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Double-Strand Break Repair in Yeast Requires Both Leading and Lagging Strand DNA Polymerases

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Summary

Mitotic double-strand break (DSB)-induced gene conversion at *MAT* in *Saccharomyces cerevisiae* was analyzed molecularly in mutant strains thermosensitive for essential replication factors. The processivity cofactors PCNA and RFC are essential even to synthesize as little as 30 nucleotides following strand invasion. Both PCNA-associated DNA polymerases δ and ε are important for gene conversion, though a temperature-sensitive Polε mutant is more severe than one in Polδ. Surprisingly, mutants of lagging strand replication, DNA polymerase α (*pol1-17*), DNA primase (*pri2-1*), and Rad27p (*rad27Δ*) also greatly inhibit completion of DSB repair, even in G1-arrested cells. We propose a novel model for DSB-induced gene conversion in which a strand invasion creates a modified replication fork, involving leading and lagging strand synthesis from the donor template. Replication is terminated by capture of the second end of the DSB.

Introduction

Repair of double-strand breaks (DSBs) by homologous recombination requires new DNA synthesis, but its relation to normal DNA replication is unknown. In yeast, as in all eukaryotes, origin-dependent DNA replication requires three DNA polymerases, Polα, Polδ, and Polε (reviewed by Baker and Bell, 1998). However, most models of DSB-initiated recombination, such as that proposed by Szostak et al. (1983), or various synthesis-dependent strand annealing models (Nasmyth, 1982; Hastings, 1988; McGill et al., 1989; Nassif et al., 1994; Ferguson and Holloman, 1996), envision that both 3′ ends generated by the DSB act as primers to initiate DNA replication exclusively by leading strand synthesis; consequently, there should be no requirement for the Polα-primase protein complex in DSB repair.

However, in some situations observed primarily in bacteria and bacteriophages, recombination occurs by primase-dependent, origin-independent DNA replication (George and Kreuzer, 1996; Mueller et al., 1996; Kogoma, 1997). This type of replication appears to be critical in repairing broken replication forks and may play a general role in other types of DSB repair. A similar break-induced replication (BIR) pathway operates in *Saccharomyces* to repair broken chromosome ends, creating nonreciprocal translocations (Dunn et al., 1984; Walmsley et al., 1984; Voelkel-Meiman and Roeder, 1990; Malkova et al., 1996; Morrow et al., 1997; Bosco and Haber, 1998).

A unifying hypothesis would be that DSB repair by gene conversion and by BIR are alternative outcomes of a single process in which one end of a DSB invades into a donor sequence and establishes a replication fork. This fork could proceed all the way to a chromosome end, as in BIR (Figure $1A_3$), or could be captured by the second end of the DSB, terminating replication and yielding a gene conversion (Figure $1A_4$). If this were true, gene conversion events in *Saccharomyces* would also require both leading and lagging strand DNA synthesis.

The DNA polymerase α-primase complex is believed to initiate replication on the leading and lagging strands in a coordinated fashion; then, by a process of polymerase switching, Polδ or Polε finishes elongation with the help of replication factor C (RFC) and the clamp protein PCNA (Waga and Stillman, 1994). In yeast, *POL1* (Polα), *POL2* (Polε), and *POL3* (Polδ) are three essential DNA polymerase genes, as defined by temperature-sensitive (ts) mutations in their catalytic domains, that prevent DNA synthesis at nonpermissive temperatures (Sugino, 1995; Baker and Bell, 1998). The Polα complex associates with the primase complex to initiate leading and lagging strand synthesis (Francesconi et al., 1991; Longhese et al., 1993). In yeast, Polδ and Polε are both essential (Budd and Campbell, 1993). Several genetic studies suggest that one of these two polymerases replicates the leading strand, while the other functions on the lagging strand (Gordenin et al., 1992; Shcherbakova and Pavlov, 1996). Rad27p, the *S. cerevisiae* homolog of mammalian DNase IV (FEN-1), is a 5′ to 3′ flap endo/exonuclease that processes the Okazaki fragment intermediates by removing the last 5′ ribonucleotide (reviewed by Lieber, 1997). Loading of leading strand polymerases onto the DNA in yeast and higher eukaryotes is accomplished with PCNA (O′Donnell et al., 1993). Efficient loading of PCNA to the primer terminus requires RFC (Tsurimoto and Stillman, 1991; Podust et al., 1995).

All of these essential components in replication were tested for their roles in HO endonuclease–induced mating-type (*MAT*) switching, the best-studied example of DSB-induced gene conversion (Strathern et al., 1982; McGill et al., 1989; Raveh et al., 1989; White and Haber, 1990; reviewed by Haber, 1998). HO endonuclease creates a site-specific DSB in *MAT*, which is repaired by gene conversion, using one of two unexpressed donor loci, *HML*α and *HMR***a**, to provide the template for new DNA synthesis. Normally, *MAT***a** recombines with *HML*α and *MAT*α interacts with *HMR***a**, so that the gene conversion replaces approximately 700 bp of Y**a** or Yα sequences (Figure 1B).

To investigate the roles of DNA replication proteins in gene conversion, we have analyzed synchronous HO endonuclease–induced recombination, by Southern blots or PCR (White and Haber, 1990), in cells carrying conditional-lethal mutations of the various DNA replication proteins. Mutant cells were arrested at their nonpermissive temperature and then recombination was initiated using a galactose-inducible HO endonuclease gene, so

