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Conservative Inheritance of Newly Synthesized DNA in Double-Strand Break-Induced Gene Conversion▽

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To distinguish among possible mechanisms of repair of a double-strand break (DSB) by gene conversion in budding yeast, *Saccharomyces cerevisiae*, we employed isotope density transfer to analyze budding yeast mating type (*MAT*) gene switching in G_2/M-arrested cells. Both of the newly synthesized DNA strands created during gene conversion are found at the repaired locus, leaving the donor unchanged. These results support suggestions that mitotic DSBs are primarily repaired by a synthesis-dependent strand-annealing mechanism. We also show that the proportion of crossing-over associated with DSB-induced ectopic recombination is not affected by the presence of nonhomologous sequences at one or both ends of the DSB or the presence of additional sequences that must be copied from the donor.

Mitotic double-strand breaks (DSBs) are efficiently repaired by homologous recombination. Repair can occur by several mechanisms, including gene conversion, break-induced replication, or single-strand annealing (reviewed in references 18 and 26). In wild-type cells, where the DSB is flanked by sequences that are homologous to intact sequences located on a sister chromatid, a homologous chromosome, or an ectopic donor, repair occurs mostly by gene conversion. Several models have been advanced to explain how gene conversion occurs (Fig. 1). In the seminal DSB repair model of Szostak at al. (35) (Fig. 1A), the ends of a DSB are resected by a 5′-to-3′ exonuclease, producing 3′-ended single-stranded DNA (ssDNA). Recombination proteins from the Rad52 epistasis group assist the ssDNA ends to search for an intact homologous sequence where the ssDNA end can invade and prime new DNA synthesis (reviewed in reference 18). The invasion of both ends produces a symmetric structure linked together by two Holliday junctions (HJs), whose cleavage by a Holliday junction resolvase can yield gene conversions either with an associated crossing-over or without crossover. Hereafter we will refer to this model as the double Holliday junction, or dHJ, model (Fig. 1A). Support for this mechanism has come predominantly from studies of meiotic cells, where key molecular steps of this process have been identified by the physical monitoring of DNA undergoing recombination (12, 32).

In the past few years it has become evident that there are additional mechanisms of gene conversion besides that described by Szostak et al. (35). First, the dHJ model postulates the formation of an equal number of crossover and noncrossover outcomes, but very low proportions of mitotic recombination events show crossing-over in budding yeast, *Saccharomyces cerevisiae*, including mating type (*MAT*) switching (<1%) (17), interchromosomal recombination (generally about 5%) (6, 14, 20, 24, 36), and Rad51-dependent intraplasmid recombination (8%) (13). A similar constraint appears in interhomolog and intersister mitotic recombination in mammalian cells (<3%) (34). Second, heteroduplex-containing DNA involved in mitotic recombination between slightly divergent sequences is found only at the recipient, but not also at the donor locus as would be predicted by the dHJ model (22, 29, 37). Finally, genetic and physical analyses of both mitotic and meiotic recombination intermediates suggest that crossover and noncrossover outcomes may result from different recombination intermediates (2, 14).

Gene conversions where there is little or no accompanying crossing-over have been explained by several versions of synthesis-dependent strand-annealing (SDSA) mechanisms (5, 7, 24, 26, 30, 34) (Fig. 1B). Here, resection of DSB ends occurs as in the dHJ mechanism, but after strand invasion and the initiation of new DNA synthesis, the newly synthesized DNA is displaced from the template. Alternatively, intermediates similar to those of the dHJ model are transiently formed but are unwound by helicases/topoisomerases (10, 22, 24) (Fig. 1C). Support for this idea comes from the demonstration in budding yeast that the Sgs1 helicase and its associated Top3 topoisomerase suppress crossing-over (14) and that human homologs of Sgs1-Top3 (Blm/Topo3α) resolve a covalently closed synthetic dHJ into noncrossovers (38). Two basic features of SDSA are that recombination is generally not associated with crossing-over and that the donor locus remains unchanged while the recipient locus receives all newly synthesized DNA. In yeast and in *Drosophila melanogaster*, evidence supporting SDSA has come from so-called tripartite recombination experiments where sequences homologous to each double-strand break end are located on different chromosomes (27, 33). The completion of these events requires the invasion and unwinding of newly synthesized DNA from both template sequences and the annealing of these newly copied strands. In the dHJ model, where there is no strand displacement, repair would be impossible if the two ends invaded homologous template sequences on different molecules. Further support for the SDSA

