in Table 1 were constructed by transforming strains tNS1368, YFP103, and YFP255 with plasmids pFP122, pFP120, pFP130, pFP131, and pMC9. Yeast cells were transformed using the one-step transformation method of Chen *et al.* (1992).

**Media:** For the time course assays, cells were grown in synthetic complete medium lacking uracil (Sherman *et. al.* 1986) and in YEP medium (1% w/v yeast extract, 2% w/v Bactopeptone) supplemented with lactic acid (3.7% YEPL, pH 5.5). Galactose (2% w/v) was added as a 20% solution to YEPL medium. Bacto-agar (2%) was added to YEP medium supplemented with dextrose (2% w/v YEPD) and synthetic media lacking uracil (SC-URA) for the plating assays.

**DNA analysis:** DNA was extracted before HO induction (0 hr) and at intervals following the induction as described in Sugawara and Haber (1992). The DNA was then restriction-endonuclease digested and run in either 0.5% or 0.7% nondenaturing agarose gels. The kinetics of repair were analyzed by Southern blots and probed with *lacZ* sequences (Rudin *et al.* 1989). The levels of gene conversion with and without crossovers were analyzed in two ways. First, DNA was extracted from 30 to 60 individual colonies that scored as $Ura^+$ after 24 hr of induction. These samples were restriction digested with *Pst*I and assayed by Southern blots probed with *lacZ* sequences to detect crossover and noncrossover products. In parallel, the 24-hr lanes of the Southern blots corresponding to time courses involving large populations of cells were quantitated by phosphorimager analysis. The intensity of the smaller crossover band (c.o.2) was divided by the sum of the intensities of this smaller crossover band with the noncrossover band (g.c.). The percentage crossover is therefore (c.o.2)/(c.o.2 + g.c.). An average from quantitations of three to five Southern blots for each strain is presented in Table 2.

**Measurement of DSB repair efficiency:** Plasmid retention was scored as a measure of efficiency of repair by plating cells at intervals before and after HO endonuclease induction. Cells were first plated in YEPD and then replica plated on SC-URA. The percentage of plasmid retention was calculated as the fraction of colonies retaining the repaired plasmid on SC-URA divided by the total number of colonies on YEPD. A minimum of 1100 colonies per strain were examined. After 24 hr of induction, all HO cut sites were cleaved and repair was complete as can be observed through Southern blots by the disappearance of the restriction fragment carrying the HO cut site (disappearance of band P2 in Figures 3A, 4A, 5A, and 6A). We note that the levels of plasmid retention in these experiments were consistently lower than the ones observed

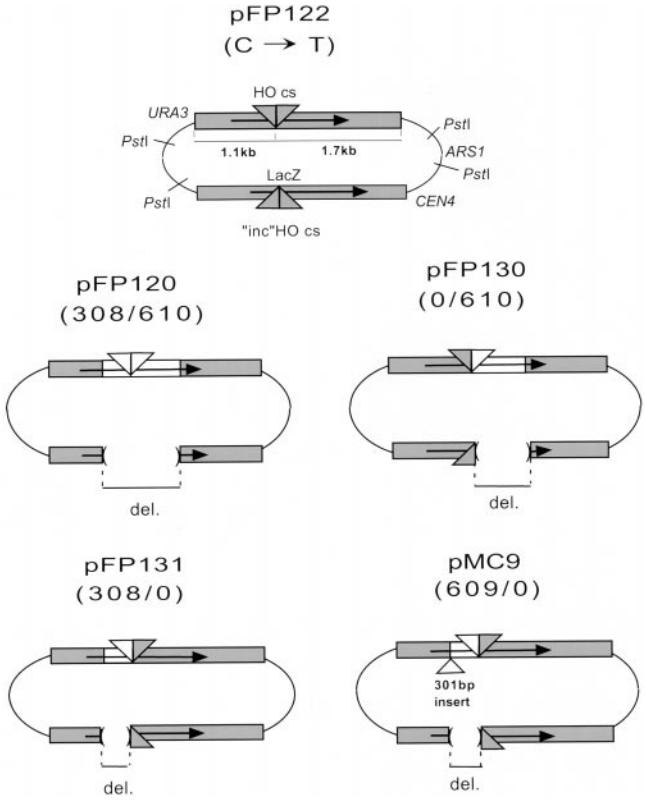


Figure 2.—Plasmid structures. All the plasmids depicted above were built in the same backbone (see materials and methods). They carry two copies of *lacZ* in inverted orientation. Homologous sequences are presented in gray while nonhomologous sequences are in white. One copy carries the active HO cut site (HO cs; inverted triangle). In the case of pFP122, the other copy carries an "inc" HO cut site inactivated by a C to T change. In pFP130, pFP131, and pMC9, the template *lacZ* copy carries only a half cut site (gray half triangle). To generate nonhomologous sequences flanking the DSB in pFP120, pFP130, pFP131, and pMC9, regions labeled del. were deleted from the copy of LacZ that does not carry the HO cut site. In pMC9, in addition to the deletion, an extra 301 bp of phage λ DNA were inserted adjacent to the nonhomologous end. The values in parentheses indicated underneath the name of the plasmids represent the length (in base pairs) of the nonhomologous sequences as well as the side of the DSB at which they are present.

by Pâques and Haber (1997). However, the overall proportions of the decrease in plasmid retention levels between wild type and the mutant strains were the same. In that case, cells grown on selective liquid media underwent galactose induction on plates, while in the present study cells were induced in nonselective liquid media, which increased the probability of plasmid loss.

## RESULTS

**Repair via gene conversion when the DSB has one nonhomologous end:** The *URA3*-containing plasmids illustrated in Figure 2 were transformed into wild-type strain tNS1368 and into derivatives of JKM146 deleted for *rad1* (YFP103) or *msh2* (YFP255). In each case, cells were pregrown in lactate medium to which a final concentration of 2% galactose was added to induce *HO* expression (see materials and methods). Twenty-four hours after HO induction, aliquots were removed and plated on dextrose-containing YEPD plates, where HO expression is shut off, to measure plasmid cell survival. Plasmid retention, representing almost exclusively homologous recombinational repair of the DSB (Rudin *et al.* 1989), was measured by replica plating the colonies on YEPD to SC-URA.

As shown previously (Sugawara *et al.* 1997), recombinational repair of a plasmid in which the donor sequence is essentially perfectly homologous to the HO-cut ends (plasmid pFP122) was equivalent among wild-type, *rad1*, and *msh2* mutant cells (Table 2). Southern blot analysis established that these deletion mutations did not affect the proportion of gene conversions that were accompanied by crossing over in the case of pFP122. Also, when the HO-cut plasmid has two nonhomologous ends of 308 and 610 bp (plasmid pFP120), repair was reduced ~44% in the wild-type strain, but fell >88-fold in the absence of either *RAD1* or *MSH2*, which confirms our previous results (Sugawara *et al.* 1997). We examined a small number of pFP120 plasmids that were retained after HO induction in *rad1* and *msh2* strains through Southern blot analysis and confirmed that they were indeed gene convertants and not the product of nonhomologous end-joining.

