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# Dynamics of Homology Searching During Gene Conversion in *Saccharomyces cerevisiae* Revealed by Donor Competition

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**ABSTRACT** One of the least understood aspects of homologous recombination is the process by which the ends of a double-strand break (DSB) search the entire genome for homologous templates that can be used to repair the break. We took advantage of the natural competition between the alternative donors *HML* and *HMR* employed during *HO* endonuclease-induced switching of the budding yeast *MAT* locus. The strong mating-type-dependent bias in the choice of the donors is enforced by the recombination enhancer (RE), which lies 17 kb proximal to *HML*. We investigated factors that improve the use of the disfavored donor. We show that the normal heterochromatic state of the donors does not impair donor usage, as donor choice is not affected by removing this epigenetic silencing. In contrast, increasing the length of homology shared by the disfavored donor increases its use. This result shows that donor choice is not irrevocable and implies that there are several encounters between the DSB ends and even the favored donor before recombination is accomplished. The increase by adding more homology is not linear; these results can be explained by a thermodynamic model that determines the energy cost of using one donor over the other. An important inference from this analysis is that when *HML* is favored as the donor, RE causes a reduction in its effective genomic distance from *MAT* from 200 kb to ~20 kb, which we hypothesize occurs after the DSB is created, by epigenetic chromatin modifications around *MAT*.

ONE of the least-understood aspects of homologous recombination is how a *Rad51*-coated nucleoprotein filament of single-stranded DNA can search the entire genome for homologous sequences with which to effect repair of a double-strand break (DSB). A well-studied model system of DSB repair is mating-type gene (*MAT*) switching in budding yeast (Figure 1A) (reviewed by Haber 1998; Haber 2007). This homothallic process allows haploid cells to grow into isogenic diploid colonies. Switching is initiated by the site-specific *HO* endonuclease, which creates a single DSB within the *MAT* locus adjacent to *Ya* or *Y $\alpha$*  sequences that encode cell-type-specific regulators. The *HO*-cut *MAT* se-

quences engage in homologous recombination (a gene conversion event without crossing over) in which either the *HML* or *HMR* sequences, located 200 and 100 kb from *MAT*, respectively, are used as templates to repair the DSB (Figure 1A). Although *HML* and *HMR* harbor equivalent *HO* cleavage sites to that at *MAT*, they are not cleaved, because of the heterochromatin structure of these sites. In most strains *HML* carries *Y $\alpha$*  and *HMR* carries *Ya*. Following *HO* cleavage, the ends of the DSB are resected by 5'–3' exonucleases to produce long, 3'-end single-stranded DNA (ssDNA), which is coated first by the ssDNA-binding protein complex RPA and then by a filament of the *Rad51* recombinase (Figure 1B). Collisions between the nucleoprotein filament and other DNA sequences results eventually in the recognition of homologous sequences, which can be opened up within the filament to allow the ssDNA to base pair with its complementary strand, creating a D-loop strand-invasion intermediate. These steps have been well studied *in vitro* (Sung *et al.* 2003; Krogh and Symington 2004). *In vivo*, chromatin immunoprecipitation techniques can be used to demonstrate the association of *Rad51* first with the ssDNA tail at *MAT* and later with donor

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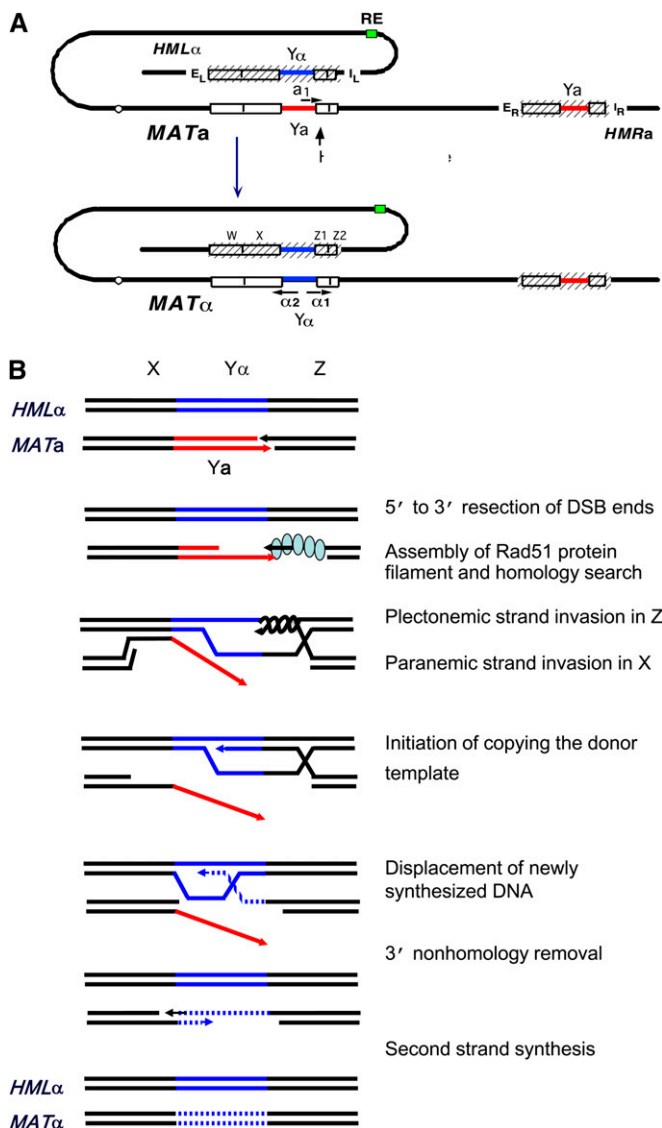
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**Figure 1** *MAT* switching in *Saccharomyces cerevisiae*. (A) Chromosomal arrangement of *MAT* and its donors on chromosome III. An HO endonuclease cleavage at the boundary between *Y $\alpha$*  and *Z1* sequences at *MAT* initiates replacement of the ~650-bp *Y $\alpha$*  sequences by ~700 bp *Y $\alpha$*  sequences copied from the heterochromatic *HML $\alpha$*  locus. HO cleavage sites in *HML* or *HMR* are not cleaved because these regions are untranscribed and heterochromatic (indicated by hatching); silencing depends on *cis*-acting *E* and *I* sites adjacent to these loci. Regions of homology *W*, *X*, *Z1*, and *Z2* shared by *HML* and *MAT* are indicated, along with the *X* and *Z1* regions shared by *MAT* and *HMR*. In *MAT $\alpha$*  cells, the recombination enhancer (RE) (shown as a green square) increases to 90% the choice of *HML*; when RE is deleted or in *MAT $\alpha$*  cells, where RE is inactivated, *HML* usage drops to 10%. (B) Molecular events during *MAT* switching. The ends of a DSB are resected, allowing Rad51 filament formation. Homology searching by this filament engages the donor (here, *HML $\alpha$* ). On the right side (region Z) of the DSB, strand invasion can form a fully interwound plectonemic structure, which can recruit PCNA and other DNA replication proteins. However, on the left side, the paired *W* and *X* regions may form only a weak, side-by-side paranemic joint, because the first 650 nt of the 3' end of the left end of the DSB is not homologous to the donor. Conversion of this joint to an interwound plectonemic joint requires either the action of a topoisomerase or the removal of the nonhomologous tail by the Rad1–