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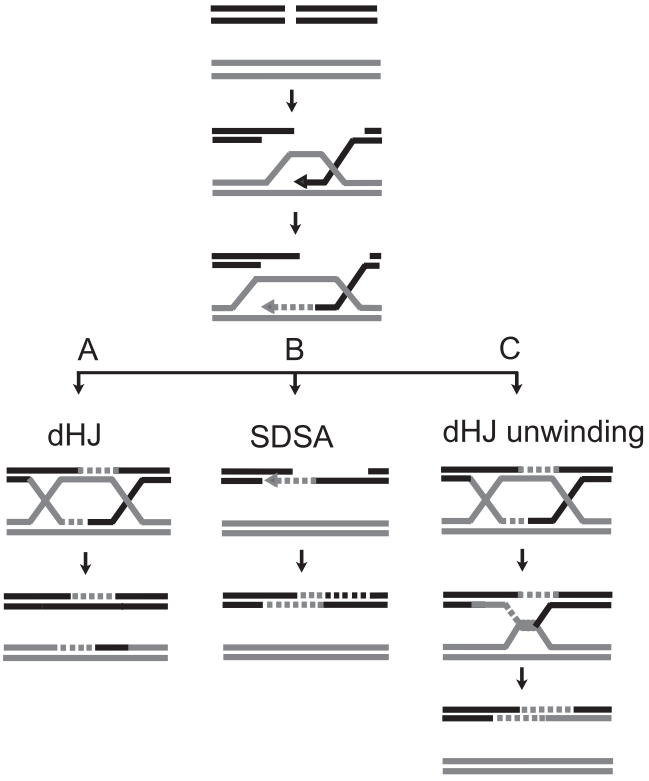

FIG. 1. Models of double-strand break repair. Newly synthesized DNA is represented by a dashed line. (A) dHJ model. Both 3′ ends invade the template and form two HJ structures that are cleaved by a resolvase. The resolution leaves newly synthesized DNA in both the recipient locus and the donor locus. (B) SDSA model. The invading strand is displaced from the template sequence after new DNA synthesis has been initiated and anneals to a second, resected end of the DSB. The second strand is then synthesized, using the first, newly copied strand as the template. Both newly synthesized strands are therefore located at the recipient locus. (C) dHJ unwinding model. The dHJ is unwound by concerted helicase/topoisomerase activity. The two newly synthesized strands anneal to each other and end up in the recipient locus.

model comes from gene conversion experiments in which the donor sequence contains a series of repeated sequences; changes in the number of repeats are seen frequently in the recipient locus and rarely in the donor (1, 25, 27).

Looking for a definitive way to determine the predominant mechanism of DSB repair in mitotic cells, we examined the fate of newly synthesized DNA during gene conversion using the isotope density transfer method initially used by Meselson and Stahl (23) to demonstrate semiconservative DNA replication. We examined gene conversion in *Saccharomyces cerevisiae* during *MAT* gene switching, a well-characterized intrachromosomal mitotic gene conversion event (reviewed in reference 8). *MAT* switching is an intrachromosomal ectopic gene conversion event induced by the site-specific HO endonuclease, in which normally about 700 bp of either Y**a** or Yα sequences at *MAT* are replaced by a similar-size copy of the opposite mating type copied from either the *HML*α or *HMR***a** donor loci (Fig. 2). Previous studies have shown that the genetic requirements for *MAT* switching are similar to those for other ectopic and allelic recombination events (26); however, *MAT* switching differs from some DSB gene conversion events in that one DSB end has the Y region that is not homologous to the donor and must eventually be removed to complete gene conversion using the more-distant, homologous X region. Recombination is initiated by strand invasion of the 3′-ended ssDNA in the Z region, where the donor and the recipient are perfectly matched. The 3′ end of the invading strand primes new DNA synthesis that copies the donor Y region and continues into the X region. The joining of the X region at *MAT* to the newly copied DNA occurs only about 30 min after strand invasion (37). Using isotope density methods, we show here that both of the newly synthesized strands created during gene conversion are inherited at the repaired recipient molecule, further supporting SDSA models of recombination.