When we examined the three plasmids that had nonhomology on only one side of the DSB, it was clear that they were repaired far more proficiently than pFP120, with two nonhomologous ends (Table 2). In wild-type strains, these plasmids were repaired about as efficiently as pFP122. In both *rad1* and *msh2* strains, the repair of the one-nonhomologous-ended plasmids was reduced on average to 20 or 26%, respectively, of the levels found with the perfect-ended pFP122. This still represents a >20-fold increase relative to pFP120. Comparing pFP-131 to pMC9, there does not seem to be any significant effect of the length of the nonhomologous tail that had to be excised but there may be a small effect of the side on which the nonhomologous tail is found.

These results strongly suggest that the excision of a single nonhomologous end, when recombination has been initiated by a homologous end, can occur by a *RAD1*- and *MSH2*-independent process. On the basis of the observation that, in the one-nonhomologous-ended cases, recombination could occur on average 20–25% of the time compared to perfect ends in the same mutants, we would have predicted that two nonhomologous ends removed in the same way would have led to recombination ~4–6 % of the time, whereas repair occurred 10-fold less frequently (0.6%).

**Physical monitoring of the kinetics of gene conversion repair:** Recombination in synchronously induced cultures can be followed by Southern blot analysis of *Pst*I digests of DNA purified at intervals after the expression

**TABLE 2**

**Levels of gene conversion associated with or without crossing over**

| | Plasmid retention levels at 24 hr[a](%) | | | Gene conversion with crossing over (%) | | | Aberrant events (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| Plasmid | Wild type | *rad1* | *msh2* | Wild type | *rad1* | *msh2* | Wild type | *rad1* | *msh2* |
| pFP122 (C→T) | 52 | 56 | 50 | 30 (10/33)<br>22[b] | ND<br>20[b] | ND<br>18[b] | 6 (2/33) | ND | ND |
| pFP120 (308/610) | 23 | 0.6 | 0.6 | 30 (17/56)<br>31[b] | 42 (5/12)<br>ND[c] | 11 (1/9)<br>ND[c] | 5 (3/56) | 0 (0/12) | 11 (1/9) |
| pFP130 (0/610) | 42 | 9 | 7 | 19 (12/63)<br>28.5[b] | 7 (2/29)<br>10.5[b] | 3 (1/30)<br>9.5[b] | 5 (3/63) | 0 (0/29) | 7 (2/30) |
| pFP131 (308/0) | 43 | 13 | 16 | 19 (12/64)<br>32.5[b] | 20 (6/30)<br>27[b] | 12 (7/60)<br>35[b] | 9 (6/64) | 0 (0/30) | 7 (4/60) |
| pMC9 (609/0) | 37 | 10 | 17 | 23 (7/31)<br>29.5[b] | 12 (4/33)<br>23[b] | 15 (5/33)<br>30.5[b] | 6 (2/31) | 0 (0/33) | 6 (2/33) |

Cells were grown in YEPL medium with galactose (2% w/v) for 24 hr. These cells were then plated on YEPD and replica plated to SC-URA plates. A total of 30–60 individual colonies that scored as $Ura^+$ were assayed in Southern blots to determine how they had repaired their HO-induced DSBs. The table does not indicate the class of gene conversions without crossovers, which comprise all those not shown. ND, not determined.

[a] Levels of plasmid retention were obtained from the genetic analysis of a minimum of 1100 colonies. The percentages presented here represent an average of three or more individual experiments, each of which was normalized to the 0-hr time point data.

[b] Phosphorimager quantitation data of the 24-hr lanes of the Southern blots shown in Figures 3–6 and from additional data not shown.

[c] Cases in which levels of repair were below the level of detection of the assay.

of *HO*. For example, in a wild-type strain carrying plasmid pFP131, with a nonhomology at only one side of the DSB, one can first see the appearance of the two products of HO cleavage (HO cut 1 and HO cut 2) of the 5.2-kb parental fragment (P2), at 0.5 hr (Figure 3A). Because each of the one-nonhomologous-ended plasmids has a large heterology that is removed during the repair, the size of a gene conversion product is distinctly different from the parental *lacZ* fragment containing the HO cleavage site. This allows us to determine the appearance of both gene conversions with and without crossing over. Both crossover bands, c.o.1 (8.2 kb) and c.o.2 (4.0 kb), were observed along with the 4.9-kb noncrossover gene conversion product (g.c.) at 1 hr. When the same plasmid was studied in *rad1* and *msh2* hosts, there was a distinctive difference in the kinetics of repair. The *Pst*I restriction fragment characteristic of gene conversion without crossover (g.c.) appeared at 1 hr along with one of the two crossover products (c.o.2); however, the other crossover product (c.o.1) appeared 1 hr later. The band with the delayed appearance corresponds to the crossover that involves the nonhomologous end. To clearly indicate the first time point of appearance of the crossover and the gene conversion products in the interval between 0 and 5 hr of induction, an average of two to three Southern blots from independent experiments were quantitated by phosphorimager analysis for every strain. These data are presented as graphs along with examples of the Southern blots used in the analysis.

A very similar result was obtained for the two other plasmids, pFP130 and pMC9, with the nonhomology on only one side of the DSB. Again, in the wild-type host, the gene conversion products with and without crossing over all appear at 1 hr (Figures 4A and 5A). In *rad1* and *msh2* strains, there is a dis-coordination of the crossover products, with a 1-hr delay in the crossover product that involves the nonhomologous end (c.o.2 in Figure 4, B and C, and c.o.1 in Figure 5, B and C); the restriction fragment expected for a gene conversion without exchange appeared simultaneously with the first visible crossover product.

With plasmid pFP120 in a wild-type strain (Figure 6A) with nonhomologies on both sides of the DSB, a 4.3-kb *Pst*I fragment characteristic of a gene conversion (g.c.), along with a 3.4-kb crossover product (c.o.2), is visible 2 hr after HO induction while the 7.6-kb crossover product (c.o.1) is visible an hour earlier. Thus, in the wild-type case, gene conversions without crossover and those with an exchange of flanking markers do not appear simultaneously. As shown previously (Sugawara *et al.* 1997), recombination of pFP120 in *rad1* and *msh2* strains is not detected (Figure 6, B and C). We also noted a retardation in the rate of degradation of the HO-cleaved fragments (HO cut 1 and HO cut 2) in these and other *rad1* and *msh2* strains. We speculate that this retardation reflects an interaction between Rad1p and Msh2p with the 5′–3′ exonuclease, ExoIp (Tishkoff *et al.* 1997).