sequences (Sugawara *et al.* 2003; Wolner *et al.* 2003; Wang and Haber 2004; Hicks *et al.* 2011).

Strand invasion is followed by the initiation of new DNA synthesis using the 3' end of the invading strand as a primer (White and Haber 1990; Hicks *et al.* 2011). Because switching to the opposite mating type replaces *Y* sequences to the left of the DSB, strand invasion and primer extension initially occur in the Z region, where the end of the DSB is perfectly matched to the donor. In contrast, primer extension from the WX region can occur only after a longer resection has taken place and the nonhomologous 700-bp *Y* region is clipped off to expose a functional primer end (White and Haber 1990). Eventually the first strand copied is displaced from the template and is itself used as the template for the rightward copying of the second strand, leaving all the newly synthesized DNA at the *MAT* locus and the donor unchanged (Ira *et al.* 2006).

It is important to note that the amount of homology shared by *HML* or *HMR* with *MAT* is sufficient to ensure highly efficient recombination. When both donors contain the same *Y* region, more than 90% of cells complete *MAT* switching, and this number rises to nearly 100% when the competing nonhomologous end ligation of the HO-cut ends is eliminated (Valencia *et al.* 2001). As noted above, recombination is initiated with the *MAT*-Z end, which shares only 230 bp homology with the distal side of *HMR* or 320 bp at *HML*. Replacement of *HML* with *HMR* does not alter the left donor usage in *MAT $\alpha$*  cells (Wu and Haber 1995), so that 230 bp to the right of the DSB is sufficient at either donor to effect highly efficient recombination.

A remarkable aspect of *MAT* switching is its donor preference, such that *MAT $\alpha$*  usually recombines with *HML $\alpha$*  (i.e., *MAT $\alpha$*  is replaced by *MAT $\alpha$* ) and, similarly, *MAT $\alpha$*  is usually replaced by *MAT $\alpha$* , by preferentially copying *HMR $\alpha$* . Preference is not dictated by the *Y* sequences at the donors nor differences in the sequences in and around the donors, because the choice remains the same if the *Y* regions are replaced or if *HML $\alpha$*  is replaced by *HMR $\alpha$* ; choice is dictated by chromosome location. Donor preference is enforced by a 275-bp *cis*-acting recombination enhancer (RE) sequence, located 17 kb from *HML $\alpha$*  (Wu and Haber 1996). In *MAT $\alpha$*  cells, RE binds several proteins including *Mcm1*, *Fkh1*, and *Swi4/Swi6*, whereas in *MAT $\alpha$*  cells the *Mat $\alpha$ 2–Mcm1* repressor binds to RE and establishes a region of highly positioned nucleosomes that prevent binding of other proteins (Wu *et al.* 1998; Coic *et al.* 2006a,b). How RE works has not yet been precisely elucidated, but it clearly influences how *HML* is used as a donor; when RE is deleted in *MAT $\alpha$*  cells, they behave like *MAT $\alpha$*  cells, using *HML* less than 10% of the time. We have proposed that the chromosome III architecture is different in *MAT $\alpha$*  and *MAT $\alpha$*  cells in a way that favors

Rad10 flap endonuclease. Initiation of new DNA synthesis from the 3' Z end produces a single strand of newly copied DNA that is displaced and can anneal with the second end, allowing that end to copy the new *MAT* sequences.

*HML* or *HMR* accessibility (Bressan *et al.* 2004; Coic *et al.* 2006a).

The presence of two donors allows us to assay competition between these donors, to examine key steps leading up to recombination. We have previously used competition assays to demonstrate that an intrachromosomal donor outcompetes an interchromosomal donor, a result that is supported by observing the kinetics of DSB repair (Wu *et al.* 1997; Ira *et al.* 2003; Keogh *et al.* 2005). These observations and others (Agmon *et al.* 2009) suggest that the search for homologous sequences by the *Rad51* filament is more efficient intrachromosomally. Here we wished to determine if the use of *HML* and *HMR* is affected by changing the length of homology shared between one donor and *MAT*. We note again that, in this situation, the natural length of homology at each donor is sufficient to accomplish nearly 100% gene conversion, so that we are not looking at a net increase in recombination efficiency as one might do if each donor were inherently inefficient. Such increases in recombination efficiency (in the absence of competition) can be seen if the initial lengths of homology are close to the minimum efficient processing segment (MEPS) and allow only inefficient repair of a DSB (Shen and Huang 1989). In the case of budding yeast, MEPS appears to be about 70 bp (Ira *et al.* 2003).