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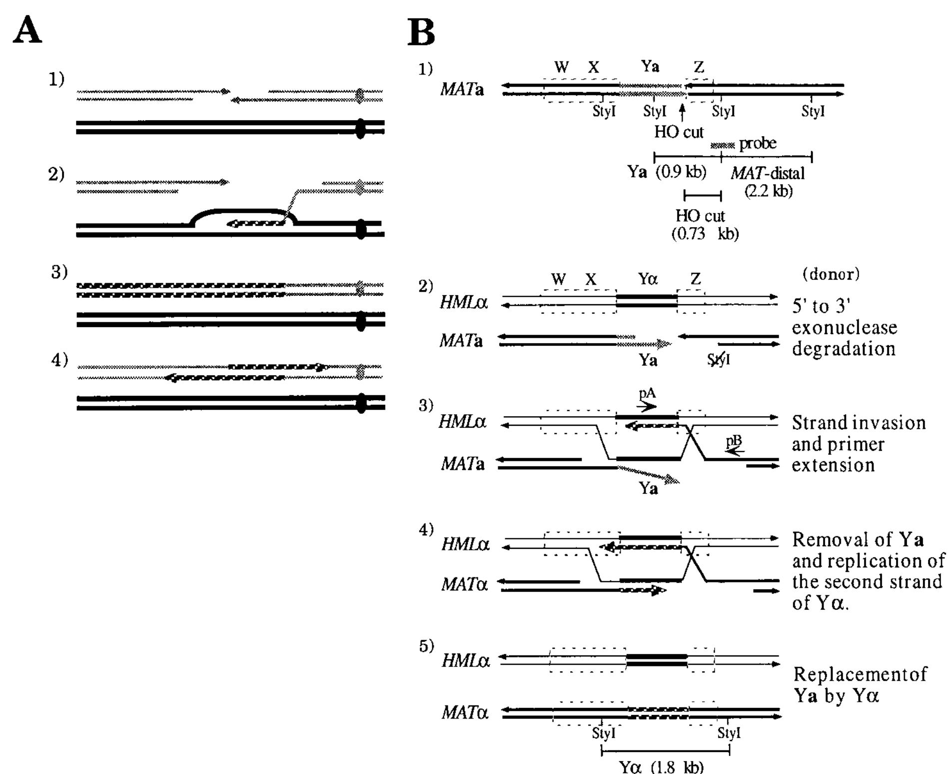

Figure 1. Models for BIR and *MAT* Switching

(A) A diploid cell with a DSB on one chromosome can be repaired from its homolog by strand invasion and the initiation of new DNA synthesis (2). This can lead either to extensive replication to the end of the chromosome during break-induced replication (3) or to gene conversion (4), depending on whether the second end of the DSB becomes engaged in the repair process.

(B) A molecular model of *MAT* switching, based on the DSB repair model of Szostak et al. (1983). A DSB is induced at the Y/Z junction by HO endonuclease (1). 5′ to 3′ exonucleolytic degradation creates a 3′ single-stranded tail that invades the homologous silent donor sequence, *HML*α, and initiates new DNA synthesis (2, 3). After removal of the nonhomologous Y region, the second end of the DSB can copy the displaced donor strand, again using a 3′ end as the primer (4). Resolution of Holliday junctions leads to gene conversion without crossing-over (5). The kinetics of repair can be monitored by Southern analysis of StyI-restricted genomic DNA, using a *MAT* distal probe, as shown. Cells switching from *MAT*a to *MAT*α will lose the diagnostic StyI site within Ya (0.9 kb), creating a larger Yα StyI fragment (1.8 kb). The initiation of DNA synthesis after strand invasion can be monitored using a unique set of primers, shown as pA and pB (3), located within *HML*α, and distal to *MAT*.

that nearly all cells in the population experienced a DSB at the same time. Genomic DNA was then isolated from samples taken from the culture maintained at the nonpermissive condition. We report that HO-induced switching of the *MAT* locus requires all three DNA polymerases. We propose a novel model in which a replication fork, similar but not identical to an ARS-dependent replication fork, is established during gene conversion.

Results

PCNA and RFC

We first analyzed the cold-sensitive alleles of two essential DNA polymerase accessory proteins, PCNA (*pol30-52*) and the largest subunit of RFC (*cdc44-8*). Cells grown at the permissive temperature of 30°C were shifted to the nonpermissive temperature of 14°C and grown 3 to 4 hr until the characteristic large budded cell replication arrest phenotype was observed. Two percent galactose was added for 1 hr to induce a DSB at *MAT*, followed by the addition of 2% glucose to repress further HO expression. DNA collected at regular intervals was analyzed on Southern blots to monitor the kinetics of recombination. The amount of recombined product visible at each time point was then measured relative to the amount of HO cut fragment produced after 1 hr of galactose induction, with each sample normalized to the total amount of DNA in each lane (see Experimental Procedures). In the wild-type strain at 30°C, product begins to appear within 1 hr, and the process is 100% efficient (Figure 2A). At 14°C, the wild-type strain recombines as efficiently, but with a 4 to 6 hr delay. *cdc44-8* is able to complete *MAT* switching at 30°C with 51% ± 7% efficiency but is completely deficient in repair at 14°C. At 14°C, a similar result is seen for *pol30-52*. At its permissive temperature of 30°C, the *pol30-52* mutant completes *MAT* switching with only 41% ± 5% efficiency, with a delay of 2 to 3 hr. This *pol30-52* strain has a doubling time of about 150 min at 30°C, 30 min longer than wild type (Ayyagari et al., 1995). Thus, replication (which requires PCNA) is much less impaired than gene conversion, which entails less than 1 kb of new DNA

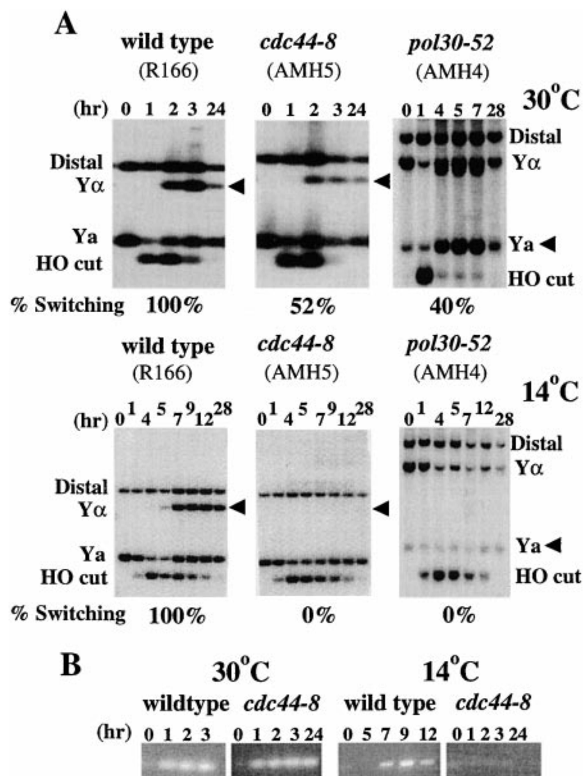

Figure 2. Southern Blot and PCR Analysis of the Requirement for PCNA and RFC

(A) DNA extracted at intervals after HO cutting was digested with StyI and separated by gel electrophoresis on a 1.4% native gel. Southern blots were probed with a ^{32}P-labeled *MAT*-distal fragment homologous to the first two StyI fragments distal to the HO cut site (Figure 1B). The 1 hr point represents 1 hr of induction of the HO endonuclease, after which time its further expression is repressed (see Experimental Procedures). The switched product is indicated by an arrowhead. The percent switching for the wild-type, *cdc44-8*, and *pol30-52* strains are indicated for analyses performed at the permissive (30°C) and nonpermissive temperatures (14°C). Percent switching is indicated for the particular Southern blot shown and was calculated from the ratio of the amount of HO cleavage at 1 hr compared to the amount of final product, normalized for total DNA in each lane (see Experimental Procedures). Both *cdc44-8* and *pol30-52* strains are unable to switch mating type at 14°C and also show approximately 2× less product at 30°C, compared to the wild-type strain. The *pol30-52* strain carries an integrated *ade3::GAL::HO*, which is slightly leaky, leading to a small amount of switching in cells prior to galactose induction. This background of switched product was subtracted from the final product.