**Proportion of gene conversions associated with crossing over:** The proportion of gene conversions accompanied by crossing over was ascertained in two ways. First,

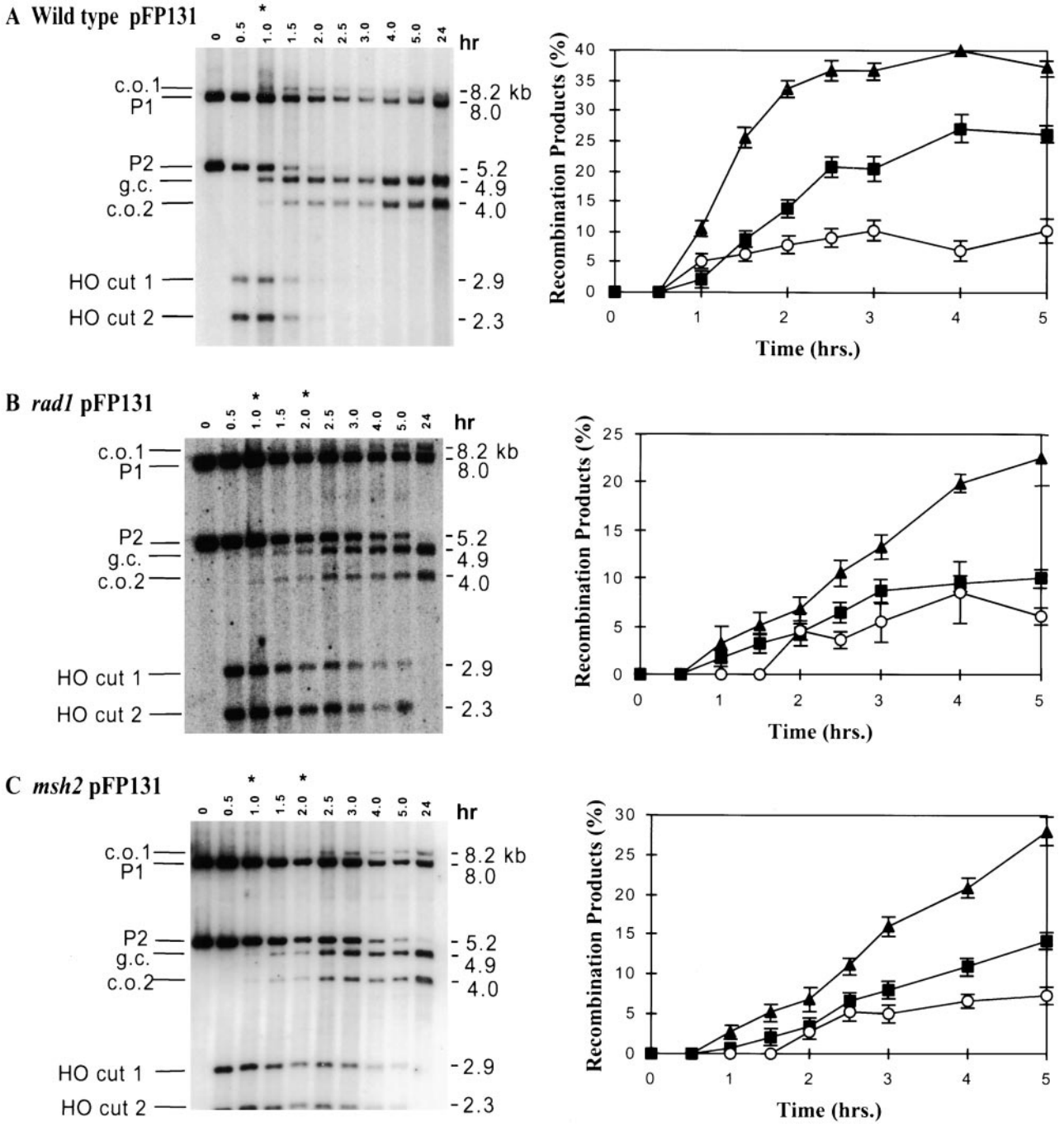


Figure 3.—Physical analysis of the kinetics of DSB repair in plasmid pFP131. DNA extracted from strains at intervals before and after galactose induction was restriction digested with *Pst*I and separated by gel electrophoresis in 0.5% agarose gels at 34 V for 24 hr. The Southern blot was probed with *lacZ* sequences. As shown in Figure 7, P2 is the parental *Pst*I fragment containing the HO cut site, whereas P1 is the donor *lacZ*-containing *Pst*I fragment. C.o.1 is the crossover fragment containing *URA3* and *CEN4* sequences, and c.o.2 is the reciprocal crossover fragment. The gene conversion product (g.c.) is derived from P2, with the loss of the HO recognition site and the loss and/or adddition of any sequences copied from the donor *lacZ* sequence in P1. (A) In wild-type strain tMC48 carrying pFP131, both crossover products along with the gene conversion product are visible synchronously at 1.0 hr. (B and C) The same analysis was performed for *rad1* (YFP126) and *msh2* (YFP376) strains carrying pFP131. The fragment corresponding to the crossover event involving the perfectly homologous 3′ end (c.o.2) appeared at 1.0 hr along with the gene conversion band (g.c.). The second fragment corresponding to the crossover event that required processing of the nonhomologous end appeared faintly at 2.0 hr (c.o.1, 8.2 kb). The plotted data were obtained by phosphorimager analysis of the recombination products between the 0- through 5-hr interval depicted in the Southern blots. These data correspond to the average of two or three independent experiments. Standard deviations are indicated by the bars. The intensity of each band corresponding to a recombination product was divided by the sum of the intensities of all the bands present in that same lane. The results were plotted as a percentage of overall DNA content. ▲, gene conversion product; ○, c.o.1; ■, c.o.2; *, first time point at which the products are visible.