MATERIALS AND METHODS

Strain tGI268 was derived from strain RM14-3a provided by M. K. Raghuraman (University of Washington) and has the genotype *MAT***a** *hml*Δ::*NAT HMR*α::λ *bar1*::*ADE3 cdc7-1 his6 leu2 trp1 ura3* carrying the centromeric YCp50 plasmid with *GAL1*::*HO* marked by *LEU2* (pJH727). Plasmid pGI6 contains a modified *HMR*α locus marked by *URA3* (plasmid pXW172 [39]) and by the insertion of a 2,918-bp phage λ fragment at a BamHI site introduced in Yα 96 bp 5′ from the HO cut site (Fig. 2A). A linear HindIII fragment was used to replace *HMR***a**. Cells were grown in ^{13}C- and ^{15}N-containing medium (heavy medium; 0.1% potassium acetate and 0.01% ammonium sulfate) for over seven generations, and then growth was arrested with nocodazole (15 μg/ml) in the G_2/M phase of the cell cycle, so that there was no ongoing DNA replication. Cells whose growth was nocodazole arrested were washed and resuspended in light medium (containing ^{12}C and ^{14}N) plus nocodazole. Thirty minutes after the isotope shift, 2% galactose was added to induce the DSB at *MAT***a**. To repress HO expression, 2% glucose was added after 1 h, and the cells were harvested 6 h after *HO* induction. Purified DNA was digested with BamHI and StyI restriction enzymes and fractionated by CsCl equilibrium density sedimentation as described previously (21). The refractive index of each sample was measured, and then DNA was separated by agarose gel electrophoresis. Hybridization with a phage λ-specific probe showed sedimentation of both the donor and recipient DNA fragments (Fig. 3).

Three ectopic recombination substrates were created by a modification of our previously described assay (14) in which an HO-induced break at *MAT* on chromosome III is repaired by recombination with *MAT* sequences inserted at the *arg5,6* locus on chromosome V. All three *ade3*::*GAL*::*HO* strains contain *MAT***a** and have *HML* and *HMR* deleted. The chromosome V donor contains either *MAT***a**-*inc*, differing from *MAT***a** by a single base pair that prevents HO cleavage (pGI354); MATα-*inc* (pGI439); or 117 bp of the BglII-HincII region covering the HO cleavage site in *MAT***a** into which a 708-bp bacterial *hisG* segment was introduced (pGI438).

RESULTS AND DISCUSSION

MAT gene switching in budding yeast can be induced synchronously in a population of cells by induction of a galactose-regulated HO endonuclease gene. For strain tGI268, when an HO-induced DSB is created in *MAT***a**, the Y**a** sequence is replaced by the 3.6-kb Yα-λ sequence copied from *HMR*α-λ. Thus, this gene conversion event requires the synthesis of >3.6 kb of new DNA. The insertion of these additional sequences was needed in order to generate appropriately sized restriction fragments whose density would be well resolved by equilibrium density gradient centrifugation. The insertion of phage λ DNA did not affect the silencing of the *HMR* locus, as HO endonuclease did not cut its cleavage site, which is occluded by positioned nucleosomes in this locus (28; data not shown). To assess the nature of repair DNA synthesis, it was necessary to carry out the isotope incorporation under conditions in which normal DNA replication was not occurring. We studied HO-

