

Saccharomyces forkhead protein Fkh1 regulates donor preference during mating-type switching through the recombination enhancer

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Saccharomyces mating-type switching results from replacement by gene conversion of the *MAT* locus with sequences copied from one of two unexpressed donor loci, *HML* or *HMR*. *MAT*_a cells recombine with *HML*_α ~90% of the time, whereas *MAT*_α cells choose *HMR*_a 80%–90% of the time. *HML* preference in *MAT*_a is controlled by the cis-acting recombination enhancer (RE) that regulates recombination along the entire left arm of chromosome III. Comparison of RE sequences between *S. cerevisiae*, *S. carlsbergensis*, and *S. bayanus* defines four highly conserved regions (A, B, C, and D) within a 270-bp minimum RE. An adjacent E region enhances RE activity. Multimers of region A, D, or E are sufficient to promote selective use of *HML*. Regions A, D, and E each bind in vivo the transcription activator forkhead proteins Fkh1p and Fkh2p and their associated Ndd1p, although there are no adjacent open reading frames (ORFs). Deletion of *FKH1* significantly reduces *MAT*_a's use of *HML*, as does mutation of the Fkh1/Fkh2-binding sites in a multimer of region A. We conclude that Fkh1p regulates *MAT*_a donor preference through direct interaction with RE.

[Key Words: *Saccharomyces cerevisiae*; donor preference; recombination enhancer; *FKH1*; *FKH2*; mating-type switching]

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Mating-type gene switching in *Saccharomyces cerevisiae* has provided one of the best-studied examples of DNA double-strand break (DSB)-induced recombination in mitotic cells. Recombination is initiated by cleavage of the *MAT* locus with the sequence-specific HO endonuclease. The ends of the DSB can recombine with one of two silenced and heterochromatic donor loci, *HML* and *HMR*, located near the extremities of the same chromosome (Fig. 1A). Gene conversion occurs without crossing-over, often resulting in the replacement of *MAT*-Y_a or -Y_α sequences that regulate whether cells will be a- or α-mating type (Strathern 1989; Haber 1992, 1998a,b; 2002). In most strains of *S. cerevisiae*, *HML* carries Y_α sequences (*HML*_α), whereas *HMR* carries Y_a (*HMR*_a). One of the remarkable aspects of this process is the phenomenon of donor preference, in which *MAT*_a cells preferentially recombine with *HML*_α, ensuring that recombination will usually result in a change of mating type; similarly, *MAT*_α cells preferentially select *HMR*_a (Klar et al. 1982; Weiler and Broach 1992; Wu and Haber

1995; Wu et al. 1996). Donor selection depends on the location of the sequences but not their content, as *MAT*_a cells will preferentially recombine with *HML*_a even versus *HMR*_α (Weiler and Broach 1992). Moreover, *MAT*_a's choice of a left-arm donor occurs even if the entire *HML* region is replaced by *HMR* (Weiler and Broach 1992) or if the donor is placed at other locations along the left arm (Wu and Haber 1995). *MAT*_α cells continue to choose a right-arm donor even if *HMR* is replaced by *HML* sequences, again regardless of whether the donor carries Y_a or Y_α (Wu et al. 1996).

The control of donor preference depends on a small cis-acting sequence, the recombination enhancer (RE), which acts as a locus-control region to control recombination along the entire left arm of chromosome III (Wu and Haber 1996). RE lies in a 2.5-kb intergenic region. It is located ~29 kb from the left end of chromosome III (i.e., 17 kb centromere-proximal to *HML*) between *KAR4* and *SPB1* genes, but it retains its ability to influence donor choice even if it is inserted 43 kb further toward the centromere (G.-F. Richard and J.E. Haber, unpubl.). Deletion of the entire RE region causes a profound change in *MAT*_a donor preference, so that *HML* is used only 10% of the time, compared with 85%–90% when RE is present (Wu and Haber 1996). These studies indi-