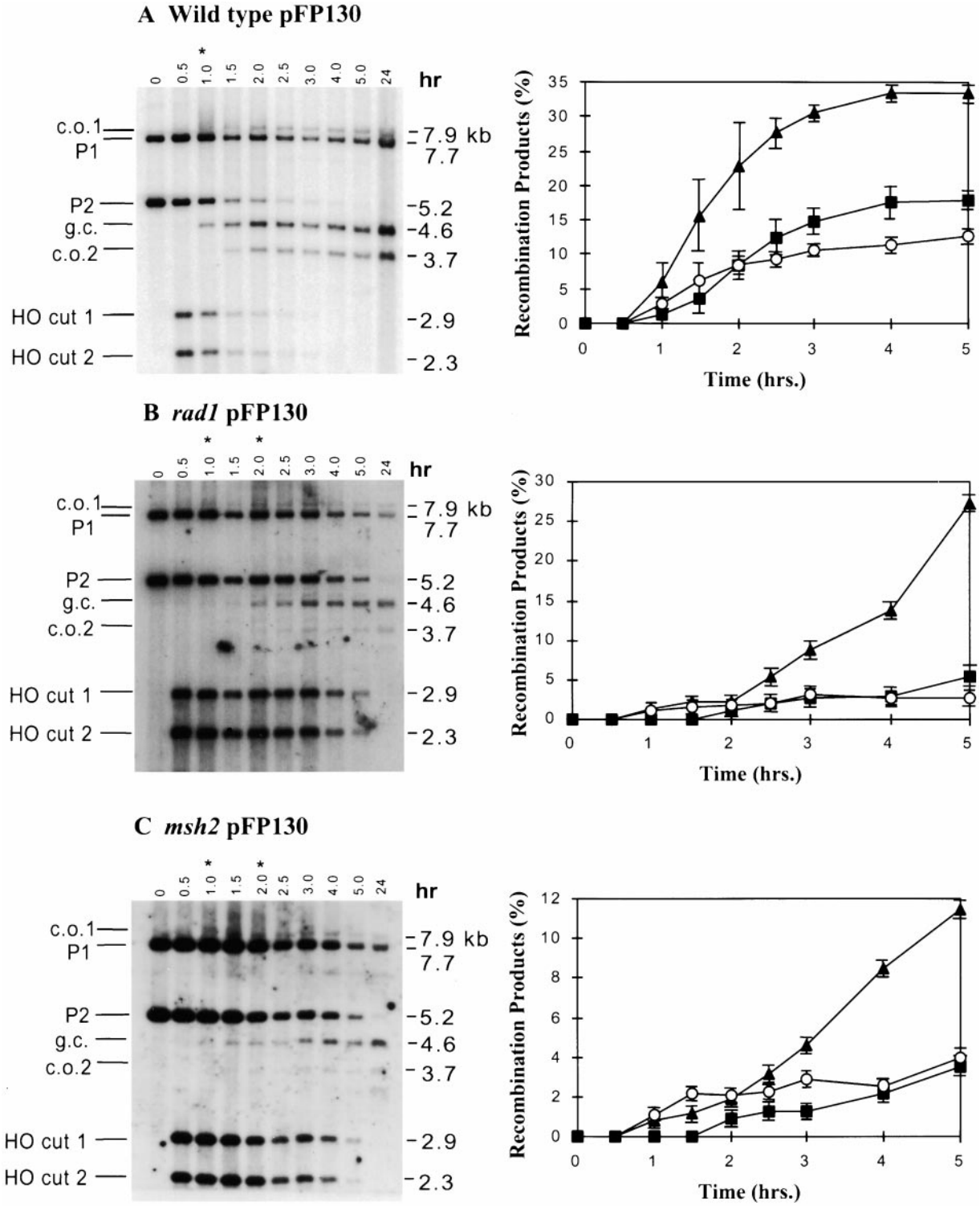


Figure 4.—Physical analysis of the kinetics of DSB repair in plasmid pFP130. Experimental procedure is as described in the legend to Figure 3. (A) In wild-type strain tMC47 carrying pFP130, both crossover products along with the gene conversion product are visible synchronously at 1.0 hr. (B and C) In both *rad1* (YFP124) and *msh2* (YFP375) strains carrying pFP130, the crossover products were faintly visible. One crossover fragment appeared at 1.0 hr (c.o.1) along with the gene conversion product (g.c.), while the crossover fragment carrying the nonhomologous 3′ end appeared at 2.0 hr (c.o.2, 3.7 kb). The crossover products are distinctively visible upon overexposure of the blots. Phosphorimager analysis of the recombination products is based on an average of two to three independent experiments. ▲, gene conversion product; ○, c.o.1; ■, c.o.2; *, first time point at which the products are visible.

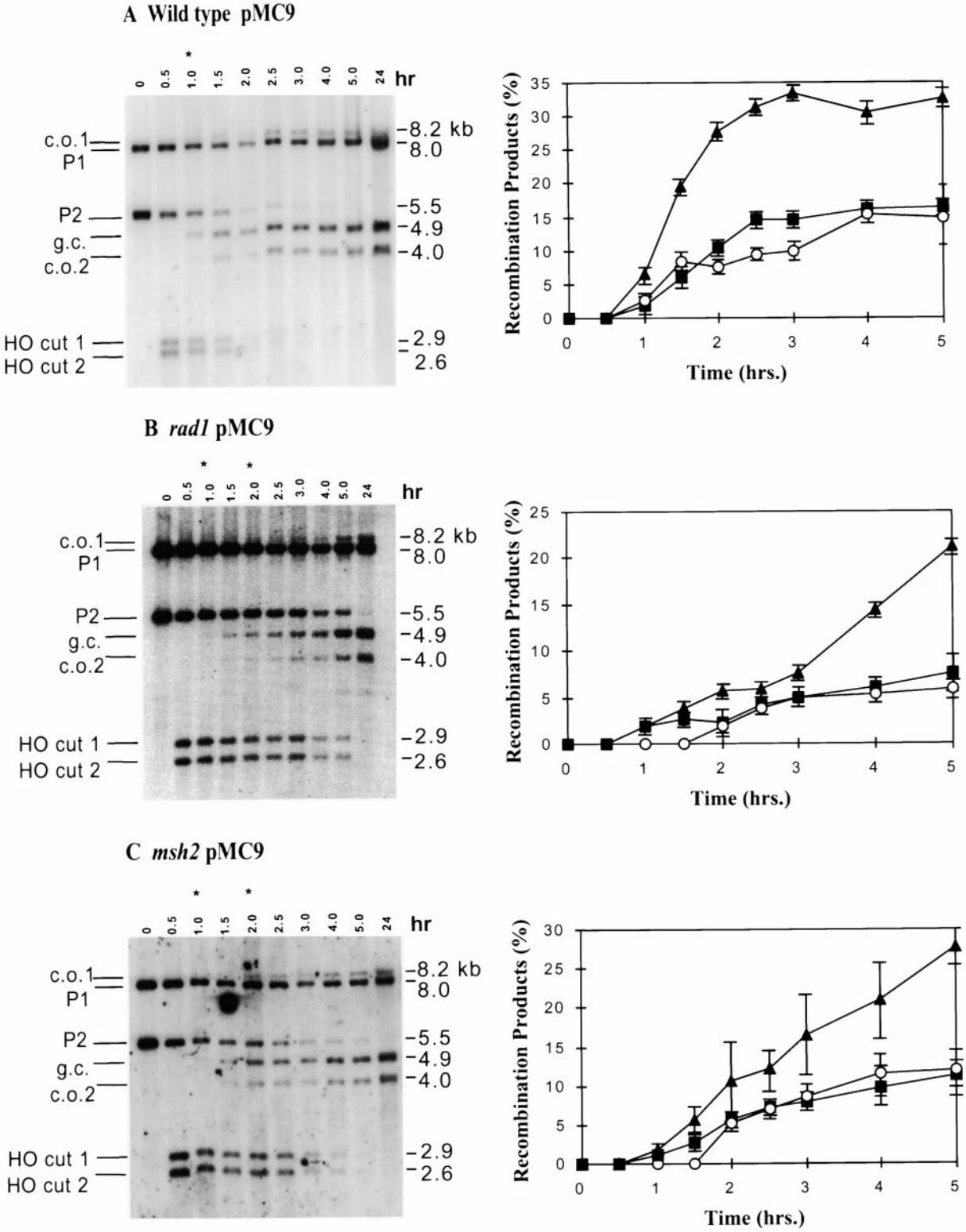


Figure 5.—Physical analysis of the kinetics of DSB repair in plasmid pMC9. Experimental procedure is as described in the legend to Figure 3. (A) In wild-type strain tMC90 carrying pMC9, both crossover products along with the gene conversion product are visible synchronously at 1.0 hr, as observed for pFP131 and pFP130 in the wild-type background. (B and C) The same analysis was performed for *rad1* (tMC96) and *msh2* (tMC93) strains carrying pMC9. The average of three independent experiments is presented along with a representative Southern blot. ▲, gene conversion product; ○, c.o.1; ■, c.o.2; *, first time point at which the products are visible.