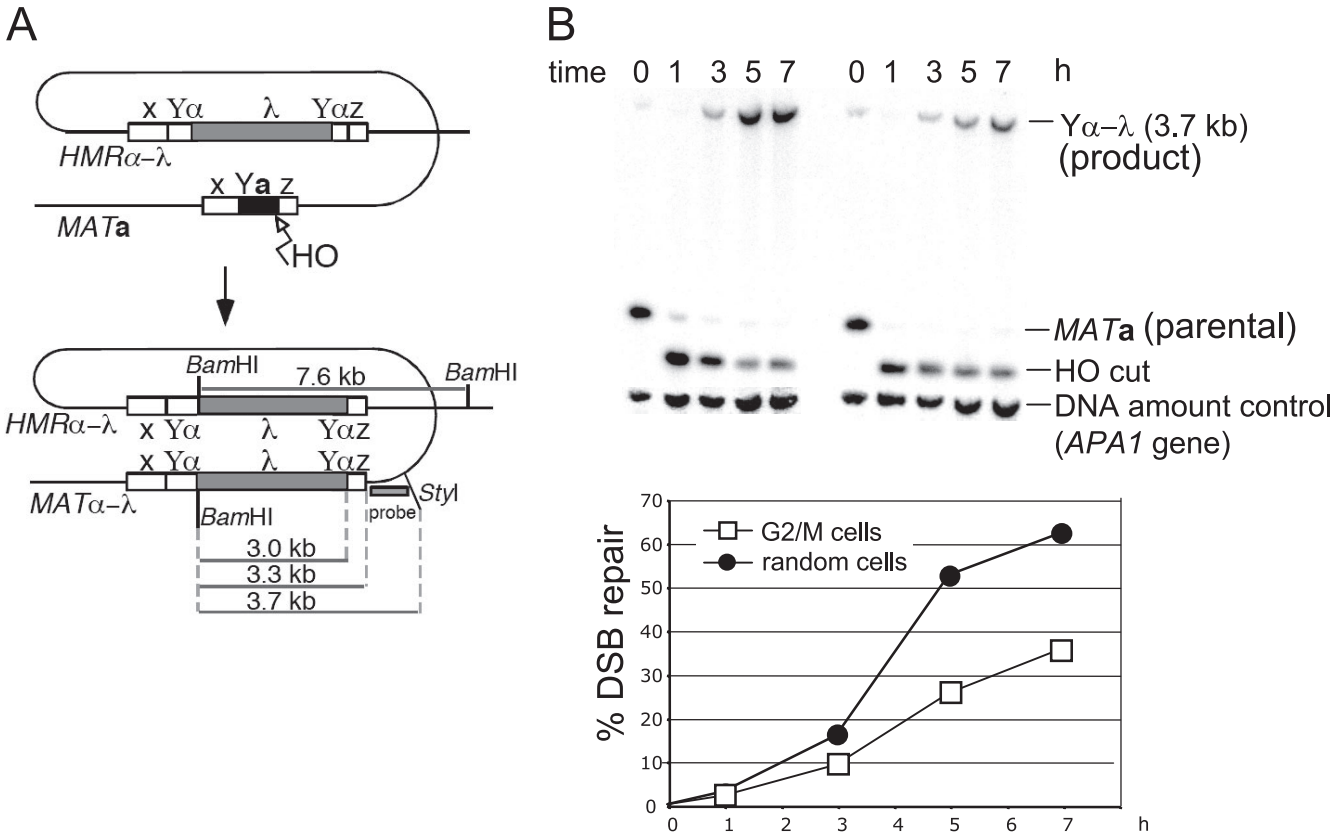

FIG. 2. Recombination assay used to study the fate of newly synthesized DNA. (A) A 2,918-bp phage λ fragment was inserted in the Yα region of *HMR*. When HO endonuclease creates a DSB at *MAT***a**, the Yα-λ sequence (3.0 kb) is copied from the *HMR*α-λ donor locus, creating a novel BamHI-StyI fragment. A fate of newly synthesized DNA in this BamHI fragment (3.7 kb) and in the BamHI fragment (7.6 kb) from the donor locus was tested. A *MAT*-distal probe is shown as a shaded rectangle. (B) Kinetics of DSB repair in randomly growing cells and in cells arrested in G_2 with nocodazole. Two percent galactose was added to cells to induce expression of HO endonuclease at 0 h; expression was repressed by the addition of 2% glucose at 1 h.

induced recombination in G_2-arrested cells, after replication was complete, rather than in G_1, prior to DNA replication, because Cdk1 kinase, which is required for completing HO-induced recombination, is inactive in G_1 (Fig. 2B) (15). Cells were grown in medium containing heavy isotopes of carbon and nitrogen (^{13}C and ^{15}N); after growth was arrested in G_2/M with nocodazole, the cells were shifted to medium containing normal, light isotopes. Thirty minutes after the shift in the medium, the HO endonuclease gene was induced by adding galactose to a final concentration of 2%, to induce a DSB at *MAT***a**. Most of the cells (>90%) remained blocked in G_2 during the 6-h duration of the experiment; therefore, incorporation of new nucleotides synthesized from light isotopes was limited to DNA synthesis during the gene conversion event. Southern blot analysis showed that the replacement of Y**a** by Yα-λ DNA sequences at *MAT* was completed within 6 h (Fig. 2B). Repair efficiency is only about 50% of that observed for HO-induced cycling cells.