(B) PCR analysis of DNA samples from the *cdc44-8* mutant strain was performed using unique primers distal to the HO cut site and within *HML*-Yα (Figure 1B). To form a PCR product, the 3′ end generated after HO induction must invade and replicate at least 30 nucleotides of the template donor sequence. *cdc44-8* and *pol30-52* (data not shown) yield no PCR product and therefore do not form these early intermediates at 14°C.

synthesis. Thus, *MAT* switching requires some form of PCNA-associated synthesis.

PCR analysis can be used to detect an early intermediate of *MAT* switching (White and Haber, 1990). An oligonucleotide primer specific for Yα in *HML*α (used for the *cdc44-8 MAT*a strain) or for Ya in *HMR*a (used for the *pol30-52 MAT*α strain) and a second primer that hybridizes distal to *MAT* can only amplify a PCR product after the 3′-ended single strand in *MAT-Z* invades the donor and primes new DNA synthesis copied from the donor (Figure $1B_3$). Not even 30 nt of new DNA synthesis could occur at 14°C for mutants of RFC (Figure 2B) and PCNA (data not shown).

Polε Is the Major PCNA-Associated Polymerase at *MAT*

DNA polymerases δ and ε are both essential in replication to extend the primers made by the Polα-primase complex. *MAT* switching in *pol3-14*, a ts mutant of Polδ, occurs almost as well in the wild-type strain at both permissive (23°C) and nonpermissive (37°C) temperatures. The amount of recombined product formed at 37°C was 60% ± 4% at 2 hr, with no further increase over time and with no delay relative to wild type. In contrast, *pol2-18*, the mutant allele of Polε, exhibits delayed and reduced (39% ± 3%) *MAT* switching within 3 hr of HO induction (Figure 3B). By 20 hr, gene conversion increases to 61% ± 4%. PCR analysis of *pol3-14* and *pol2-18* yields a similar conclusion (Figure 3C). Therefore, Polε appears to be the major PCNA-associated polymerase involved in this process, but there is some functional redundancy between the two polymerases in DSB repair.

Mutations in the Lagging Strand Complex Are Defective in *MAT* Switching

The Polα-primase complex includes proteins encoded by *POL1*, *POL12*, *PRI2*, and *PRI1* (Foiani et al., 1994, 1995). *POL1* encodes the catalytic polymerase subunit, and *PRI1* and *PRI2* encode the DNA primase heterodimer complex (Longhese et al., 1993). Figure 4A shows the effects of the temperature-sensitive alleles *pol1-17* and *pri2-1* on *MAT* switching. Both mutant strains switch less efficiently than wild type, with at least a 3 to 4 hr delay at the nonpermissive temperature of 37°C, with *pri2-1* producing 18% ± 2% and *pol1-17* producing 20% ± 1% overnight. The kinetics of recombination are also delayed, even at 23°C, but eventually reach wild-type levels of product.

To support the idea that lagging strand synthesis is required for *MAT* switching, we tested the role of *RAD27*, involved in processing Okazaki fragments. A *rad27* deletion is slow growing at 23°C and inviable at 37°C. Once again, *MAT* switching was defective, as demonstrated by a 1 hr delay at 23°C and 37°C, with only 52% ± 4% final product formation at 23°C and 49% ± 6% at 37°C (Figure 5A).

To understand if leading strand synthesis could still occur in the absence of lagging strand synthesis, PCR analysis was also performed to analyze the initial strand invasion and extension intermediates (Figure $1B_3$). Our results, using 20 PCR cycles (within the linear range under our PCR conditions), indicate that strand invasion and/or initial repair synthesis is compromised compared to wild-type cells, since there is a 10-fold decrease in the overall amount of PCR product for *pri2-1* and *pol1-17* mutant strains (Figure 4B). We observe the same delay when we amplify the mutant samples even more, with 23 and 26 PCR cycles, to levels comparable to wild-type samples at 20 cycles; the initial appearance

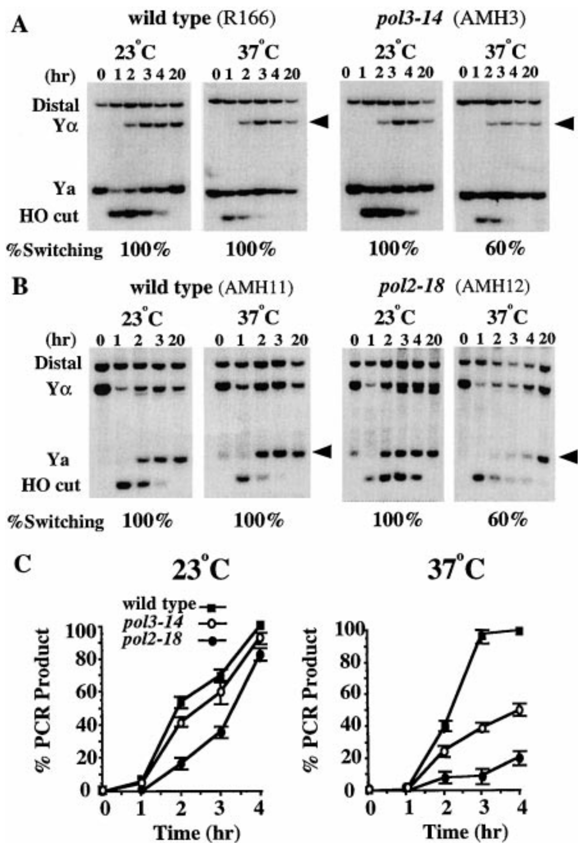

Figure 3. Southern and PCR Analyses of Polδ and Polϵ Mutants

Methods are as described in Figure 2 and Experimental Procedures.