In the case we study here, the bias in usage of one donor is enforced by RE and we are interested in whether donor preference is so strongly dictated that the ratio of the preferred to the excluded donor cannot be changed. The strong exclusion of the “wrong” donor can be partially relieved by increasing the homology shared with *MAT* on the distal side of the DSB from ~150 bp to  $\geq 2$  kb. A series of donors with increasing amounts of homology distal to the DSB reveals that use of the disfavored donor increases, but not linearly, to about one-third of all switches. These data argue that the choice of the preferred donor is not irrevocable. We conclude that successful recombination usually results only after multiple encounters between the donor and the *Rad51* filament covering DSB ends at *MAT*, so that the rare encounters of the “wrong” donor with the DSB ends become more likely to be successful when the size of homology increases. Greater homology between the disfavored donor and *MAT* leads to a higher chance that each encounter will progress from an initial, transient strand association to a successful recombination event.

These data, combined with data from the kinetic analysis of the early steps in homologous recombination, allow us to construct a thermodynamic model for donor preference that accounts quantitatively for the effect of changing the homology length. The free energy cost for pairing each of the two donors with the *Rad51*-coated *MAT* sequences can be calculated and used to infer how RE acts to facilitate the use of the more distant *HML* donor. We conclude that RE markedly lowers the free energy of association with *MAT* equivalent to moving *HML* from its normal genomic distance of 200 kb to a proximity that is equivalent to having a donor

only 23 kb away. This prediction of the thermodynamic model suggests that there is a dramatic change in the architecture of chromosome III after induction of the DSB, which could be explained by RE associating with the DSB region.

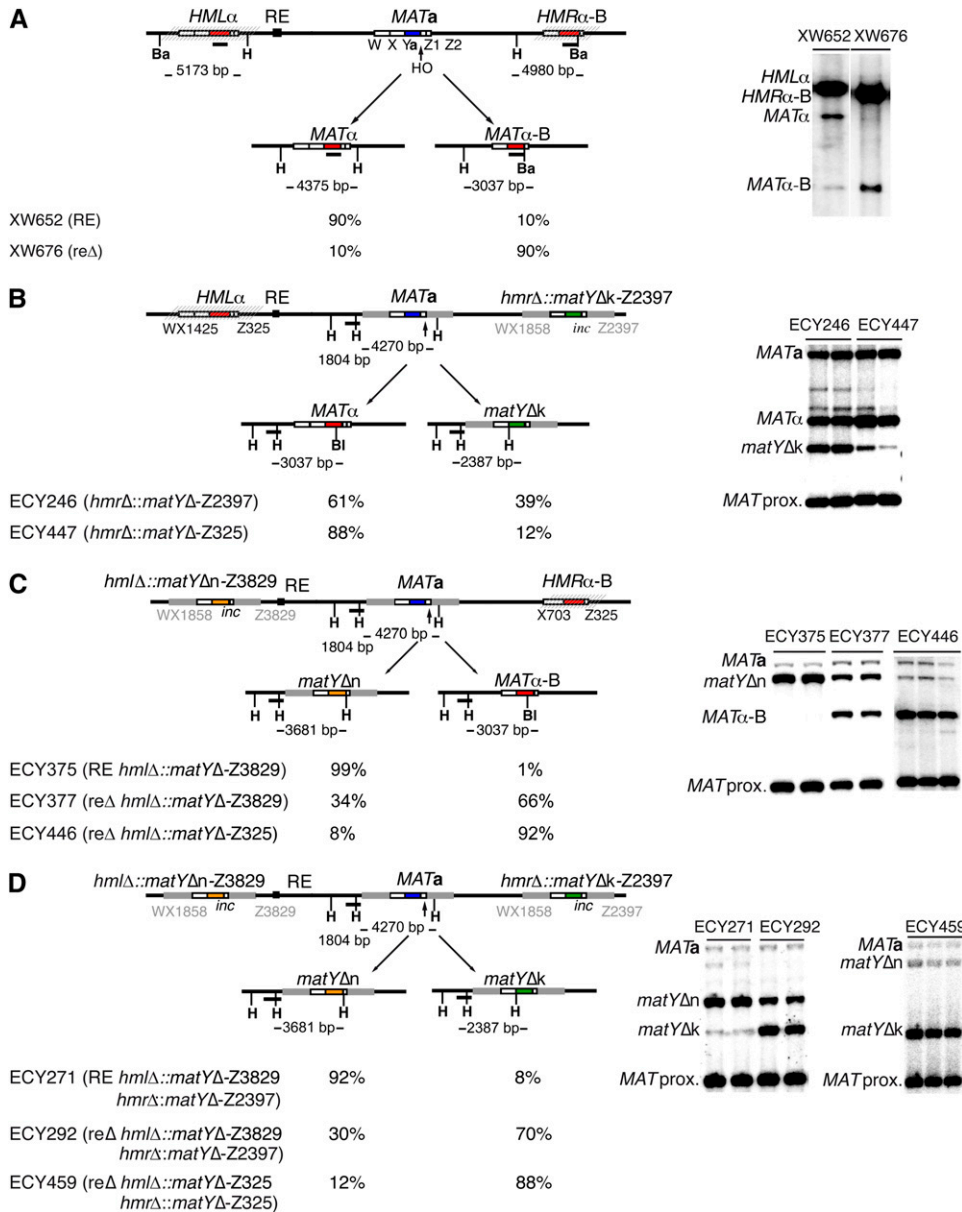
## Materials and Methods

### Strains

Donor preference was measured in derivatives of DBY745 (*ho MATa ade1-100 ura3-52 leu2-3,112*). The replacement of *HML* and its associated silencers sequence E and I have already been described (Wu and Haber 1995). A 3.4-kb region including *HMR* and its adjacent silencers was replaced with a 4.8-kb *EcoRI*–*Bam*HI *MATa-inc-URA3* (uncuttable by the *HO* endonuclease) fragment that lacks the silencers E and I. To avoid the expression of mating-type genes from the additional *MAT* copies introduced, the Y sequences were deleted at these loci.  $Y\alpha$  was replaced by a *NAT* cassette obtained from pAG25 (Goldstein and McCusker 1999) by transformation with a PCR fragment containing the *NAT* cassette flanked by the 50 first and last sequence of  $Y\alpha$  with the primers:  $Y\alpha$ NATU (5'-tat gtc tag tat gct gga ttt aaa ctc atc tgt gat ttg tgg att taa aag cat agg cca cta gtg gat ctg-3') and  $Y\alpha$ NATL (5'-cga agt agt ccc ata ttc cgt gct gca ttt tgt ccg cgt gcc att ctt cac agc tga agc ttc gta cgc-3'). Equally,  $Y\beta$  was replaced by a KanMX4 cassette by transformation with a PCR fragment obtained from pFA6-KanMX4 and primers MATKANp1 (5'-tag gta aat tac agc aaa tag aaa aga gct ttt tat tta tgt cta gta cag ctg aag ctt cgt acg c-3') and MATKANp2 (5'-caa cca ctc tac aaa acc aaa acc agg gtt tat aaa att ata ctg ttg cgc gaa gta gtc cca gca tag gcc act agt gga tct g-3'). These alleles are referred to as *matY $\Delta$ n* or *matY $\Delta$ k*, respectively.