By using CsCl equilibrium density centrifugation, we determined whether newly incorporated light-isotope-labeled (LL) DNA was incorporated at *MAT* and/or at *HMR*α::λ. The results of Southern blot analyses of fractions taken from the CsCl gradient are shown in Fig. 3. The position of LL DNA was determined from *MAT*α::λ cells grown in normal ^{12}C ^{14}N medium, and the position of semiconservatively replicated DNA from the same cells but grown first in heavy (^{13}C ^{15}N) medium and then allowed to go through one round of DNA synthesis in light (^{12}C ^{14}N) medium (HL DNA) was determined. It appears that all the newly synthesized DNA is located in the recipient, because the *MAT*α::λ cell DNA sediments at nearly the same position as LL DNA isolated from cells grown in normal medium (Fig. 3A). The small shift of the newly created *MAT*α-λ fragment toward the HL peak probably results from the fact that the 3.7-kb BamHI-StyI fragment that we tested is 0.7 kb longer than the newly synthesized 3.0-kb Yα-λ sequence (Fig. 2). The *MAT* Z region and more-distal sequences are partially resected and later will be filled in by light (^{12}C ^{14}N) nucleotides, but the strand ending 3′ at the HO cut site is not replaced (37); thus, the BamHI-StyI fragment would be expected to be about 17% heavier than a fully LL segment.

In contrast, after completion of *MAT* switching, the *HMR*α-λ fragment remained fully heavy isotope labeled (HH) and sedimented in the same fractions as it did before repair, implying that no newly synthesized DNA was inherited by the donor locus (Fig. 3B). The donor fragment analyzed is 7.6 kb long, and only about 3.3 kb participates in recombination (Yα-λ plus Z), so that only about half of the region was likely to have been replicated during repair (Fig. 2). Therefore, about 50% of one