(A) The mutant of Polδ, *pol3-14*, is able to switch mating type as well as the wild-type strain at 23°C. At 37°C, the efficiency drops to 60%, with no delay and increase in product over time.

(B) The mutant of Polϵ, *pol2-18*, also switches normally at 23°C, but shows a 3 hr delay in switching, reaching only 60% of wild-type levels.

(C) PCR analysis detecting 70 nucleotides of repair synthesis. Error bars represent the range of values from two independent PCR analyses from independent experiments. These results confirm the Southern blot analysis in (A).

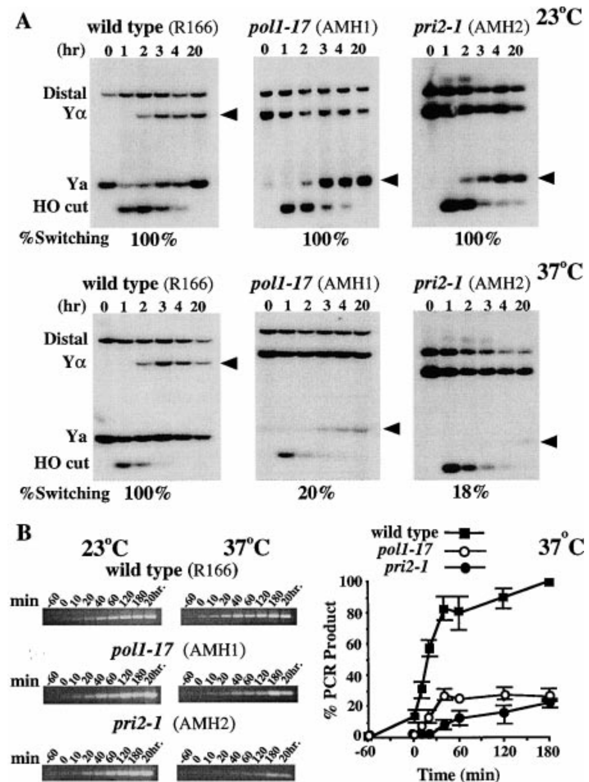

Figure 4. Southern and PCR Analyses of *pri2-1* and *pol1-17*

(A) Methods are as described in Figure 2 and Experimental Procedures. At 23°C, the *pri2-1* and *pol1-17* mutant strains switch normally, but at 37°C, *pol1-17* and *pri2-1* are reduced and delayed, producing at most about 20% recombinant product at the overnight time point.

(B) PCR analysis was performed on DNA from two independent experiments using unique primers distal to the HO cut site and within *HMR*-Ya. *pri2-1* and *pol1-17* are reduced about 20% for the initial repair synthesis of at least 70 nucleotides, similar to final product formation.

of product is delayed 20 to 40 min (data not shown). Therefore, the delay we observe in these mutants is not due to the level of detection, but reflects a real defect in strand invasion and extension of the leading strand in these lagging strand mutants.

The defect in the lagging strand mutants could be due to an indirect effect of blocking replication in S phase, thus causing the entire replication machinery to be sequestered away from *MAT*. To address this, we arrested the *pri2-1* mutant strain at the G1 stage of the cell cycle with α factor at 23°C, shifted the cells to 37°C to inactivate primase, and then induced HO while maintaining G1 arrest. *MAT* switching occurs efficiently in α factor arrested G1 cells (Raveh et al., 1989), so the cell does not need to enter S phase to carry out repair synthesis at the *MAT* locus. PCR analysis of *pri2-1* cells arrested in G1 shows that *MAT* switching occurs normally at 23°C but is deficient at 37°C, with a 10-fold decrease in product, just like *pri2-1* cells arrested at 37°C without α factor (Figure 5C). Therefore, *pri2-1* is specifically needed for repair at *MAT*.

MAT Switching Is Not Dependent on the Origin Recognition Complex

The origin recognition complex (ORC), a six-subunit protein complex necessary for initiation of replication in an ATP-dependent manner (Bell and Stillman, 1992; Liang et al., 1995), remains bound to origins throughout the cycle (Diffley et al., 1994) but exists as a larger prereplication complex (pre-RC) established in late M phase, which contains Cdc6p and the MCM family of proteins (Loo et al., 1995; Santocanale and Diffley, 1996; Aparicio et al., 1997). We asked if this initiation complex might be necessary to recognize the strand invasion intermediate produced after an HO-induced DSB and to help recruit DNA polymerases to this site for repair synthesis. The temperature-sensitive *orc5-1* mutation blocks initiation of DNA replication prior to mitosis (Dillin and Rine, 1998). At 37°C, the *orc5-1* strain, arrested in G2/M, was able to perform *MAT* switching as efficiently as the wild-type cells (Figure 5B). Therefore, the pre-RC is needed for

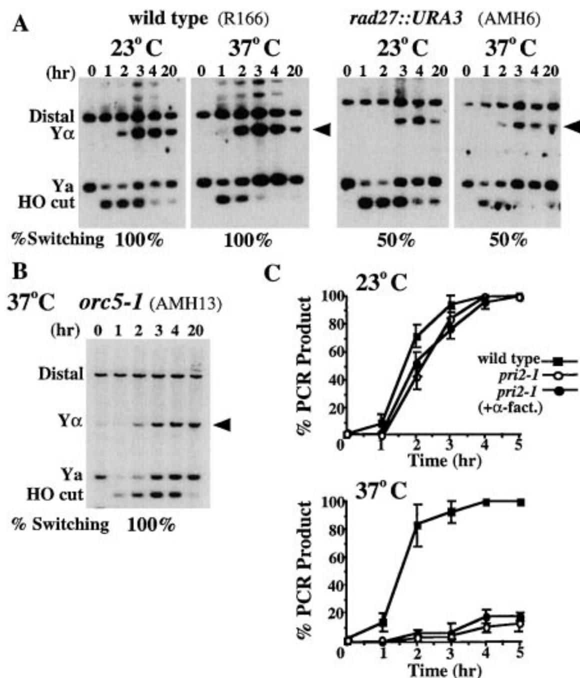

Figure 5. Southern Analysis of *rad27*Δ and *orc5-1* and PCR Analysis of α Factor Arrested *pri2-1* Cells

Methods are as described above.