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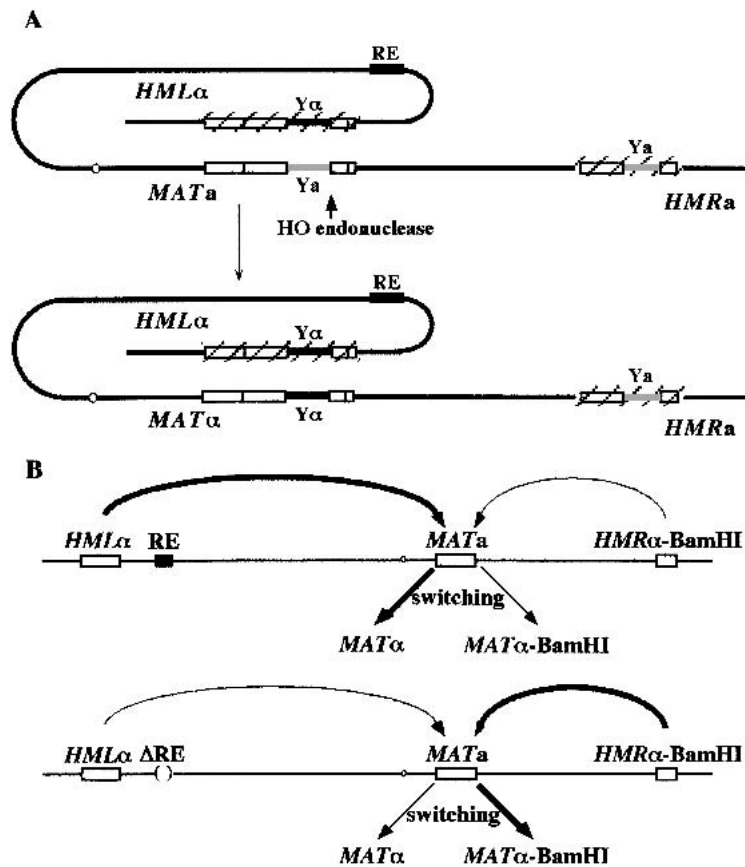


Figure 1. Mating-type switching at the *MAT* α locus. (A) Expression of HO endonuclease creates a double-strand break (DSB) at the *Y/Z1* border of the *MAT* locus and initiates repair by using the silent donor *HML* and leads to gene conversion. The two donor loci (*HML* and *HMR*) are maintained in a transcriptionally inactive chromatin structure. Other shared regions of homology are indicated. The preferential use of *HML* donor in *MAT* α cells is regulated by a cis-acting element, recombination enhancer (RE), located ~17 kb centromere-proximal to *HML*. The positions of *HML*, RE, centromere, *MAT* and *HMR* are indicated. (B) *HMR* α is replaced by *HMR* α modified to carry a point mutation that creates a *Bam*HI site, so that it is possible to distinguish which sequences have been gene converted into the *MAT* locus. Donor preference is assayed in populations of cells induced to switch from *MAT* α to either *MAT* α or *MAT* α -B (Wu and Haber 1995). *MAT* α cells recombine with *HML* ~90% of the time in the presence of RE; when RE is deleted, *MAT* α cells prefer to choose *HMR* 80%–90% of the time.

cated that RE acted to make the left arm of chromosome III “hot” for recombination in *MAT* α cells, whereas in *MAT* α cells the left arm was made unusually inaccessible for recombination (thus allowing *HMR* to be the preferred donor). These changes in recombination are not accompanied by any change in the mRNA levels of genes along the left arm (Galitski et al. 1999; A.P. Gasch, M.B. Vaze, D. Botstein, P.O. Brown, and J.E. Haber, unpubl.).

Comparison of the RE regions of *S. cerevisiae* and *S. carlsbergensis* allowed us to identify four highly conserved sequences (A, B, C, and D) within a 270-bp region (Wu et al. 1998). Deletion analysis revealed that the A, C, and D segments were required for activity, so that a minimum enhancer consisting of these three regions increased *HML* usage from 10% without RE to 45% (Wu et al. 1998). It is likely that an additional region, E (Fig. 2), which shares strong sequence homology with D, especially in *S. cerevisiae*, is also important. That some of these regions function in a redundant fashion is indicated by the finding that a 700-bp region lacking A, but containing B, C, D, and E, was nearly as functional as a 753-bp region containing all five elements (Wu and Haber 1996). Both regions, D and E, contain repeats of TTT(A/G), the only sites in the yeast genome to have such similar-length arrays of this sequence.