At the *HML* loci, the extra homology centromere-distal to the *matY $\Delta$ n* extra copy was truncated in a series of strains, in each case by replacement with a KAN cassette. In strain ECY446, replacement was accomplished by transformation with a PCR fragment containing the KAN cassette flanking by sequences surrounding the distal sequences, leaving the same 325 bp of homology that exists between *MAT* and *HML*. The PCR fragment was obtained with primers MATdistKANU (5'-tat gta ttt gta taa aat atg ata tta ctc aga ctc aag caa aca atc aac agc tga agc ttc gta cgc-3') and HMLMATdistKANL (5'-att tgc tca aaa tta tcc atg aaa gaa gta cca atg aag cac tag cct gtg cat agg cca cta gtg gat ctg-3'). A similar strategy was then used to truncate the homology between the *matY $\Delta$ n* sequences at *hml $\Delta$*  and those at *MAT* to lengths of 148 (ECY487), 653 (ECY474), 754 (ECY488), 994 (ECY489), 1320 (ECY475), 2216 (ECY490), 2717 (ECY491). The specific primers used are available upon request.

The same approach was employed to delete the distal homology of *matY $\Delta$ k* at the *HMR* locus by replacement with the *NAT* cassette. The PCR fragment suitable for transformation was obtained with primers MATdistKANU (see above) and HMRMATdistNATL (5'-tgt ggg gtt gca ttg tag ata aaa gta ata ata tta ggt ata tag aat atg cat agg cca cta gtg gat ctg-3').



**Figure 2** Alterations of the donor loci to examine competition between *HML* and *HMR*. (A) Quantification of donor preference in *MATa* strains bearing heterochromatic donors (indicated by hatching) *HMLα* and *HMRα-B* (from Wu and Haber 1995 and Wu *et al.* 1998). The proportion of the two expected products *MATα* and *MATα-B*, resulting from an HO-induced DSB at *MAT* (arrow), are determined by probing a Southern blot of *Hind*III (H)–*Bam*HI (Ba) enzyme-digested DNA with *Yα* sequences (horizontal bar). The size of the restriction fragment detected on the Southern blot is indicated. The results of the quantification is given in strains carrying or deleted for the RE. (B) The *HMR* locus was replaced by *MATa-inc* sequences, deleting the E and I silencers leading to *hmrΔ::matYΔk-Z2397*. *Ya* was replaced by the G418-resistance *KAN* gene (*matYΔk*, green), so that the unsilenced donor would not express mating-type information (strain ECY246). The replacement introduced additional sequences homologous to the *MAT* locus: 1155 bp was added upstream of the 703 bp of X sequences, increasing the homology to 1858 bp (WX1858). On the right side, the homology increased from 238 bp (Z1) to 2397 bp (Z2397). The additional homology at Z was subsequently removed by inserting the *NAT* locus, yielding to *hmrΔ::matYΔk-Z325* (strain ECY447). Following expression of a galactose-inducible HO gene for 90 min, the DSB could be repaired by recombining either with the modified, unsilenced *hmrΔ::matYΔk* locus or with the normal, heterochromatic *HMLα* locus. The ratio between these two recombination outcomes was determined by probing a Southern blot of *Hind*III (H)–*B**l**p**I* (BI) enzyme-digested DNA with *MAT* proximal sequences up-

stream of the region of extended homology (horizontal bar). In addition to the bands corresponding to the parental and products fragments, a signal corresponding to the proximal sequences of *MAT* is also revealed (*MAT* prox.) (C) An analogous replacement of *HML* and adjacent E and I sites was carried out, adding 433 bp homology to the left (WX1858) and 3504 bp homology to the right (Z3829) of *MAT* leading to *hmlΔ::matYΔn-Z3829*. *Ya* was replaced by *NAT* (*matYΔn*, yellow) in ECY375. In strain ECY446, the additional homology was removed by *KAN* insertion leading to Z325. RE was deleted in strains ECY377 and ECY446. Following induction of the HO endonuclease, the proportion of the alternative recombination outcomes was determined as in B. (D) Competition between *hmrΔ::matYΔk-Z2397* and *hmlΔ::matYΔn-Z3829* (ECY271 RE<sup>+</sup>, ECY292 reΔ). Note that the size of Z at the *HML* locus is nearly twice the size as at the *HMR* locus. Deleting the additional homology at both loci (ECY459) restores the rule of donor preference when the *HML* and *HMR* donors are unmodified. Donor usage was determined as in B for ECY459; the genomic DNA of ECY271 and ECY292 was digested with *Hga*I and probed with the same *MAT* proximal sequences. The left donor usage is the mean of at least three independent determinations in all experiments.

RE was deleted by transformation of a DNA fragment containing an *URA3* cassette surrounded by RE flanking sequences isolated from the plasmid pJH1561 (Wu and Haber 1995).

The *MATa* reΔ strains carrying *HMRα-B* and *hmlΔ::matYΔn*, with various *NAT*-marked truncations of the homology (described above) were then transformed with a *hmrΔ::LEU2* fragment from plasmid JKM106 to delete *HMRα-B*,

yielding a set of strains in which a DSB at *MAT* can be repaired only by recombination with the modified *HML* sequence. Because the *HML* construct lacks an HO cleavage site, cells that switch will not suffer further HO cleavage; hence the strains can be directly plated on rich media containing galactose (YEP-Gal plates). Standard yeast genetic and molecular techniques were used (Guthrie and Fink 1991).