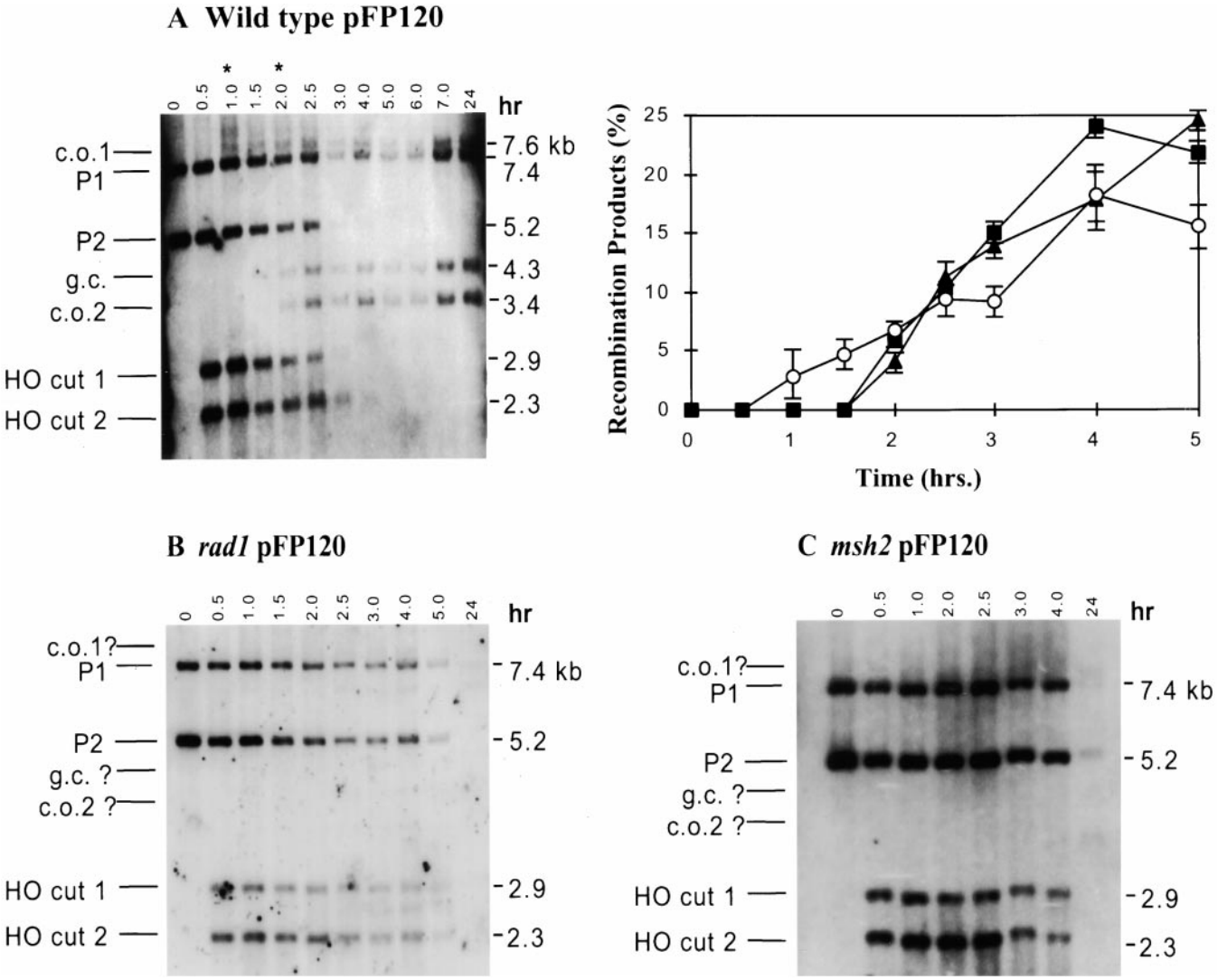


Figure 6.—Physical analysis of the kinetics of DSB repair in plasmid pFP120. Experimental procedure is as described in the legend to Figure 3. (A) In wild-type strain tMC43 carrying pFP120, both crossover products do not appear synchronously. One product is visible at 1.0 hr (c.o.1, 7.6 kb) while the second is visible between 1.5 and 2.0 hr (c.o.2, 3.4 kb) along with the gene conversion product (g.c., 4.3 kb). The HO cut fragments are no longer visible by 4.0 hr. Phosphorimager quantitation of the Southern blot data between the 0- and 5.0-hr time points is also presented. These data correspond to an average from quantitations of two to three independent experiments and are shown along with standard deviation bars. (B and C) In both *rad1* and *msh2* strains, respectively YFP112 and YFP265, none of the recombination products are visible. This was confirmed by phosphorimager analysis of an average of three independent experiments (data not shown). ▲, gene conversion product; ○, c.o.1; ■, c.o.2; *, first time point at which the products are visible. ?, the positions of undetected c.o.1, c.o.2, and g.c. in B and C.

*Pst*I-digested DNA from individual colonies was analyzed by Southern blots to detect crossover or noncrossover products. Alternatively, these proportions were determined by densitometric analysis of the 24-hr lanes of Southern blots of DNA extracted from a large population of cells, as shown in Figures 3–6. These data are summarized in Table 2. In wild-type strains, all three plasmids with one nonhomologous end had about the same levels of crossing over as both pFP122, with two homologous ends, and pFP120, with two nonhomologous ends. While *rad1* and *msh2* had no effect on the proportion of associated exchange in pFP122, there was a statistically significant reduction in the crossing-over levels of pFP130, which is evident in the densitometric analysis in Figure 4 and Table 2. However, there was no effect of these mutants on either pFP131 or pMC9 products with and without crossover. A possible explanation of this difference is discussed below.

**Analysis of aberrant recombination events:** In a wild-type host ~6% of the recombined products of pFP122 were aberrant, having neither the two *Pst*I restriction fragments expected for a gene conversion without crossover nor the two reciprocally recombined fragments when crossing over had occurred. There is a similar frequency of such events in pFP120 and in the three plasmids with one nonhomologous end (Table 2). An