How RE works to promote *MAT* α donor preference is not yet understood; to date only one trans-acting mutation, a deletion of *CHL1*, has been found that alters *MAT* α donor preference, and in this case the effect is

modest (Weiler et al. 1995). We have a better idea how RE is inactivated in *MAT* α cells, causing a phenotype of *MAT* α strain lacking RE; region C contains a binding site for the Mcm1-Mat α 2 repressor complex that is known to turn off transcription of a set of *a*-specific genes when the *MAT* α genes are expressed. In *MAT* α cells, the RE region is covered with highly positioned nucleosomes that apparently inactivate RE (Weiss and Simpson 1997; Wu et al. 1998). This inactivation is similar to that seen at the promoter of the *a*-specific *STE6* gene (Shimizu et al. 1991; Murphy et al. 1993), although no open reading frame (ORF) is found adjacent to RE. The activation of RE in *MAT* α cells depends on binding of Mcm1p. In *MAT* α cells, mutation of the Mcm1-binding site in RE also leads to the formation of a positioned array of nucleosomes and the loss of RE activity (Wu et al. 1998). At *STE6*, Mcm1p appears to be a trans-activating transcription factor. The RE does not contain an ORF, but there are several noncoding RNAs (Szeto et al. 1997). Whether Mcm1p is needed for transcription of these noncoding transcripts has not been established; moreover, it should be noted that the minimum RE and synthetic REs that lack these transcribed regions are not seriously impaired in activating *HML* in *MAT* α cells (Wu et al. 1998) (results below).

In the present study, we have further characterized the sequences that are critical for RE activity in *MAT* α cells. We have refined the identity of these sequences by sequencing the RE region from *S. bayanus*. We show that