## Analysis of donor preference

Quantification of donor preference on Southern blot was performed as previously described (Wu *et al.* 1998). Signals were quantified using ImageQuant V1.2 (Molecular Dynamics).

## Viability

Cells were grown in YEP-lactate overnight to mid-log phase and then appropriate dilutions were plated on rich media containing dextrose (YEED) and rich media containing galactose (YEP-Gal plates, where HO is induced).

## Results

### **Extra homology adjacent to the Z region, but not its heterochromatic state, increases competitiveness of the less frequently used donor**

In a *MATa* cell in which the two donors carry differently marked  $Y\alpha$  sequences, *HMR* is used only ~10% of the time to repair the DSB (Figure 2A; Wu and Haber 1995). We asked if extra homology introduced at the *HMR* locus would increase its use. In these experiments we replaced *HMR* by *MATa-inc* sequences, eliminating the *cis*-acting silencers adjacent to *HMR*, but preventing HO from cleaving the unsilenced locus, because the HO cut site is mutated by a single base pair substitution in this construct (Weiffenbach *et al.* 1983) (Figure 2B). In addition, *Ya* was replaced by the G418-resistance *KAN* gene, so that the unsilenced donor would not express mating-type information that would alter the cell's mating type. The replacement introduced an additional 1155 bp of homology shared with *MAT* to the left of the 703-bp X region (the amount of homology shared between *MAT* and the *HMR* locus is now  $WX = 1858$ ); 2159 bp of homology was also added to the right of the 238-bp Z1 region ( $Z = 2397$ ). Because the modification that is most decisive involves the length of the Z sequences (see below), we designate this locus as *hmr* $\Delta$ ::*matY* $\Delta$ k-Z2397. Following expression of a galactose-inducible HO gene for 90 min, the DSB could be repaired by recombining either with the modified, unsilenced *HMR* locus or with the normal, heterochromatic *HML* $\alpha$  locus. The ratio between these two recombination outcomes was determined by probing a southern blot with *MAT*-proximal sequences (Figure 2B).

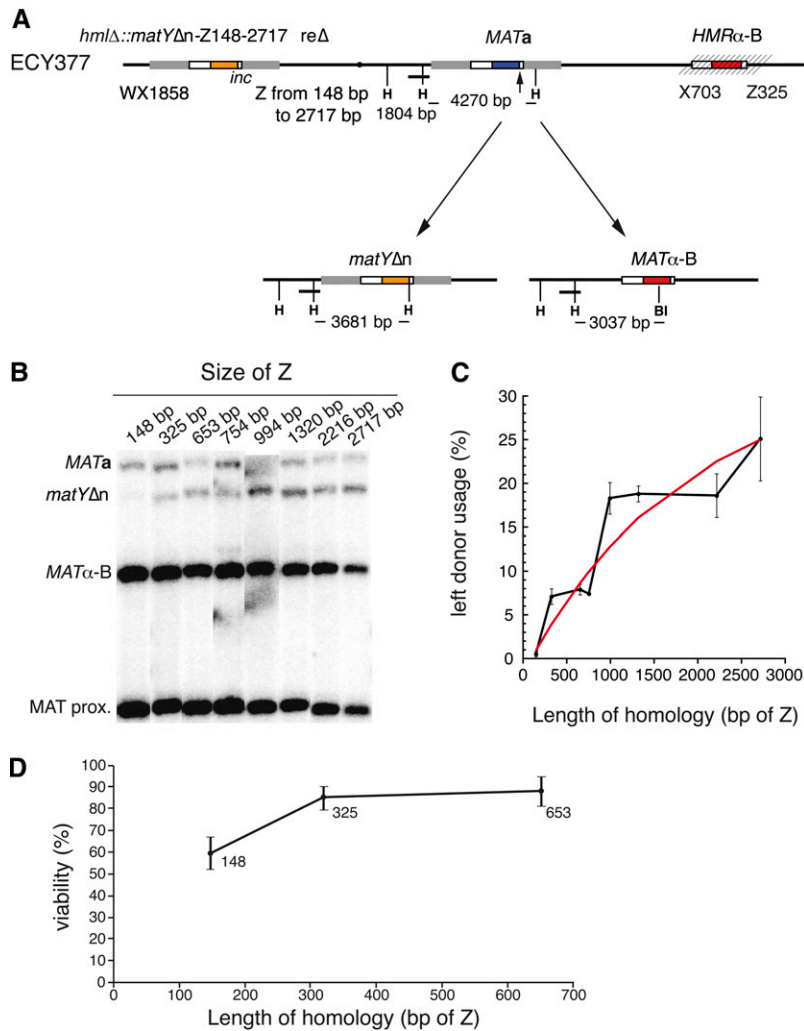
With the additional homology, *HMR* usage in this *MATa* strain increased from 10% in the unaltered strain to 39% (compare Figure 2, A and B). This increase in donor usage could have come either from unsilencing the donor locus or from the creation of greater shared homology. To distinguish between these alternative explanations, we deleted the additional *MAT*-distal sequences at *HMR* with a *NAT* (nourseothricin resistance) gene (see *Materials and Methods*) leaving only 325 bp of homology to the right of the cleavage site, the same as that found at *HML*, creating *hmr* $\Delta$ ::*matY* $\Delta$ k-Z325. In this construct, where the *KAN* gene replaced *Ya*, *HMR* usage returned to its normal level, 12%. Therefore, the improved usage of unsilenced *MAT* replacing *HMR* is not

explained by the lack of silencing, but by the increased length of homology shared between the donor and the recipient on the centromere-distal side of *MAT*.