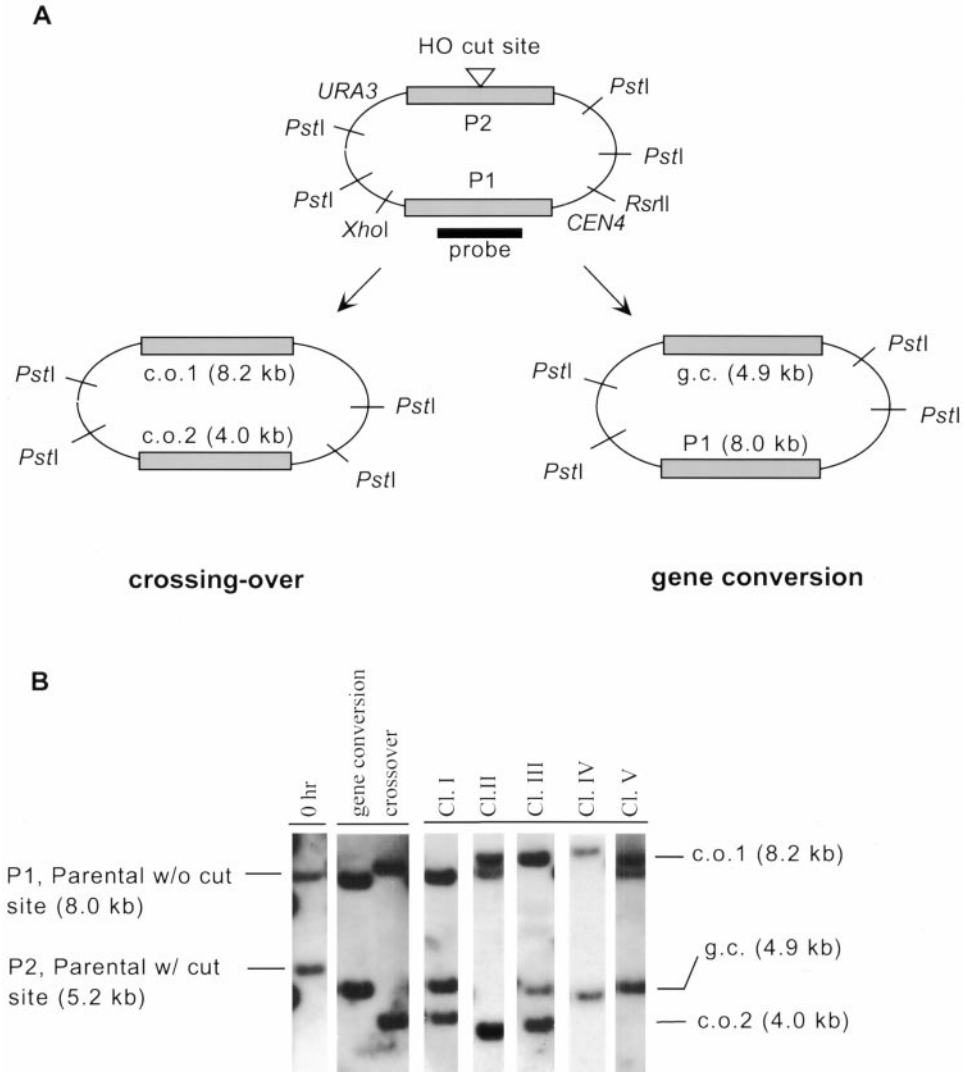


Figure 7.—Physical analysis of the aberrant events. (A) After the DSB is repaired, restriction digest of the plasmid DNA with *Pst*I generates diagnostic fragments corresponding to either a gene conversion with crossing over or a gene conversion event without exchange. These products are probed with a *lacZ* fragment spanning both sides of the HO cut site (see materials and methods). (B) DNA from the *msh2* strain carrying pFP131 (YFP376) was extracted from individual colonies plated at the 0- and 24-hr time points, digested with *Pst*I, and analyzed by Southern blots. The first lane shows DNA before galactose induction of the HO endonuclease. The two visible bands correspond to the two *lacZ*-containing fragments (P1 and P2). The other lanes analyze colonies obtained 24 hr after induction. The second lane corresponds to repair by gene conversion without crossing over in which the lower band (g.c.) is 308 bp smaller than the original fragment containing the HO cleavage site and 288 bp of adjacent nonhomologous DNA. The third lane represents repair by gene conversion associated with crossing over (c.o.1 and c.o.2). Lanes labeled Cl. I–V represent the five different classes of aberrant events observed in this study.

example of each of five unusual classes found among the recombinants of pFP131 is shown in Figure 7. Surprisingly, we found similar levels of these aberrant products in the *msh2* strains, but not in *rad1* hosts (Table 2).

Several of these aberrant products were analyzed to establish their structure. Class IV, for example, has one of the bands characteristic of a crossing over accompanied by one of the noncrossover fragments. Classes I and V have the two noncrossover products plus one of the two bands expected for a crossover. Classes II and III have two crossover bands, but also one band indicative of a noncrossover.

In an attempt to understand how these aberrant products were generated, DNA from cells containing Classes I–V was digested with *Xho*I, which cuts the plasmid only once. In Classes III–V only one band was observed, with the size expected for a repaired plasmid carrying two copies of *lacZ*. In Classes I and II two bands were observed. One had the size of a normally repaired plasmid while the second one corresponded to a plasmid that would have lost ~2.5 kb from the ~15.4-kb original plasmid. A higher-molecular-weight product that might have resulted from the recombination between two centromeric plasmids and subsequent loss of one centromere was not observed. DNA from Classes I, II, and IV was also transformed into *E. coli*, to select for ampicillin resistance. Digestion of 20 transformants of each class with *Rsr*II that cuts the original plasmid only once near the centromere sequence generated only one band. This band corresponded to the size of a repaired plasmid that has two copies of *lacZ*. This suggested that the original yeast strains carrying Classes I and II harbored two plasmids, one that repaired by generating an intact plasmid and a second smaller plasmid that lacked some of the sequences necessary for its propagation and selection in *E. coli*.

The fact that none of these events were observed in *rad1* strains argues that Rad1p/Rad10p and Msh2p/Msh3p play different roles in processing. It also indicates

that to engage in an efficient aberrant pathway of repair, the nonhomologous end must be removed by Rad1p/Rad10p.

## DISCUSSION

**Repair of an HO-induced DSB with nonhomologous sequences at one end occurs in the absence of Rad1 or Msh2:** Previous results from this lab (Sugawara *et al.* 1997) have demonstrated that Rad1/Rad10 and Msh2/Msh3 proteins were necessary for the removal of 3′ nonhomologous single-stranded DNA flanking a DSB during gene conversion, when both ends contained nonhomology. In the present study we set out to determine whether one nonhomologous end would be enough to prevent DSB repair in the absence of these proteins or whether it might affect the types of outcomes. Our results argue that there is a *RAD1*-, *MSH2*-independent pathway that can remove nonhomology from one end of a DSB, but only if recombination has been initiated by the opposite end that shares homology with the donor sequence. We suggest that this mechanism of excising the nonhomologous end is less efficient and normally serves as a backup to *MSH2*- and *RAD1*-dependent removal of this segment, on the basis of two observations. First, the efficiency of repair is reduced two- to sixfold in the absence of these genes. Second, the completion of gene conversion accompanied by crossing over is delayed by 1 hr in the absence of these genes. This delay specifically involves the formation of a crossover restriction fragment involving the nonhomologous end. The lack of delay both in completing gene conversion without exchange and in the appearance of the restriction fragment corresponding to the first of the two crossover products is discussed below.