Forkhead protein regulates yeast donor preference

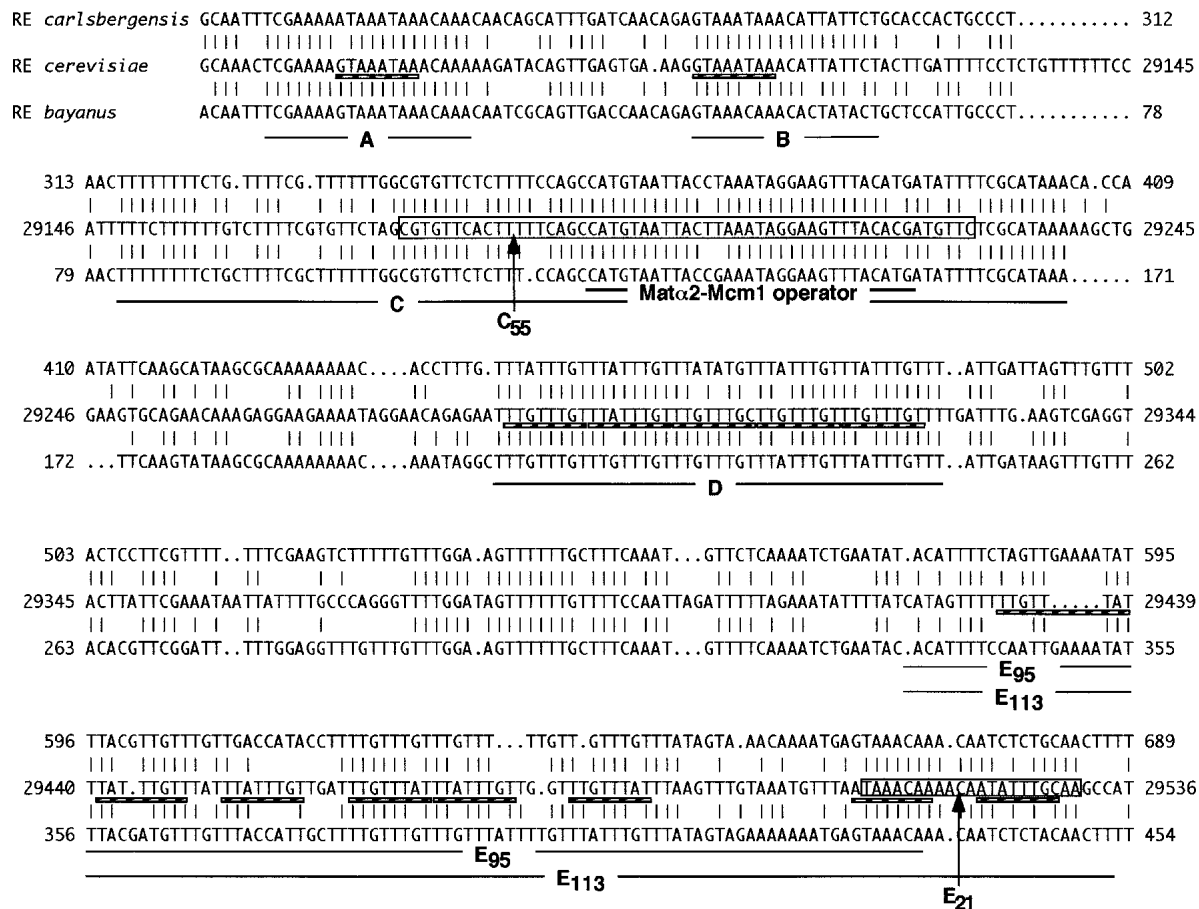


Figure 2. Comparison of RE among three species: *Saccharomyces carlsbergensis*, *S. cerevisiae*, and *S. bayanus* (analyzed in “LA-SERGENE navigator”). Identical residues in the alignment of the DNA sequences of RE *carlsbergensis* (top), RE *cerevisiae* (middle), and RE *bayanus* (bottom) are indicated by vertical lines. The four most conserved subdomains (designated as A, B, C, and D) and a second near-perfect array of TTT(G/A) repeats (designated as E₁₁₃) are underlined. Region C contains the Mat α 2-Mcm1 consensus binding site (double underlined). Regions A, B, D, and E contain a total of 15 Fkh1p and Fkh2p consensus binding sites (hatched line). The fragments used to replace the wild-type RE in donor preference assay—designated as C₅₅ (boxed), E₂₁ (boxed), E₉₅ (solid line), and E₁₁₃ (solid line)—are indicated.

there is substantial redundancy in the *S. cerevisiae* RE region, so that even multimers of A, D, or E segment have strong RE activity. Second, we show that Fkh1 and Fkh2 transcription activators bind to region A in vivo, as well as to regions D and E, and that a *fkh1* Δ strain has a marked reduction in MAT α donor preference. We conclude that, in addition to their key role in regulating expression of a class of cell cycle-regulated genes, forkhead proteins play an important role in the cell cycle-independent activation of the RE.

Results

In combination with the 270-bp minimum RE, a second region containing TTT(G/A) repeats fully restores RE activation

By comparing the sequences from originally identified 700-bp RE between *S. carlsbergensis* and *S. cerevisiae*, a 270-bp minimum RE was defined, containing four highly

conserved segments designated A, B, C, and D (Wu et al. 1998) (Fig. 2). When this 270-bp region was then inserted into a chromosome in place of a 1.8-kb sequence containing RE and other intergenic sequences between *KAR4* and *SPB1*, this minimum enhancer only retained part of the donor preference activity. A strain carrying a 753-bp RE region selected *HML* ~75% of the time, whereas a strain with 270-bp RE used *HML* ~45% of the time, which is still significantly above the 10% level seen when RE is absent (Wu et al. 1998). Further analysis showed that region B is dispensable for MAT α 's use of *HML*, but the other three regions were required (Wu et al. 1998).

We then further refined our examination of RE by including sequences obtained by sequencing the intergenic region between highly homologous *KAR4* and *SPB1* genes in *S. bayanus*. Three of the four subregions we had previously identified (A, C, and D) were strongly conserved; however, region B was less conserved among the three species (Fig. 2). We showed that this RE sequence

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was functional by inserting a 3.1-kb sequence containing the *S. bayanus* RE in place of the *S. cerevisiae* RE and showing that *HML* usage in *MATa* cells was >80% (data not shown).

A notable feature of RE region is that it contains many TTT(G/A) repeats. Conserved region D has 10 TTT(G/A) repeats. Region A and B also have two or three of such repeats. Among the sequences outside of the 270-bp minimum RE, a less well-conserved segment (E_{113}) also contains TTT(G/A) repeats (Fig. 2). The E_{113} region has been shown as a unique nuclease hypersensitive region to micrococcal nuclease digestion flanked by two foot-printed regions in *S. cerevisiae* (Weiss and Simpson 1997).

In the alignment of the DNA sequences from *S. carlsbergensis*, *S. cerevisiae*, and *S. bayanus* (Fig. 2), the overall region E is not conserved among all three species. Interestingly, included in the E_{113} region is an 18/21 match between *S. carlsbergensis* and *S. cerevisiae* and a 17/21 match between *S. bayanus* and *S. cerevisiae*, centromere-proximal to the less-conserved TTT(G/A) repeats (bp29512–bp29532, designated E_{21} , boxed in Fig. 2).