It should be noted that the increased amount of homology on the left side of the donor (increased from 703 bp in the X region of *HMR* to 1858 bp) did not alter *HMR* usage when the right side was reduced to 325 bp. This result is consistent with the idea that the initial steps of recombination are dictated by the ssDNA of the Z region, which matches the donor exactly (except for a single-base-pair substitution), whereas the left side of the DSB has a large segment of heterologous DNA at its end (in this case the *KAN* sequences and part of *Ya*) that prevent stable synapsis and primer extension (White and Haber 1990).

By a similar approach we unsilenced the *HML* donor by deleting the adjacent E and I silencer sequences and inserting an equivalent segment from a *MAT* $\alpha$  locus (Figure 2C). Nearly all of the  $Y\alpha$  region and part of the HO cleavage site was then replaced by a *NAT* gene. The initial segment of *MAT* that we inserted extended the amount of homology from 1425 bp in the W and X regions to 1858 bp and from 325 bp in the Z1/Z2 regions to 3829 bp. The resulting construct was designated as *hml* $\Delta$ ::*matY* $\Delta$ n-Z3829. Following induction of the HO endonuclease, the ratio between the alternative recombination outcomes could be determined by probing a Southern blot with *MAT* proximal sequences upstream of the region of extended homology. When RE is active, the use of the modified *HML* was significantly higher (nearly 100%, Figure 2C) compared to a normal *HML* (90%, Figure 2A). When RE was deleted, the use of *HML* also increased, from the normal level of about 10 to 34% when the Z sequences share 3829 bp with *MAT*. As with the alterations at *HMR* discussed above, the improved use of the "wrong" donor is attributable to the length of the shared homology and not to the unsilenced donor, because when the *hml* $\Delta$ ::*matY* $\Delta$ n-Z3829 sequences were truncated back to 325 bp to the right of the cut site, using a *KAN*-marked modification, *i.e.*, *hml* $\Delta$ ::*matY* $\Delta$ n-Z325, the use of this *HML* locus was again ~10% in a RE deleted strain.

Finally, we examined left donor usage in strains where both *HML* and *HMR* loci had been replaced by extended, unsilenced *MAT* sequences (Figure 2D). We observed a wild-type donor preference: 92% of the cells used the left donor when RE is active but only 30% when RE is deleted. This result again confirms that the E and I silencer sequences are not involved in donor preference. The greater use of *hml* $\Delta$ ::*matY* $\Delta$ n-Z3829 in the *MATa* re $\Delta$  strain (30%) than that seen in normal strains (10%, Figure 2A) may reflect the fact that this donor shares more homology with the right side of the HO-cut *MAT* (3829 bp vs. 2397 bp). When the extra homologies introduced at both donor loci were deleted in a *MATa* re $\Delta$  strain, so that each donor had 325-bp homology in the Z region, the left donor was largely excluded. Left donor usage (12%) was not significantly different from strains carrying *HML* $\alpha$  and *HMR* $\alpha$ -*Bam*HI (10%, Figure 2A). These data also show that the *NAT* or the *KAN* cassettes



**Figure 3** Homology length dependence on the use of *HML* in a *MATa* reΔ *HMRα-B* strain. (A) Rationale. The length of homology shared by *hmlΔ::matYΔn-Z* and *MAT* is modulated at the donor by the introduction of a *KAN* cassette containing variable amounts of *Z* sequences (from 148 to 2717 bp). (B) Donor usage in the resulting nine strains (see *Materials and Methods*) was measured on Southern blots as in Figure 2C. (C) The percentage use of the “wrong” *HML* locus, *hmlΔ::matYΔn-Z*, measured as the average of at least three independent quantifications, is plotted as a function of the length of homology shared with *MAT*. A ball-tossing model of donor preference that reflects the consequences of increasing the length of *HML* homology with *MAT* is shown as a red curve. This line is a best fit through the data, representing the case where the search is directed toward *HMR* nine times as often as *HML*, and where  $S_R/S_{L_{max}} = 0.23$ ,  $S_{L_{max}} = 1$ ,  $L_{opt} = 1800$  bp, and the minimum length of homology used by Rad51 is 100 nt. A nearly identical line is obtained if the minimum homology length is 70 pb. (D) Donors with limited homology on the *Z* side are efficient. *MATa* reΔ strains with varying extents of homology on the *Z* site of *hmlΔ::matYΔn-Z* were further modified by the *LEU2*-marked deletion of *HMRα-B*. Strains with Z148, Z325, and Z653 were grown in YEP-lactate and dilutions were plated on YEPD to determine cell number, and on YEP-Gal to measure viability after HO induction. The percentage viability plotted is the results of three independent experiments.

used both to delete the extra homology and to replace the *Y* sequences at both donors do not interfere with their usage.

#### Length dependence of the increased use of *HML*

To better understand the relationship between the length of homology shared by *HML* and *MAT* in competition with a normal, silenced *HMR* in a RE deleted strain, we constructed a series of eight truncations of the right-hand homology, leaving as little as 148 bp and ranging up to 2717 bp, in each case with the shared homology bounded by the *KAN* cassette. These strains are designated by the total homology shared between *MAT* and the modified donor (*hmlΔ::matYΔn-Z*148, *hmlΔ::matYΔn-Z*326 to *hmlΔ::matYΔn-Z*2717). The results of this analysis are shown in Figure 3. When *matYΔn* shares only 148 bp with its donor, the efficiency of repair is considerably reduced compared to the normal 325 bp. Increasing homology leads to increased use of the *HML* donor, although the increase is not strictly proportional to distance. Thus, additional homology at the “wrong” donor can partially overcome the donor preference mechanism. A best-fit line through these data is based on a “toy” model based on a model in which (1) an encounter between either the pre-

ferred or disfavored donors of normal size will result only in the completion of recombination in ~25% of attempts, and (2) the success in completing DSB repair increases as the size of the *Z* region increases (see *Discussion*).