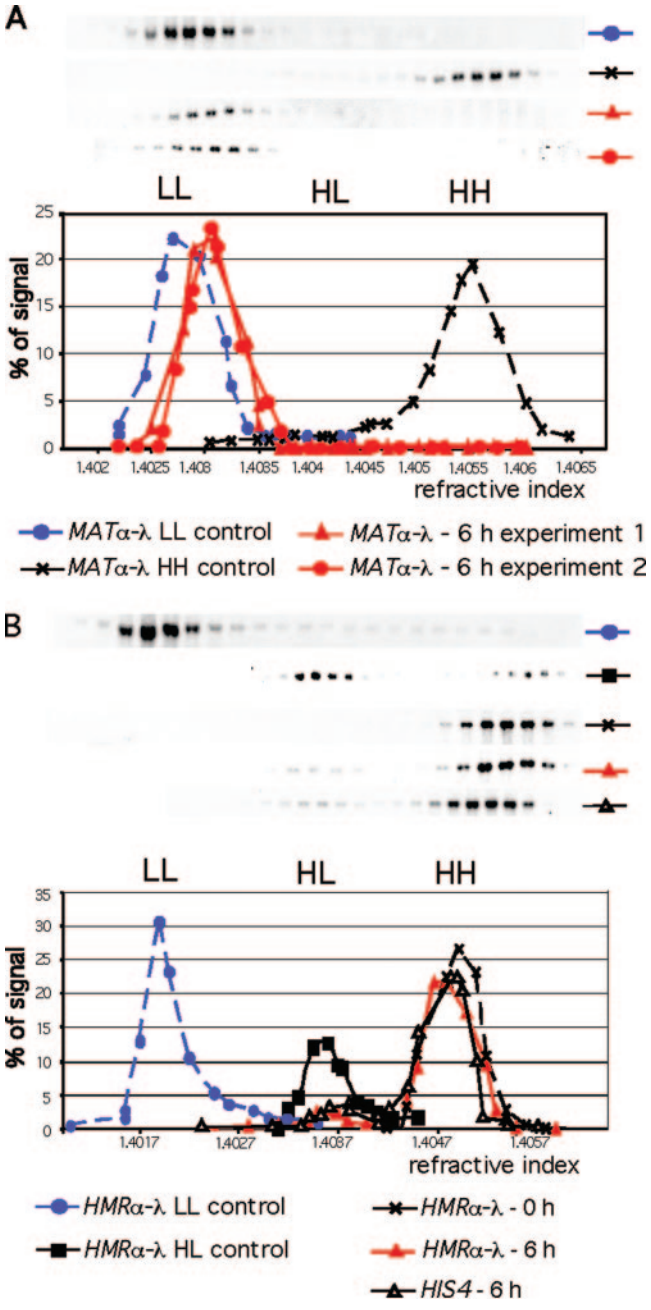

FIG. 3. Density distributions of recipient and donor fragments before and after *MAT* switching. Cells grown in ^{15}N- and ^{13}C-containing medium and whose growth was arrested with nocodazole were induced to switch *MAT***a** to *MAT*α-λ. (A) Fragments with the switched recipient locus (*MAT*α-λ) sediment in CsCl equilibrium centrifugation similarly to fragments with the same locus from cells grown in light-isotope-containing medium. The results of two independent experiments are shown. (B) Fragments with the *HMR*α-λ donor locus sediment before and after repair in the same fractions as DNA from yeast grown in heavy-isotope-containing medium. An HL control was obtained by releasing HH cells from G_2/M arrest into light medium for one replication cycle.

strand of the donor molecule would be light if *MAT* switching occurred by the dHJ mechanism. Consequently, if the dHJ mechanism were used, about 25% of the total DNA would be LL, and we should observe a peak halfway between HL and HH for the donor. Because we do not observe this shift, we conclude that the molecular weight of the donor did not change during recombination. We note, however, that nocodazole arrest of growth is not perfect, and a certain percentage of cells escape the arrest; consequently 5% of *HMR***a**-λ fragments sedimented as HL (lighter than we would expect from the dHJ model of recombination). To make sure that this was due to the escape from nocodazole arrest and is not relevant to the repair, we tested the sedimentation of DNA fragments with two other loci on the same chromosome (*THR4* and *HIS4*) by hybridizing the same membrane (sampled 6 h after DSB induction) with *THR4*- and *HIS4*-specific probes. In both cases, we observed a very small fraction of HL DNA due to escape from nocodazole arrest, similar to the results with the *HMR*α-λ fragment (Fig. 3B).

Our results show clearly that both DNA strands that are newly synthesized during DSB repair are recovered in the recipient locus. This finding is not compatible with a dHJ mechanism involving an HJ resolvase in which almost all of the resolved recombinants are recovered as noncrossovers. Such a mechanism would produce HL restriction fragments for both the donor and the recipient, contrary to what was observed. These data provide strong evidence that either SDSA or a dHJ unwinding model is the predominant repair process in mitotic cells. Based on results from previous studies, we suggest that both pathways are used but that the predominant one is SDSA. In particular, SDSA best explains both the frequent expansion/contraction of repeated sequences copied into the recipient during gene conversion (27, 31) and the tripartite recombination events, where sequences homologous to each double-strand-break end are located on different chromosomes.

We note that, in order to use density transfer methods, we were obliged to study DSB repair that includes the copying of a larger nonhomologous donor sequence than the usual 700-bp segment, so that the new DNA was incorporated into a 3-kb fragment. Moreover, *MAT* switching is inherently asymmetric, in that one side of the DSB has nonhomologous sequences that prevent the immediate engagement of an end that can prime new DNA synthesis. It is possible that when both ends are equally capable of strand invasion and initiation of new DNA synthesis and when there is no large heterologous region to be copied, a higher proportion of cells will use the dHJ mechanism. To address whether the exceptional features of *MAT* switching create a special case in which SDSA predominates, we tested the ratios of crossovers and noncrossovers in three different ectopic recombination assays between chromosomes III and V, in which we altered the donor and recipient (Fig. 4). The first assay showed that both DSB ends are almost perfectly homologous (there is a single-base-pair difference that prevents the *MAT*α-*inc* donor from being cleaved by HO). The equivalence of the two ends might promote dHJ formation and an increased level of crossovers (Fig. 4A). The second assay showed that the recipient has one nonhomologous end (the 0.65-kb Y**a** sequence) and the donor has a heterologous segment (the 0.7-kb Yα sequence) that needs to be copied to the recipient; this scenario perfectly imitates *MAT* switching, except that the donor is on a different chromosome (Fig. 4B). The third assay showed that the recipient has two nonhomologous tails, 75 and 46 bp long, and a 708-bp *hisG* insertion that needs to be copied during repair (Fig. 4C). If nonhomologous