To investigate whether the other important cis-acting elements other than A, C, and D contribute to the function of RE, a series of deletions were made in a 732-bp RE fragment that has the same activity as the 753-bp RE shown previously (Wu et al. 1998; Fig. 3). To assess donor preference, *MATa* cells carrying a galactose-inducible *HO* gene were transferred from YEP-lactate medium to YEP-galactose for 1.5 h to induce gene conversion of *MATa*, which can use either *HML* α or *HMR* α -B as a donor (Wu and Haber 1995). *HMR* α -B carries a single base-pair mutation that creates a *Bam*HI site and allows us to distinguish which sequences have been gene converted into the *MAT* locus (Fig. 1B). As shown in Figure 3, deletions removing the second TTT(G/A) repeat-rich region (E) caused a significant reduction in RE activity (Fig. 3, cf. lanes 12 and 5). A small deletion (bp29505–bp29535) including the highly conserved E_{21} region in a 732-bp RE decreased *MATa*'s use of *HML* from ~75% to ~65% (Fig. 3, #12 and #11). Further deletions in TTT(G/A) region diminished the activity from ~65% to ~55% when at least seven TTTG/A repeats remained (Fig. 3, #8). Activity fell to 40% when only three TTT(G/A) repeats were retained (Fig. 3, #6). This result argues that region E is an important contributor to overall RE activity.

Linker sequences between regions A, C, and D are dispensable

To define the active elements within RE more precisely, we ligated together various combinations of the conserved regions, A, C, D, and E. In all cases, the constructs were introduced into a 1.8-kb deletion that removed the native RE. A construct containing a 22-bp A region, a 55-bp segment of the C region including the *Mcm1*-binding site (designated C_{55} , in Fig. 2), and a 43-bp D region had approximately the same activity as the entire 270-bp minimum RE (Fig. 4A, KS226). This construct

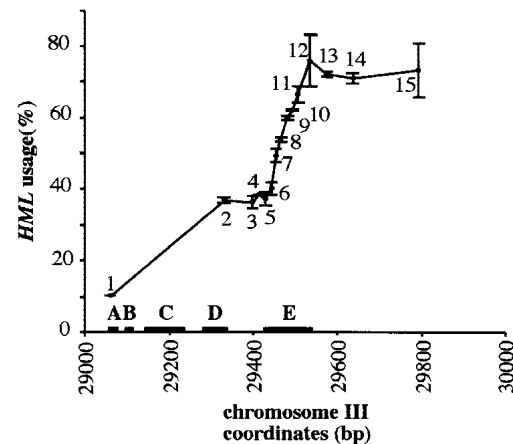


Figure 3. Identification of region E, which, in combination with A, B, C, and D, can provide full RE activity in *MATa* cells through a series of deletions from centromere-proximal end of RE region. The different RE fragments were introduced into the chromosome III to replace a 1.8-kb sequence containing RE. The strains used here (XW676, CWu81, KS210, KS209, KS208, KS207, KS206, KS205, KS204, KS203, KS202, KS198, KS199, KS184, and KS185) are indicated as 1–15, respectively. Number 1 is the strain that has no RE fragment inserted into a 1.8-kb deletion. Number 2 carries the chromosome III region from 29059 bp to 29330 bp, which was reported previously as the 270-bp RE (Wu et al. 1998). Numbers 3–14 each represent strains that carry a fragment starting from 29059 bp of chromosome III to centromere-proximal end. The numbers of TTT(G/A) repeats are #3 (0), #4 (0), #5 (0), #6 (3), #7 (5), #8 (7), #9 (11), #10 (14), #11 (16), #12 (20), #13 (20), and #14 (20). Number 15 carries the 732-bp RE starting from 29059 bp to 29790 bp. Donor preference is shown as the percentage of *HML* usage in *MATa* cells. Important region E is from ~29423 to ~29535 bp as indicated above.

was inserted in the opposite orientation as the normal RE, but previous studies had shown that RE is orientation independent (Wu and Haber 1996). Thus the regions in between A, C, and D are not important for minimum RE function, which therefore resides in these 120 bp. This result also means that the exact positioning of these elements on the chromosome is not crucial.