(A) *rad27*Δ cells are delayed for switching by 1 hr and are reduced by 50%, at both 23°C and 37°C.

(B) *orc5-1* cells are arrested in G2/M at 37°C, but are normal for *MAT* switching.

(C) PCR analysis of DNA from *pri2-1 MAT*a cells with and without α factor and wild-type *MAT*a cells at 23°C and 37°C. The assay requires at least 70 nucleotides of repair sythesis to form a PCR product. At 37°C, *pri2-1* cells either arrested in G1 with α factor or in S phase without α factor were equally deficient in repair synthesis, showing an approximate 10-fold decrease in PCR product formation. Data represent the average of two independent experiments, with the range indicated.

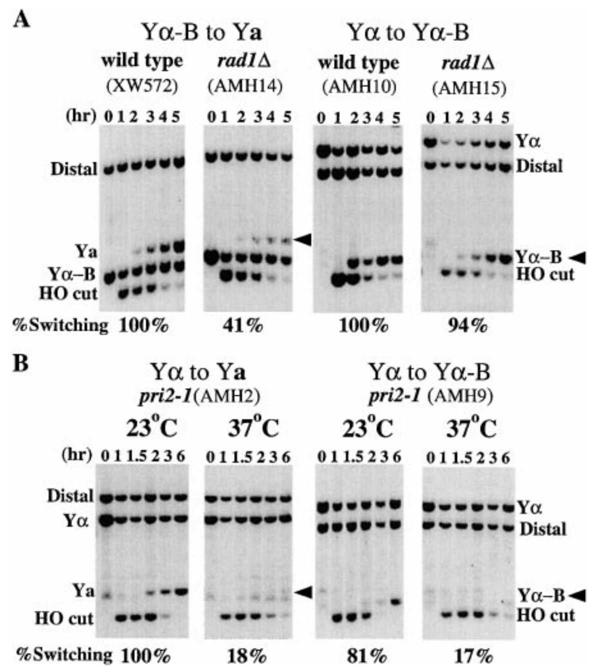

Figure 6. Southern Analysis of *rad1*Δ and *pri2-1*, with and without Perfect Homology

(A) Effect of *rad1*Δ in switching from Yα-B to Ya or from Yα to Yα-B (perfect homology) at 30°C. DNA cut with StyI and BamHI was analyzed as described above. When there is a nonhomologous tail at *MAT* (Yα-B to Ya), *rad1*Δ cells are severely delayed in switching, producing only 41% recombinant product at 5 hr. However, when perfect homology is restored (Yα to Yα-B), *MAT* switching is restored to almost wild-type levels.

(B) Analysis of *pri2-1* cells switching from Yα to Ya and Yα to Yα-B was performed at 23°C and 37°C. In contrast to the *rad1*Δ strain, where DSB repair returns to wild-type efficiency when perfect homology is restored, the *pri2-1* mutant is still reduced in both cases.

origin-dependent initiation of replication but not for DSB-induced repair at *MAT*.

Effect of Y Region Nonhomology on *MAT* Switching

DSB repair at the *MAT* locus is commonly referred to as a gap repair mechanism, since entirely new sequences are being synthesized between two regions of homology (Figure 1B). We asked if a substrate, which replaces the cut locus with perfect homology, would still require the components of the lagging strand machinery. We used a *MAT*α *hml*Δ strain carrying *HMR*α-B, in which there is a single bp mutation in Yα that creates a BamHI site about 100 bp from the HO cleavage site (Wu and Haber, 1995). In this situation, the donor is perfectly homologous to the ends of the DSB, as *MAT*α switches to *MAT*α-B. *MAT*α to *MAT*a switching is significantly delayed with a repair efficiency of only 37% ± 5% by 5 hr in a *rad1*Δ strain (Figure 6A). Rad1 is an excision repair protein that, along with Rad10 and Msh2/Msh3 mismatch repair proteins, removes nonhomologous sequences >30 nt at both ends of a DSB from the 3′ ends of the DSB (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Sugawara et al., 1997). This result indicates that the 700 bp region of nonhomology centromere-proximal to the HO cut site is normally cleaved by Rad1p. This delay disappears when perfect homology is restored in the *rad1*Δ strain switching from *MAT*α to *MAT*α-B (Figure 6A). However, we observe the same defect in the *pri2-1* mutant at the nonpermissive temperature, with or without perfect homology, and conclude that a recombination-dependent replication fork also occurs without a gap (Figure 6B).

Discussion

Lagging Strand Synthesis Is Involved in Gap Repair at *MAT*

Mutants of the Polα-primase complex are severely defective in DSB repair at *MAT*. The role of the Polα-primase complex in replication is clear: chromosomal DNA replication is completely blocked in thermosensitive mutants by preventing initiation of Okazaki fragment formation on the lagging strand and initiation on the leading strand (Plevani et al., 1985; Brooks and Dumas, 1989). We consider it unlikely that Polα and primase are required to initiate replication at the 3′ end that initially invades, as the end should serve as a primer. Instead we suggest that the requirement of Polα-primase is to

initiate Okazaki fragments on the lagging strand. This conclusion is supported by our finding that *rad27Δ* shows delayed and reduced switching even at its permissive temperature, where replication is quasi-normal.

We have ruled out an indirect effect of inactivating primase for *MAT* switching, since G1-arrested *pri2-1* cells were able to complete *MAT* switching at 23°C but were as deficient for repair in G1-arrested cells as were *pri2-1* cells arrested in S phase. Thus, inactivating *pri2-1* does not simply sequester the general replication apparatus at replication forks and away from the *MAT* locus.

A PCR analysis that detects the first new DNA synthesis after strand invasion suggests that the uncoupling of leading and lagging strand synthesis in *pol1-17* and *pri2-1* makes repair synthesis less efficient. In addition to priming replication, it is possible that Polα-primase also acts as part of a larger complex to unwind the duplex homologous donor strands. In vitro studies in *E. coli* and T7 phage demonstrate a direct physical link between the helicase-primase complex and Pol III polymerase, so the lagging strand mutants studied here may also uncouple coordinated repair synthesis (Kim et al., 1996; Lee et al., 1998). Our results also contrast previous studies that showed Polα was not necessary for repair of X-ray or UV-induced DNA damage, where repair may involve filling in of a gapped, single-stranded region (Budd et al., 1989; Budd and Campbell, 1995).