**Effect of *RAD1* and *MSH2* on the proportion of crossing over accompanying gene conversion:** In previous studies using plasmids with two homologous ends, the deletion of *RAD1* or *MSH2* had no effect on the proportion of gene conversions associated with crossing over (Sugawara *et al.* 1997). In the present study, however, when there is a nonhomologous end closer to the 3′ end of *lacZ* (pFP130), there is a dramatic reduction in the proportion of gene conversions associated with crossing over in the absence of *RAD1* or *MSH2*, but not in the case when the nonhomologous end is proximal to the 5′ end (pFP131 and pMC9). We do not know why one end should be different from the other, but we note that the *lacZ* sequences in this construct are transcribed from a composite *CYC1*/*LEU2* promoter (Rudin *et al.* 1989). Recently, Ferguson and Holloman (1996) noted a similar asymmetry in transformation experiments in *Ustilago maydis*, where the presence of nonhomology at the end of the linearized DNA distal to the promoter led to gap repair at nearly the level of a linearized plasmid with no nonhomology, but nonhomology at the promoter-proximal end led to a 100-fold reduction. A similar bias between two ends was seen in phage T4 recombination by George and Kreuzer (1996). In our case, there is not much difference in the overall efficiency of repair, but there is a significant reduction in the proportion of gene conversions accompanied by crossing over. This may mean that the backup system with which to remove nonhomology is sensitive to the transcription traversing the end of the DSB containing the nonhomology. Alternatively it may indicate that the ability of the nonhomologous-containing end to enter into the repair event is sensitive to transcription. For whatever reason, the difficulty in removing the nonhomologous end leads to a marked reduction of crossing over in pFP130. We believe this reflects a shift in the equilibrium between alternative intermediates in the repair of the DSB, favoring one that precludes crossing over.

**Separation of kinetics of gene conversions with and without crossing over suggests DSB repair uses a SDSA mechanism:** When the completion of DSB repair requires the removal of one nonhomologous end and when the efficient excision system is eliminated in *rad1* and *msh2* mutants, there is a significant change in the kinetics of the repair events. The *Pst*I fragment indicative of gene conversion without crossing over appears 1 hr earlier than a fragment signaling the completion of gene conversions with exchange. In meiosis in Saccharomyces, the *zip1* mutation causes a similar uncoupling of the timing of crossovers and noncrossovers (Storlazzi *et al.* 1996), but this is the first time that such asynchrony has been seen in mitotic cells.

We explain these results in terms of an SDSA mechanism, where the homologous end would initiate new DNA synthesis and the displacement of a replication bubble toward the second end (Ferguson and Holloman 1996; Haber 1998; Pâques *et al.* 1998; and Figure 8). If the displaced donor strand anneals to the second DSB end, a double Holliday junction would be formed, allowing crossovers (Figure 8, C1), while if the second end anneals to the newly synthesized DNA, gene conversions would be unassociated with exchange (Figure 8, C2). The coordinate appearance of crossovers and noncrossovers is not obligate in SDSA mechanisms, because the annealing process that yields noncrossovers could be finished well before the alternative process that generates Holliday junctions is completed. A difficulty in removing the nonhomologous tail from one of these intermediates might also bias resolution toward the noncrossover alternative, as was seen for pFP130 in *rad1* and *msh2* mutants. It is more difficult to explain these results in terms of the DSB repair model of Szostak *et al.* (1983), where all gene conversions arise from alternative resolution of a common double Holliday junction intermediate and both crossovers and noncrossovers should appear at the same time.

**Lack of simultaneous appearance of reciprocal recombination products in the presence of one nonhomol-**

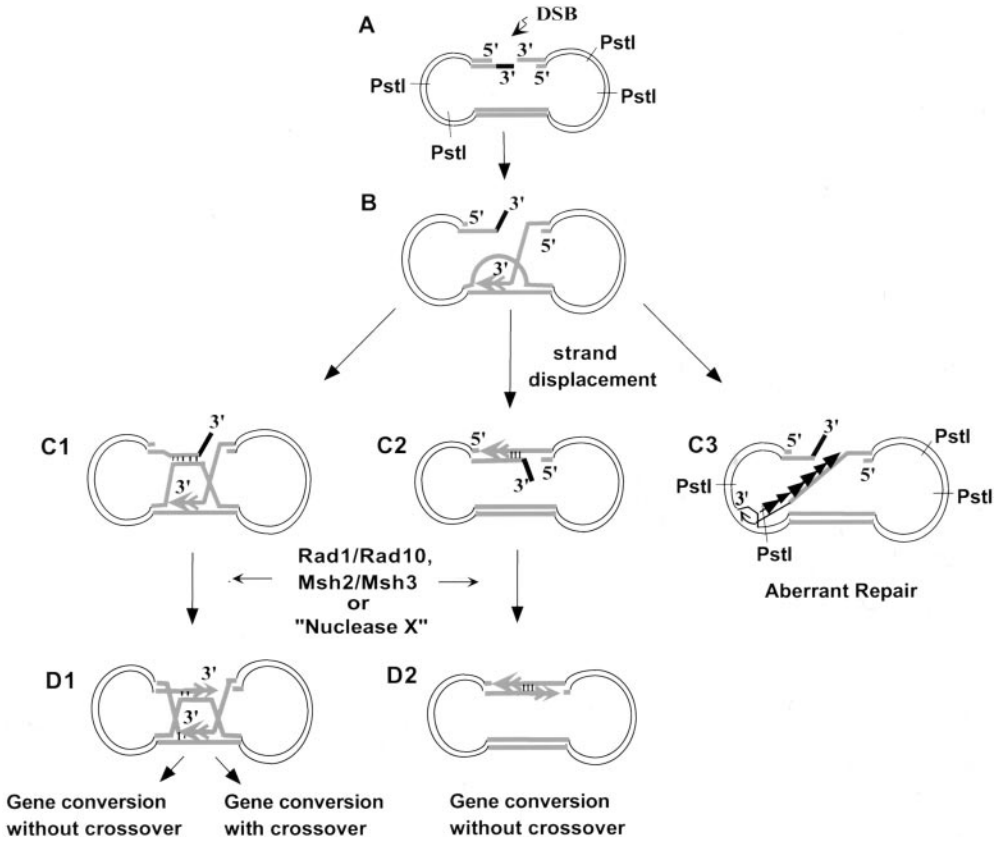


Figure 8.—Processing of a nonhomologous end in wild-type cells. (A) Upon induction of a DSB, resection of the 5′ ends produces two 3′-ended single strands, one of which contains nonhomology (depicted in boldface). (B) The homologous end (depicted in gray) is more free to invade the template, prime new DNA synthesis, and create a D-loop. Removal of the nonhomologous end is envisioned to occur once the homologous sequences adjacent to the nonhomology anneal with a strand of opposite polarity. This can occur by annealing to the D-loop (C1), creating an intermediate with two Holliday junctions that can be resolved either with or without crossing over (D1). Alternatively, the newly synthesized strand may be displaced from its template and anneal with the second end (C2), resulting in gene conversions that are always without crossing over (D2). In wild-type cells, removal of the nonhomologous end uses Rad1/Rad10 and Msh2/Msh3, but an alternative nuclease (designated as nuclease X) can also remove this end, albeit less efficiently. Engagement of the second end appears to be inefficient; hence, the DNA polymerization produced by the first end may continue beyond the border of homology between donor and recipient and lead to intermediates that are resolved by nonhomologous recombination (C3), producing aberrant outcomes that contain one noncrossover and one crossover *lacZ* fragment.