#### Efficiency of the “wrong” donor in the absence of competition

If there is no competition, will donors with limited homology on the *Z* side prove to be adequate donors? To address this question we deleted *HMRα-B* in the set of strains described above where RE is deleted. Because the modified *HML* donor in each of these strains lacks an HO cleavage site, the efficiency of switching could be assessed by plating the cells directly on a rich medium containing galactose (YEP-Gal) and comparing the viability to cells plated on rich medium containing dextrose (YEPD), Southern blot analysis being uninformative here. The results, shown in Figure 3D, demonstrate that in the absence of competition, even a donor with only 148 bp of homology is quite efficient (60% viability) compared to only ~1% usage when it is in competition with the preferred *HMRα-B* donor (Figure 3, B and C). At 325 bp (the normal size of the Z1 and Z2 regions in

*HML*) or 653 bp, the length of homology is sufficient to repair the DSB in nearly 90% of the cells. Therefore, the low usage of the modified *HML* donor can be unambiguously attributed to the competition with *HMR* $\alpha$ -B and not to a poor efficiency in recombination because of the smaller amount of homology with *MAT*.

A very important additional finding is that the primary factor regulating the use and efficiency of *HML* is the homology to the *right* of the *HO* cleavage site, where the DSB end is perfectly homologous to the donor, as we noted when extra homology was added to *HMR* (see previous section). In most of the constructs there is considerably more homology to the left of the DSB (*i.e.*, 1858 bp including the W and X regions) than to the right. As noted above, the lack of influence of the donor sequences to the left of the DSB most likely reflects the fact that these homologous sequences are separated from the DSB end by  $\sim$ 1 kb of nonhomologous sequences, so that they are unable to form a stable association with the donor (see *Discussion*).

## Discussion

### **Donor efficiency is unaffected by its heterochromatic state**

An important finding in this study is that the normally heterochromatic *HML* and *HMR* loci do not become more efficient donors if they are unsilenced. A notable feature of the silent donors is that *HO* endonuclease cannot cleave the same sequence that is readily cut at *MAT* $\alpha$  or *MAT* $\alpha$ , apparently because of highly positioned nucleosomes that occlude the cleavage site (Weiss and Simpson 1998; Ravindra *et al.* 1999). Yet the cleaved *MAT*-Z end, as part of a *Rad51* nucleoprotein filament, is capable of invading precisely the same sequences, promoting strand invasion and the initiation of new DNA sequences. *Rad51* and its associated proteins must therefore be able to overcome any chromatin-based barrier to DSB repair. These conclusions appear to be different from those reached in the study of gene conversion promoting immunoglobulin diversity in vertebrate DT40 cells (Cummings *et al.* 2007).

### **Establishment of strand invasion must involve multiple attempts**

The competition between *HMR* and a series of *HML* donors with different lengths of homology provides an opportunity to consider the dynamics of the search for a donor by the *Rad51* filament associated with the single-stranded 3' ends of the DSB at *MAT*. In a *MAT* $\alpha$  re $\Delta$  strain, *HMR* is normally used 90% of the time. If this choice was dictated prior to switching and the choice was irrevocable (*e.g.*, if the preferred donor were already held close to *MAT*), then the modifications we made at *HML* should have no effect on the preferred usage of *HMR*. But this is not the case. These results are consistent with the fact that a close association between *MAT* and its donor does not occur until after *HO* creates a DSB (Bressan *et al.* 2004; Houston and Broach

2006). It is also unlikely that the relatively small sequence that we introduced into the *HML* region changes the probability of the initial encounter between *MAT* and the donors, 100 or 200 kb distant; rather they are influenced by global constraints on chromosome movement or architecture. The fact that the excluded donor usage can be improved by increasing the size of homology with *MAT* therefore suggests that encounters between *MAT* and its favored donor are not always productive. Synapsis may result only in a paranemic joint that is not converted into an interwound, more stable plectonemic joint that can recruit *PCNA* and DNA replication factors to launch primer extension (Sugawara *et al.* 2003). Thus, even though the preferred donor will make contact with the *Rad51* filament nine times as often as the wrong donor, successful recombination is not guaranteed at any given encounter.

We postulate that the *Rad51*::ssDNA filament is likely to encounter the preferred donor several times before creating a stable recombination intermediate that will then lead to a completed switching event. Thus, although RE will ensure that the DSB in a *MAT* $\alpha$  strain will encounter *HML* nine times more often than it will collide with *HMR*, when both donors are of equivalent size, it is likely that many more than 10% of the cells will encounter *HMR* at least once before *HML* is used as the donor. Similarly, when RE is deleted, the *Rad51* filament will more frequently encounter the now-disfavored *HML* donor than is evident in its 10% usage.

We suggest that when the homology length of the wrong donor is increased, the frequency of *productive* collisions between *MAT* and the normally disfavored donor increases. This will lead to an overall increase in stable strand invasion and the initiation of new DNA synthesis with this donor, thus compensating for its infrequent collisions with the *Rad51*::ssDNA filament. A toy model that illustrates the changes in donor preference imagines a pail of ping-pong balls that are thrown nine times as often at a box in the right corner of a room than at an equivalently sized box in the left corner. The ball is hard to throw accurately, such that one gets the ball in a box only a small fraction of the time. Here we use the example that a throw succeeds 25% of the time. Therefore, on average, 75% of the balls will be on the floor and there will be nine times more balls in the right box. If one increases the width of the disfavored box by a factor of 2, now the tosses toward this box will have a twofold higher probability of landing in the bucket, while the number of throws to the right still exceeds the number of throws toward the left box by a factor of 9. From this model the success of using the “wrong” box can be calculated in the following manner:  $F_L$  and  $F_R$  are the fraction of times the ball is tossed toward left and right boxes ( $F_L + F_R = 1$ ), while  $S_L$  and  $S_R$  are the probabilities that a toss in the left or right direction is successful ( $S_L = S_R = 0.25$  in the above example). The probability of a ball landing in the box on the left ( $p_L$ ) is given by

$$p_L = F_L S_L / [F_L S_L + F_R S_R] = 1 / [1 + F_R / F_L (S_R / S_L)]. \quad (1)$$

Thus when  $F_R/F_L = 9$  and  $S_R = S_L$  the use of the left donor will be  $p_L = 1/[1 + 9 \times 1] = 0.1$ , as is observed in the unperturbed case. If, on the other hand,  $S_L$  is increased to 1.0, then the left box usage rises to  $p_L = 1/[1 + 9 \times 0.25] = 0.31$ , which is approximately the maximum value seen in our data when  $Z$  at *HML* increases  $>2700$  bp (Figure 3C).