Role of PCNA-Associated DNA Polymerases in DSB Repair

Of the two PCNA-associated polymerases, Polϵ is certainly very important for DSB repair, as evidenced by the severe delay of *pol2-18* at 37°C. Residual repair may depend on Polδ. Although *pol3-14* appears to be as strongly inhibited for replication at 37°C as *pol2-18* (Giot et al., 1995), it does not have as severe an effect on switching. This could mean that *pol3-14* is more leaky for repair or that Polϵ is better able to compensate in the absence of Polδ than Polϵ can without *pol2-18*. We believe Polδ is also important for gene conversion, as previously suggested (Fabre et al., 1991). At 42°C, *MAT* switching in *pol3-14* is completely eliminated (data not shown). We favor the idea that Polϵ is unable to compensate for Polδ at this unusually high temperature. In any case, both Polϵ and Polδ play a role in DSB repair.

It is still not clear which polymerase is acting on the leading or lagging strand in origin-dependent replication or during *MAT* switching, since the inhibition we observe by Southern and PCR analyses could reflect a disruption of repair synthesis on the leading strand, the lagging strand, or both strands. In previous genetic studies, Polδ and ϵ appeared to act on opposite strands of the replication fork (Shcherbakova and Pavlov, 1996). Further genetic studies implicate Polδ as the lagging strand polymerase because mutations of Polα, Polδ, and Rad27p (but not Polϵ) increase certain instabilities, which are explained by the displacement of Okazaki fragments in these mutants (Gordenin et al., 1992; Kokoska et al., 1998). If Polδ acts as the lagging strand polymerase in *MAT* switching, finishing the elongation of the Okazaki fragment initiated from Polα (Waga and Stillman, 1994), then in the absence of Polδ, Polϵ most likely is able to replace it and elongate both strands. However, in the converse situation, loss of Polϵ catalytic function results in a dramatic delay in *MAT* switching, with only 39% recombinant product appearing after 3 hr.

PCNA and RFC: Processivity or Initiation Factors in DSB Repair?

Yeast PCNA interacts with Polδ and Polϵ to stimulate active replication complexes (Burgers, 1991). In *MAT* switching, the clamp and the clamp loader are essential for recombinant product formation, since at the nonpermissive temperature, no switching is observed, and there is a significant decrease in product at the permissive temperature for the clamp mutant.

PCNA has emerged as a major player in many aspects of DNA metabolism, including DNA replication, nucleotide excision repair, postreplication mismatch repair, base excision repair, and DNA replication (Jónsson and Hübscher, 1997). PCNA may form different protein–protein and/or protein–DNA interactions at an ARS from those at DSB sites; therefore, different mutant alleles of PCNA may affect these processes differently. In yeast, PCNA is needed to fill in a small 30 base pair ssDNA gap (Torres-Ramos et al., 1996). Therefore, loading of PCNA onto the DNA may be necessary for recruiting or signaling the proper DNA polymerase and/or repair proteins to the site of damage, and not necessarily for processive replication by Polϵ. Further support for this idea comes from our PCR analysis of the clamp and clamp loader. Unlike the lagging strand mutants, these mutants are unable to synthesize as little as 30 bp at the nonpermissive temperature, again suggesting that PCNA may be necessary to recruit recombination proteins and polymerase(s) to the site of repair.

Repair Replication Fork-Capture Model of Gene Conversion

We propose a novel model for gene conversion events during *MAT* switching, shown in Figure 7A. This model draws from many previous suggestions concerning the sequence of events envisioned in both the Szostak et al. (1983) model and in several versions of SDSA mechanisms (Nassif et al., 1994; Pâques et al., 1995, 1998; Ferguson and Holloman, 1996). This model accounts not only for our data concerning both leading and lagging strand DNA polymerases, but for a variety of genetic observations suggesting that both newly synthesized strands of DNA are inherited at the recipient *MAT* locus, while the donor remains unchanged (McGill et al., 1989; Ray et al., 1991; Haber et al., 1993; Strathern et al., 1995; Pâques et al., 1998). The key element of this mechanism is that invasion of one end of a DSB establishes a modified replication fork that can either proceed to the end of the chromosome, as in BIR (Figure $1A_3$), or be "captured" by annealing the second end of the DSB to the moving replication fork. We favor a model in which the replication bubble remains small, allowing both newly synthesized DNA strands to anneal and eventually end up in the recipient locus (Figure 7A). This unwinding and annealing step is presumably catalyzed by a branch migration complex that may resemble the RuvAB complex in *E. coli* that is especially important for origin-independent, recombination-dependent replication (Asai