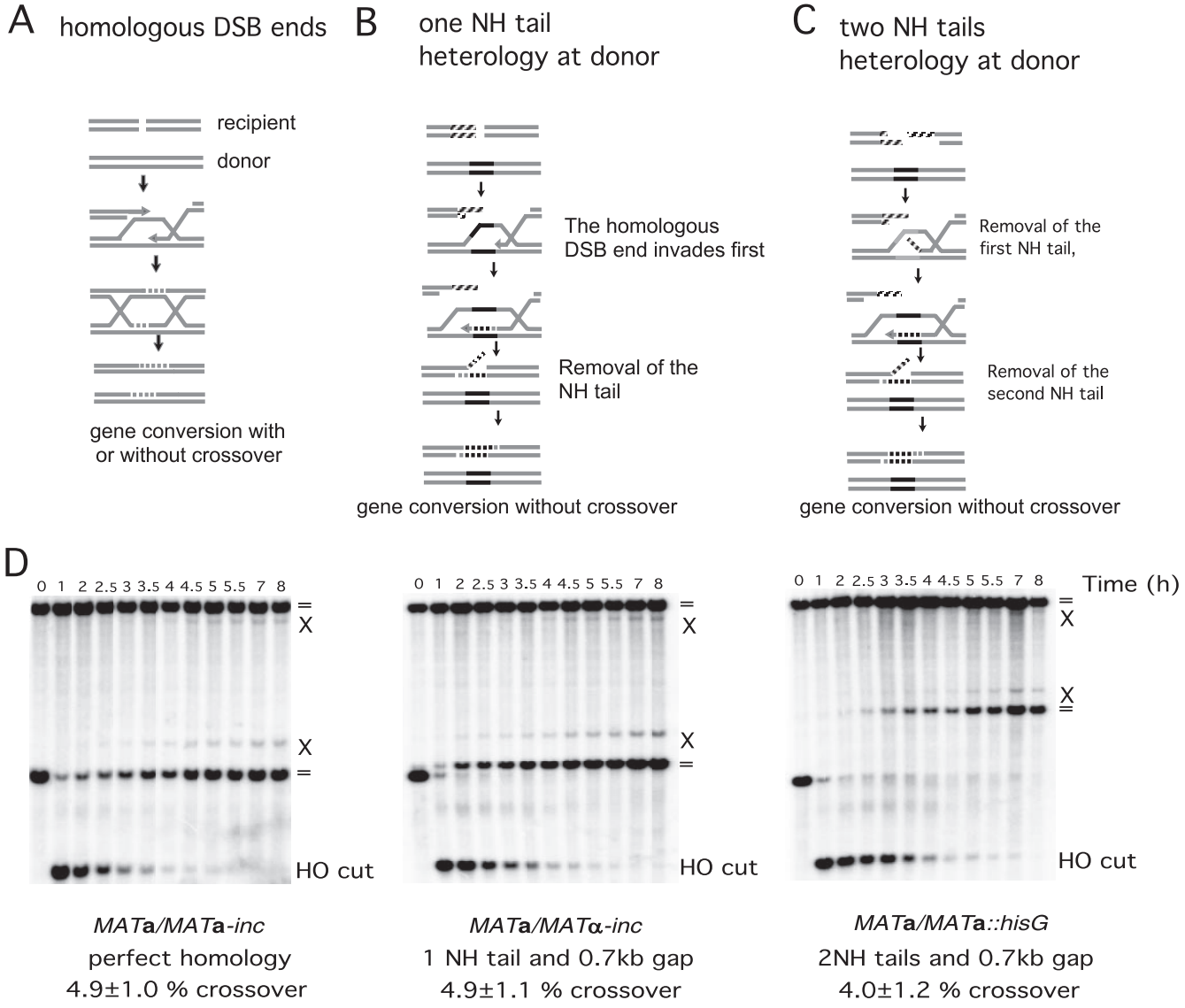

FIG. 4. Terminal nonhomology at the DSB ends and the presence of an insertion in the donor sequence have no impact on crossover and noncrossover pathway choice. Three different recombination scenarios were studied to check if terminal nonhomology (NH) or heterology of the donor sequence may affect the usage of crossover and noncrossover pathways. (A) Perfectly homologous DSB ends are equally likely to engage in recombination and might facilitate dHJ formation and produce a high level of crossover products. (B) With a 0.65-kb NH tail present at one end, the perfectly homologous end is more likely to invade and prime new DNA synthesis while the other end is being resected. This delay on one end could promote SDSA. In addition, 0.7 kb of new DNA synthesis is required. (C) Coordination of the invasion of two ends may be even more disturbed when short NH tails must be removed from each end of the DSB; in addition, there is a 1-kb insertion in the donor. (D) The break was repaired by recombination with the homologous *MAT* sequence on chromosome III. The results of Southern blot analyses of DSB repair kinetics are shown. ×, crossover products; =, noncrossover products. In strain pGI354 (left panel), the *MAT***a**-*inc* donor sequence is almost perfectly homologous to *MAT***a**. (This panel was previously published by Ira et al. and is reprinted from reference 14 with permission of the publisher.) In strain pGI439 (center panel), the donor sequence is *MAT*α-*inc*, thus (like normal *MAT* switching) requiring removal of the Y**a** NH tail and copying of Yα. In strain pGI438 (right panel), the donor sequence contains a 117-bp deletion surrounding the HO cleavage site and a 708-bp insertion of bacterial *hisG* sequences. Thus, two NH tails of 75 and 46 bp need to be clipped off the DSB ends before new DNA synthesis can be initiated.

segments at the ends or a large heterologous segment at the donor locus would have any impact on the engagement of the two DSB ends and the formation of a dHJ crossover intermediate, then we would expect to see significant differences in crossover frequency in these three cases. The results of Southern blot analyses following DSB repair after HO induction are shown in Fig. 4D. There is a delay in recombination when the two ends are nonhomologous, as we have seen before (4). However, in all cases the