**ogous end:** One is still faced with accounting for the lack of simultaneous appearance of the two crossover bands. We suggest that the product that appears earlier does not represent a completed recombination event, but is in fact an important intermediate that is especially visible when a nonhomologous end cannot be removed efficiently. We imagine that the perfect end of the DSB invades its template and begins to copy the donor template. If the presence of the nonhomologous end interferes with normal annealing steps, then the polymerase may continue to copy the template, traversing *lacZ* and copying part of the adjacent plasmid backbone, including the next *Pst*I site (Figure 8, C3). Thus there will be a continuous "crossover" DNA strand from the *Pst*I site adjacent to the perfect end of the DSB to the now-copied *Pst*I site. For this fragment to migrate as a double-stranded molecule identical to the final crossover product, the newly synthesized DNA must have also filled in any DNA resected away from the DSB. Recently we have found evidence that the second strand may be copied by lagging-strand DNA polymerase components, including Polα, primase, and Rad27 (Holmes and Haber 1999). The intermediate structure generated in this fashion may never be converted to an intact, circular plasmid; indeed this may explain why the efficiency of repair of one-nonhomologous-ended plasmids is reduced two- to sixfold in *rad1* and *msh2* strains. We note that it is necessary that both *Pst*I sites originally flanking the DSB be cleaved to detect the noncrossover fragment; hence it seems unlikely that one could explain the appearance of one crossover band an hour before the other because one *Pst*I site remained single stranded and thus could not be cleaved to yield the appropriate fragment.

**An SDSA mechanism of DSB repair:** For these reasons we favor an SDSA mechanism to describe how gene conversion occurs after the initiation of a DSB. There is now a substantial body of evidence favoring SDSA as the predominant mechanism in *S. cerevisiae.* For example, in mitotic cells when the donor sequence contains repeated sequences, DNA synthesis that copies these sequences frequently leads to expansion and contraction of the repeats, and essentially all of the rearrangements are found in the recipient locus (Pâques *et al.* 1998). Similarly, heteroduplex DNA formed during *MAT* switching is found only at the recipient locus, even though it should have initially formed during the inva-

sion of the DSB end into the donor (McGill *et al.* 1989; Ray *et al.* 1991). Supporting data have also been reported in studies of meiotic gene conversion (Gilbertson and Stahl 1996; Porter *et al.* 1996). As shown in Figure 8, the specific version of SDSA that we favor begins with the invasion of one end of the DSB to initiate DNA replication. This strand does not remain associated with its template, but is displaced as the replication structure moves. Lagging strand synthesis may also occur (Holmes and Haber 1999). As the polymerase(s) progresses, the second end of the DSB can anneal in one of two ways. If it anneals with the newly synthesized, displaced strand, no Holliday junction is formed (Figure 8, C2) and, after further processing (which includes excision of any nonhomologous 3′ tail of the second end), gene conversions exclusively lacking crossing over are recovered (Figure 8, D2). If, however, the second DSB end anneals with the D-loop that is created as the polymerase(s) moves, a double Holliday junction is produced (Figure 8, C1). This structure can, of course, be resolved to produce crossovers or noncrossovers (Figure 8, D1). Depending on the sequences involved, the presence of a nonhomologous end, and other factors, the proportion of gene conversions accompanied by exchange of flanking markers can range from 50% (Rudin *et al.* 1989) to nearly 0% (Sugawara *et al.* 1995).

Although we believe that a SDSA mechanism is the most likely explanation for these results, we cannot completely rule out the possibility that the completion of recombination, to yield a circular plasmid, might be accomplished by ARS-dependent replication of an intermediate (such as C1 or C2 in Figure 8). However, such a mechanism would require either template switching by a DNA polymerase, or other complex steps, or would again require removal of nonhomologous tails.

**Formation of aberrant events and the different roles of Rad1 and Msh2 proteins:** In this article we also describe five classes of aberrant events. In two classes cells appear to have produced deletions during the process of DSB repair that leave a plasmid without either the ampicillin-resistance gene or its bacterial origin of replication. In these two instances, the cells also contained another plasmid that had undergone crossing over. In Class IV, the plasmid appears to contain one crossover product and one noncrossover product. We suggest that these rearrangements must have involved the formation of an illegitimate (nonhomologous) junction in the regions flanking *lacZ* during the process of repair, perhaps similar to the aberrant events in *E. coli* described by Takahashi *et al.* (1997) and in mammalian cells described by Sakagami *et al.* (1994). The frequency of these events is much higher than would be expected for the simple nonhomologous end-joining seen after generating an HO-induced DSB (Moore and Haber 1996).

Although in general the roles of Msh2p and Rad1p appear to be equivalent in the removal of nonhomologous tails, they appear to play quite different roles in the production of these aberrant events, because they are eliminated by a *rad1* mutation but not by *msh2*. This result is reminiscent of the different effects Rad1 and Msh2 proteins have on an alternative homologous recombination process that can repair a DSB. In single-strand annealing, where exonucleases expose complementary homologous regions flanking a DSB, Rad1p is always required, but Msh2p becomes dispensable when the length of the homologous region that anneals is ~1 kb (Sugawara *et al.* 1997). Possibly Msh2/Msh3 proteins recognize and stabilize a branched DNA structure that Rad1/Rad10 endonuclease can then cleave, but Msh2p/Msh3p are not needed when the length of homology is great enough to form a stable structure. However, in gene conversions, where the strand containing the nonhomologous tail is part of a triplex structure, both Msh2p and Rad1p are required, even when homologies extend 2 kb in length. This suggests to us that the aberrant events arose by a process in which a nonhomologous tail was generated, but was stabilized in an unknown way. Some of these events may have an intermolecular origin in cells carrying more than one copy of the centromeric plasmid.

**Effect of two nonhomologous ends in HO-induced recombination of wild-type cells:** In wild-type cells, the presence of one nonhomologous end does not affect the kinetics of recombination. Both crossover and noncrossover products appear at the same time. In contrast, when there are two nonhomologous ends in a wild-type cell, there is a striking delay in the appearance of both the noncrossover and one of the two crossover fragments. The early appearance of one of the two crossover bands occurs both in pFP120, with at least 308 bp of nonhomology on either side of the DSB, and in other inverted-repeat *lacZ* centromeric plasmids in a different plasmid backbone, with only 45 and 72 bp of nonhomology on the two ends (Rudin *et al.* 1989). The crossover band that appears early is always involving the DSB end adjacent to the 5′ end of *lacZ* and is independent of the orientation of the HO cleavage site, the presence of a promoter adjacent to *lacZ*, the proximity of the DSB ends to the centromere, or the length of the plasmid sequences on either side of the *lacZ* regions (Rudin *et al.* 1989; M. Colaiácovo and J. E. Haber, unpublished results). Moreover, the same results are found when sequences are deleted from both ends of the recipient *lacZ*, so that only 387 bp of homology instead of 3 kb surrounds the HO cleavage site (M. Colaiácovo and J. E. Haber, unpublished results). This might suggest that there are some sequences close to one end of the DSB that make that end more efficient in strand invasion and/or in the removal of the nonhomologous end. However, in pFP120, all of these sequences close to the DSB are deleted, yet the same end continues apparently to invade first. Further studies will be needed to unravel this conundrum.

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