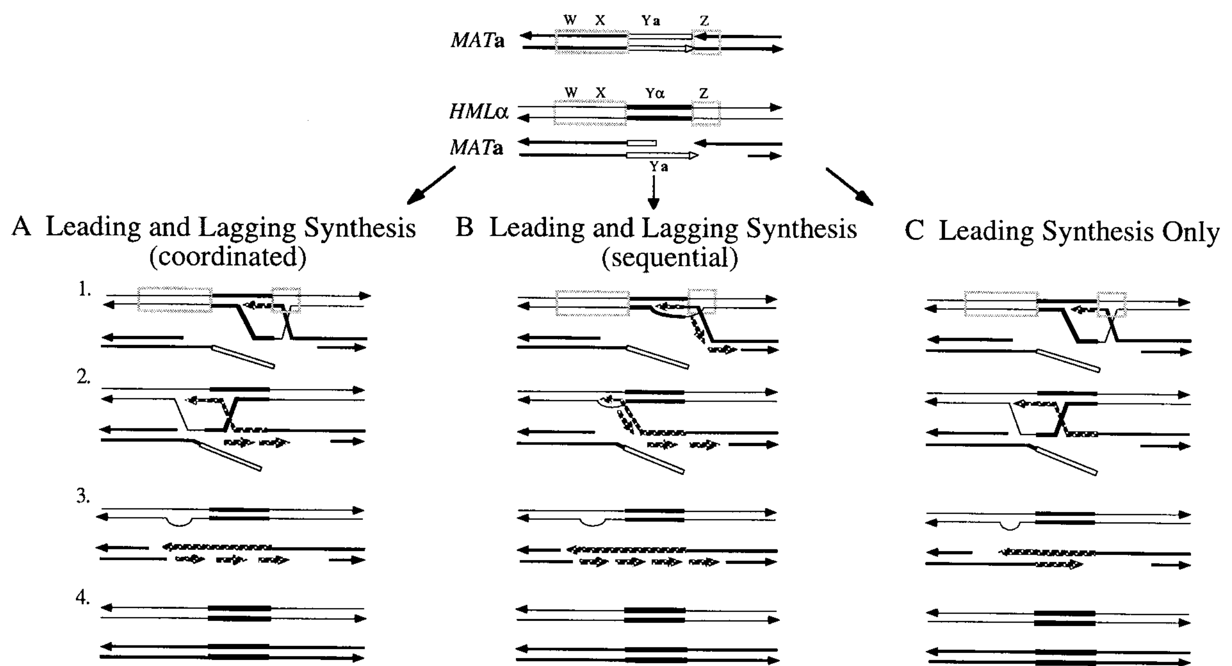

Figure 7. Alternative SDSA Models for *MAT* Switching

Three models showing initiation of DSB repair with 5′ to 3′ resection and strand invasion of the distal 3′ end. Each model represents a variation on SDSA mechanisms, where both newly replicated strands are found at the *MAT* locus, leaving the donor unchanged. In contrast to the Szostak et al. (1983) model, which proposes extensive unwinding of the donor locus to form a large D loop, these mechanisms envision the donor strands unwound as a small replication bubble (A and C) or as a migrating D loop (B).

(A) DSB repair by coordinated leading and lagging strand DNA synthesis. Consistent with data in this paper, leading strand synthesis would not occur efficiently without concomitant lagging strand synthesis. As the replication bubble progresses, presumably by the forward unwinding of the donor duplex by a helicase, branch migration might also occur, leaving the size of the progressing replication fork unchanged and bringing the newly synthesized leading and lagging strands together at *MAT*. Efficient resolution requires Rad1 and presumably also Msh2, Msh3, and Rad10 to clip off the nonhomologous Ya tail in step 3, or less efficiently by a Rad1-independent mechanism. Finally, filling in–synthesis and ligation is required.

(B) DSB repair by sequential leading and lagging strand DNA synthesis. This model differs from (A) in that lagging strand synthesis is not associated with replication fork progression, but instead occurs as the newly synthesized leading strand is displaced from the migrating replication bubble. Although our data cannot formally exclude this possibility, the effect of the lagging strand mutants suggests that efficient repair synthesis is coordinated, and previous genetic data concerning heteroduplex formation in the Z1 region indicate that both donor strands are used as templates (Ray et al., 1991).

(C) DSB repair by leading strand DNA synthesis only, similar to that proposed by Ferguson and Holloman (1996).

et al., 1993; Parsons et al., 1995). The Holliday junction produced after strand exchange and displacement of the donor strand could branch migrate, in a coordinated manner, behind a replicative helicase that is associated with Polα and primase and unwinds DNA. This step accounts for the inheritance of both new strands at *MAT*. It also explains the formation of heteroduplex DNA at the *stk* locus in the absence of mismatch repair (Ray et al., 1991), such that the second new strand of DNA in the Z region is not simply copied from the first newly synthesized strand (Figure 7B), but from the displaced donor strand. Leading and lagging strand polymerization distinguishes the model in Figure 7A from an SDSA mechanism involving only leading strand synthesis in Figure 7C, which is otherwise similar.

Although almost all of the components necessary for genomic replication are also necessary for DSB repair at *MAT*, we do not believe that replication at *MAT* is identical to origin-dependent replication. First, Polε can compensate for the loss of Polδ during DSB repair, yet both polymerases are essential for genomic replication. Second, repair synthesis at *MAT* can occur in the absence of the pre-RC and independent of S phase functions. Third, other studies of HO-induced gap repair found that as the length of the template for gap repair increases, the efficiency of repair decreases, suggesting that repair synthesis is not intrinsically as efficient or processive as normal replication (Pâques et al., 1998). One explanation for this reduced efficiency is that the replication fork associated with DNA repair may lack an efficient helicase that opens up the duplex for replication. In normal replication, the helicase is likely composed of the six proteins Mcm2-7p (Aparicio et al., 1997; Sherman et al., 1998) that have been suggested to play a role similar to DnaB in *E. coli* replication (Baker et al., 1986). Chromatin immunoprecipitation experiments by Aparicio et al. (1997) have demonstrated that these Mcm proteins load at origins and then move away with the replication complex. It is possible that these Mcm proteins cannot assemble at the repair fork and that the helicase function is carried out by another, less processive, set of proteins.

When the Y region is different between the donor and recipient, it is necessary to clip off a 3′-ended single-stranded tail before the captured replication fork can be resolved. This process normally requires Rad1 and presumably Rad10, Msh2, and Msh3 (Fishman-Lobell and Haber, 1992; Sugawara et al., 1997; Colaiácovo et al., 1999). Our genetic data, showing the requirement of the lagging strand polymerases in this process, suggests that the model in Figure 7C is the less efficient "default" pathway. As shown in Figure $7C_3$, the nonhomologous tail must be removed in order to provide an end before priming the second round of leading strand synthesis. When we both remove the endonuclease Rad1 and inactivate the lagging strand machinery, we see no residual *MAT* switching, presumably because both pathway A and C are now inactivated (data not shown). The fact that *MAT* switching is rarely accompanied by crossing-over (Klar et al., 1984) may either mean that a pair of Holliday junctions formed during the capture of the replication fork are almost always resolved without exchange, or it may imply that the newly synthesized leading strand dissociates and anneals to the second end, such that Holliday junctions are not formed, as shown in all three models of Figure 7_3. There may be sequences at *MAT* that specifically preclude crossing-over compared with other sequences induced to undergo an HO-induced gene conversion.

Experimental Procedures

Strains

Most strains were isogenic derivatives of R166 (*ho*Δ *MAT***a** *leu2-3 leu2-112 his4-519 ade1-100 ura3-52* carrying pJH132 (*GAL::HO*). The *pol1-17*, *pri2-1*, and *pol3-14* conditional alleles were introduced into the R166 by integration and excision of a YIp5 (*URA3*-containing) plasmid, producing AMH1 (*MAT*α *pol1-17*), AMH2 (*MAT*α *pri2-1*), and AMH3 (*MAT***a** *pol3-14*), respectively. Temperature-sensitive Ura^- colonies were sequenced to confirm the presence of the single base pair substitution. The YIp*pol1-17* integration plasmid, linearized with XhoI, was a gift from Judith Campbell; YIpA16 (*pri2-1*), was linearized with HpaI for integration (Francesconi et al., 1991); and pMJ14 (*pol3-14*), was linearized with KpnI (Giot et al., 1995). AMH4 (*MAT*α *pol30-52 ade3::GAL::HO*) was constructed using pBL245-52 as described (Ayyagari et al., 1995). Ura^- colonies were tested for cold sensitivity at 14°C. A galactose-inducible *GAL::HO* gene was integrated at *ADE3* using YIPade3HO constructed by Sandell and Zakian (1993). The YIPade3HO plasmid was also integrated into R166 to create AMH11 (*MAT*α *ade3::GAL::HO*).