exchange frequencies were the same, indicating that the selection of a noncrossover SDSA pathway or a crossover-generating dHJ pathway is not affected by nonhomologous tails or heterology in the donor. These new results are in agreement with our previous studies of HO-induced recombination when both ends are homologous to those of the donor (14, 19). Thus, it seems that *MAT* switching is a representative function for studying DNA synthesis during DSB

repair. Nevertheless, we acknowledge that it is still possible that recombination between fully homologous sequences, such as between two sister chromatids, can occur more often by the dHJ mechanism than by SDSA.

The low level of crossing-over in mitotic cells suggests that dHJs are formed only infrequently, and probably about half of them are removed by Sgs1-Top3 activity (14). In interchromosomal ectopic HO-induced recombination, only about 4% of cells repair the DSB with an accompanying crossover (14). In *MAT* switching, the proportion of crossovers that generate deletions between *MAT* and *HMR* is also small (9, 17). Such a small proportion would not have been detected in these experiments. Crossing-over might reflect the operation of the dHJ mechanism, or it might indicate that sometimes SDSA mechanisms can "trap" HJs, as has been suggested for some variations of the SDSA mechanism (2, 7, 26). Two enzymes that promote the noncrossover SDSA pathway are the Srs2 and Mph1 DNA helicases (14; our unpublished data). We are currently investigating the roles of these two helicases in promoting noncrossover recombination pathways.

Previously, Arcangioli (3) used density transfer to study *mat* gene switching in *Schizosaccharomyces pombe*. Approximately 25% of fission yeast cells switch mating types in any generation, and correlating with this proportion, about 25% of the newly synthesized *mat* DNA was LL, suggesting that an SDSA mechanism was involved. This gene conversion event is fundamentally different from that examined here, in that there is no HO-like nuclease to make the DSB; rather, a DSB is generated during the S phase itself from a preexisting nick, and the repair process is dependent on cells being in S phase (11, 16). In the case we studied here, repair is initiated outside S phase and both strands of the Y region of *MAT* must be copied de novo.

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