The model can be modified to reflect some of the realities of homologous recombination in budding yeast. As homology increases,  $S_L$  will increase linearly until a point at which additional homology will not be as efficiently used is reached. First, the probability of success cannot exceed 1. A decline in further efficiency with homology length may happen because the amount of *Rad51* in the cell is limited to about 3500 molecules, so that only  $\sim 5000$  bp on either side of the DSB can be covered if the filament is continuous (Sugawara *et al.* 2003). Moreover, if strand pairing is initiated far from the 3' end of the filament, the probability that the remaining filament will “zipper up” as a paranemic joint to engage the 3' end in the D loop, and thus have a chance to become plectonemic, is likely to diminish with distance. We can describe this mathematically by stating that  $S_L$  will vary with homology size ( $H_L$ ) according to the equation

$$S_L = S_{L \max}(1 - \exp(-(H_L - m)/L_{\text{opt}})), \quad (2)$$

where  $S_{L \max}$  is the maximum value of  $S_L$ , which is reached at the largest homology lengths,  $m$  is the minimum size of a *Rad51* filament that can be used in repair (70–100 nt) (Ira and Haber 2002), and  $L_{\text{opt}}$  is the point at which the efficiency of the filament's size becomes nonlinear (here we estimate that it is 1800 bp). This equation has the property that it increases linearly with  $H_L/L_{\text{opt}}$  when  $H_L$  is much smaller than  $L_{\text{opt}}$ , and goes smoothly to  $S_{L \max}$  when  $H_L$  is much larger than  $L_{\text{opt}}$ .

The data in Figure 3C are well described by this model, as shown for the best fit, where  $S_R/S_{L \max} = 0.23$ . From these values we also can estimate that *HMR* will, on average, encounter *MAT* four times before completing recombination, assuming  $S_{L \max} = 1$ . If, at large values of homology at *HML*, the probability of a *MAT-HML* collision leading to a successful recombination event is less than one ( $S_{L \max} < 1$ ), then the corresponding value for productive collisions with *HMR* will be equally reduced ( $S_R = S_{L \max} \times 0.23$ ); *i.e.*, the number of unsuccessful trials will go up. We estimate that in about 40% of cells, *HML* will have been encountered at least once.

Given all the previous experimental evidence, we believe it likely that *HML* accessibility in cells where RE is active is dependent on a spatial reorganization of chromosome III induced by the formation of the DSB at *MAT*. If we further assume that the chromatin strand can be treated as a simple Gaussian polymer chain, then the ratio of probabilities that one or the other donor will make contact with *MAT* is equal to the ratio of their genomic distances to *MAT* raised to the  $-3/2$  power (Rippe 2001). Simply based on their distances from *MAT* with no other constraints, *HMR* usage should be favored over *HML* by a factor of 3:  $[G(\text{RM})/G(\text{LM})] \wedge (-3/2) =$

$[100 \text{ kb}/200 \text{ kb}] \wedge (-1.5) = 3$ ; however, in *MATa* cells, *HML* is used 9 out of 10 times. This preference could be achieved if *HML* were brought close to *MAT* so that its effective distance were only 23 kb. This then leads to a preference, based on polymer theory, of  $[100/23] \wedge (-1.5) = 1/9$ . Although our data and that of others show that *HML* is not constitutively much closer to *MATa* than is *HMR* in the nucleus (*i.e.*, in the absence of *HO* cleavage) (Dekker *et al.* 2002; Bressan *et al.* 2004; Miele *et al.* 2009), we suggest that such a reorganization will occur after DNA damage is created. If RE is itself tethered to the DSB, then *HML* will be about 17 kb away, consistent with the estimate of 23 kb. This proximity could be accomplished if RE promoted a chromosome remodeling in response to the DSB. For example, RE binds multiple copies of the *Fkh1* protein that contains a FHA domain that can bind phosphopeptides (Sun *et al.* 1989; Coic *et al.* 2006b). If *Fkh1* binds to the checkpoint kinase-modified chromatin around the DSB (Shroff *et al.* 2004; Kim *et al.* 2007), it would recruit RE—and also *HML*—very close to the DSB at *MAT*. This possibility is being investigated.

### **Nonhomologous sequences prevent adjacent homology from dictating donor usage**

A very important additional finding is that the primary factor regulating the use of *HML* is the homology to the right of the *HO* cleavage site, where the DSB end is perfectly homologous to the donor. In *hmlΔ::matYΔn-Z<sub>325</sub>* and *hmrΔ::matYΔk-Z<sub>325</sub>*, where  $Z$  is truncated to its normal size at *HML* (325 bp), the large amount of homology to the left of the DSB (*i.e.*, 1858 bp, including the 1425-bp W and X regions) is unable to increase the use of these donors. We believe the lack of influence of the donor sequences to the left of the DSB reflects the fact that these homologous sequences are separated from the DSB end by approximately 1 kb of nonhomologous sequences, so that they are unable to form a stable association with the donor (Figure 1). We believe that this side of the DSB does make a less stable, paranemic joint with the donor, although it cannot form a fully interwound, plectonemic structure until the nonhomologous region is removed. These sequences must be clipped off by the *Rad1-Rad10* nuclease (White and Haber 1990; Fishman-Lobell and Haber 1992; Colaiacovo *et al.* 1999). Our recent studies have shown that there is *Rad51*-mediated synapsis on this side of the DSB before the nonhomologous sequences are removed (Hicks *et al.* 2011). Moreover, *MAT* switching remains efficient even if the W/X region is truncated to the same 327 bp as found on the right side (Hicks *et al.* 2011). So, although sequences distal to the left end of the DSB do make contact with the donor, the probability that *Rad51*-mediated homology searching will lead to repair is dictated by homology to the right of the cleavage site. We propose that the size of homology to the right of the *HO* cut site was selected in *S. cerevisiae* consistent with the architecture of chromosome III, to assure a strong exclusion of the wrong donor and the easy repair of the break with the preferred one.

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