The *cdc44-8* cold-sensitive allele was integrated into R166 to make AMH5 (*MAT***a** *cdc44-8*) by the one-step gene replacement method (Rothstein, 1983) using a 4.6 kb Kpn1-Sac1 *URA3*-containing fragment in the noncoding region of the *cdc44-8* gene from pCH1362, received from Connie Holm. The resulting Ura^+ colonies were examined for cold sensitivity at 14°C and confirmed by Southern analysis. AMH6 (*MAT***a** *rad27::URA3*) was also constructed by one-step gene replacement method into R166 using pMR*rad-26*Δ*::URA3* as described (Reagan et al., 1995). Strains AMH5 and AMH6 were transformed with the centromeric plasmid pJH727 (*GAL::HO LEU2*). Construction of AMH7 (*MAT***a** *rad1::LEU2*) was performed with one-step integration using the plasmid pL962 (*rad1*Δ) as described (Zehfus et al., 1990).

AMH9 (*MAT*α *pri2-1 HMR*α-B) was constructed by transforming AMH2 (*MAT*α *pri2-1*) by one-step replacement using an *HMR*α-B, *URA3*-containing HindIII fragment from pXW172 as described (Wu and Haber, 1995). *HMR*α-B contains a one base pair substitution mutation, creating a BamHI mutation in Yα. AMH10 was constructed by crossing a *MAT*α and an *HMR*α-B *MAT***a** strain to obtain segregants with the following genotype: *ho hml*Δ::*ADE1 MAT*α *HMR*α-B::*ura3 ade1 ade3::GAL::HO leu2 trp1 ura3-52*. AMH10 was transformed with the *rad1::LEU2*-containing plasmid as described for AMH7 to create AMH15 (*hml*Δ*::ADE1 MAT*α *HMR*α-B *rad1::LEU2*). Strain XW572 [derivative of DBY745: *ho hml*Δ*::ADE1 MAT*α-B *HMR***a** *ade1 leu2 trp1 ura3-52* pJH132 (*GAL::HO URA3*)] was transformed with the *rad1::LEU2* plasmid as described for AMH7 to create AMH14 (*hml*Δ*::ADE1 MAT*α-B *HMR***a** *rad1::LEU2* pJH132 (*GAL::HO::URA3*).

AMH12 (*pol2-18 ade3::GAL::HO*) was constructed by crossing YHA301 (Araki et al., 1992) with AMH11. AMH13 (*MAT***a** *orc5-1 ade3::GAL::HO*) was constructed by integrating the YIPade3HO into a derivative of JRY4249 (Jasper Rine), received from Stephen Bell (*MAT***a** *orc5-1 ade2-1 his3-11 leu2-3,112, trp1-1 ura3-1*).

Induction of *MAT* Switching

MAT switching was performed in the thermosensitive strains as described (White and Haber, 1990), with the following modification. For temperature-sensitive strains, cultures were pregrown at the permissive temperature, and at 1×10^7 cells/ml, the culture was split. One-half was maintained at the permissive temperature, while the other half was incubated at the nonpermissive temperature. After 3 to 4 hr, when >95% of cells were arrested at the nonpermissive temperature, 2.0% galactose was added for 1 hr to induce HO endonuclease, followed by 2.0% glucose to repress cutting by HO.

The effect of α factor G1 arrest in the *pri2-1* mutant strain was analyzed as described above, except the overnight cultures were grown at 23°C until 5×10^6 cells/ml and 2.5 μg/ml of pheremone was added in the morning. Pheremone (2.5 μg/ml) was added every 1.5 hr to maintain G1 arrest throughout the time course. After 4 hr, when the cells were completely arrested in G1, the culture was divided. One-half of the G1-arrested cells were maintained at 23°C, while the other half was shifted to 37°C. Cells were incubated another 3 hr to allow for inactivation of primase at the nonpermissive temperature, and then galactose induction and repression of HO was performed as described above.

DNA Analysis

Purified DNA was digested with StyI, separated on a 1.4% native gel, and probed with pCW6-1 (White and Haber, 1990). For experiments testing the effect of perfect homology in the Y region (strains XW572, AMH14, AMH10, AMH15, and AMH9), DNA was digested with StyI and BamHI to distinguish Yα from Yα-B. The Southern blots were scanned by PhosphorImager, and the repair efficiency was calculated as follows. First, the percentage of the HO cut fragment, relative to all fragments produced within the same lane, was determined after 1 hr of galactose induction. Second, the ratio of the switched product fragment, relative to total counts in the lane at various times after the 1 hr time point, was determined. Finally, the percent of switched product was divided by the percent of HO cut fragment to give the repair efficiency. Repair efficiency was then determined as the average of at least three independent time course experiments and Southern blot analyses performed for each mutant strain.

PCR Analysis

The PCR conditions used were 20, 23, or 26 cycles at 94°C for 30 s, 50°C for 1.5 min, and 72°C for 1 min, followed by a final extension step of 72°C for 7 min (PTC-100TM Programmable Thermal Controller, from MJ Research Inc). The primers for all PCR analyses were synthesized by GIBCO-BRL custom primers; sequences are available upon request. Analysis of the extension intermediates produced from a cell switching from *MAT***a** to *MAT*α was performed with oligonucleotide sequences within the Yα region, either 30 nucleotides (Yα-30) or 70 nucleotides away from the HO cut site (Yα-70), along with a *MAT* distal primer, pB or pB′. Likewise, primers within the Ya region were used for PCR analysis of cells switching from *MAT*α to *MAT***a** and were either 30 (Ya-30) or 70 (Ya-70) nucleotides away from the HO cut site. The amount of PCR product was quantified by Molecular Analyst directly from an agarose gel and was normalized by dividing by the intensity of genomic DNA used for each PCR reaction. At least two PCR reactions were performed for each mutant strain from DNA prepared from two independent time course analyses.

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