Regions A, D, and E function in a redundant fashion and the requirement of *Mcm1* binding is bypassed in synthetic REs

To determine if there were redundancies among regions A, C, D, and E, we tested the function of multimers of individual subdomains. We found that trimers of C_{55} alone were unable to activate the use of *HML* as a donor; in this case, *HML* usage was <10% (KS181 in Fig. 4B). Similarly dimers of A, D, or E alone had no activity (data not shown). However, dimers of A, D, or E combined with one C_{55} domain increased the use of *HML* (Fig. 4C, 19.3% in KS246, 32.5% in KS222, and 30.0% in KS339). Again, the orientation and precise position of these additional elements did not seem to be important. A strain

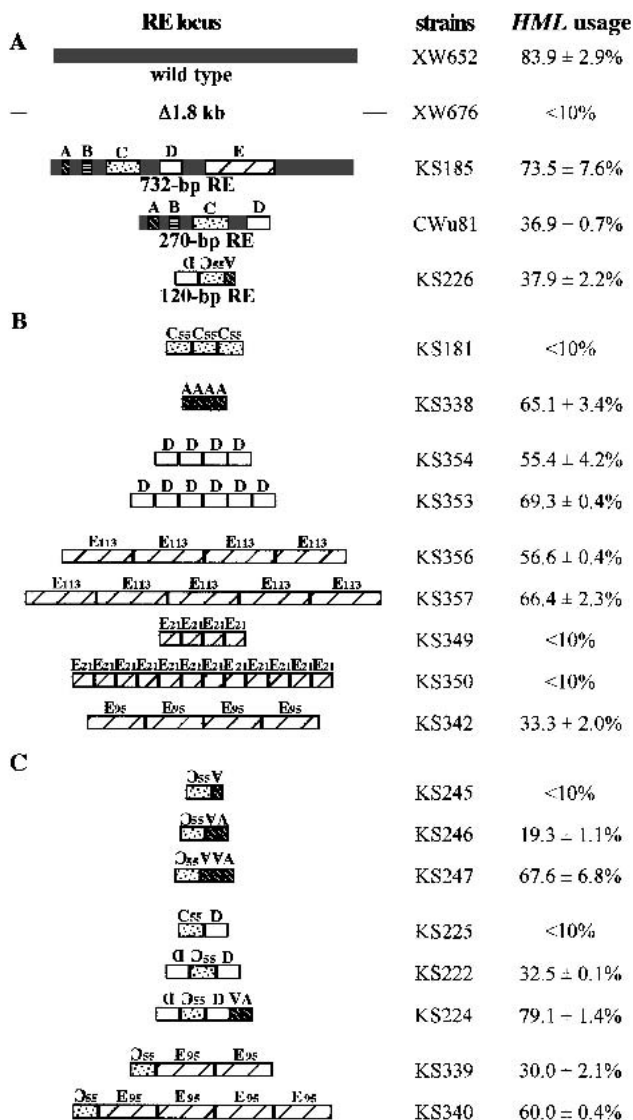


Figure 4. Effect of different combinations of regions A, C, D, and E on the percentage of *HML* usage in *MATa* cells. The different synthetic RE fragments were introduced into the chromosome III to replace the 1.8-kb sequence containing RE. C₅₅, E₂₁, E₉₅, and E₁₁₃ are indicated in Fig. 2. All other regions, A, C, and D, used here contain full length of consensus sequences.

with a synthetic RE consisting of A-A-C₅₅-D-D used *HML* at nearly wild-type levels (Fig. 4C, 79% in KS224).

Surprisingly, tetramers of A (KS338), D (KS354), and E (KS356) were able to substantially activate *HML* as a donor (50%–65% *HML* usage). Adding more copies of D (KS353) or E (KS357) gave still further increases (66%–69%), approaching the level of activity seen with the native RE (Fig. 4B).

By a similar approach, we found that the conserved E₂₁ region lacking TTT(A/G) repeats cannot by itself activate the use of *HML* as a donor (Fig. 4B, KS350). In contrast, a tetramer of the TTT(A/G)-containing part of region E (E₉₅) can provide an activation of *HML* (Fig. 4B, 33% in KS342), although not as strongly as the more

well-preserved TTT(A/G)-containing D region (60%). We conclude that region A, D, or E each is sufficient to provide at least some RE function.

In *MATa* cells, the binding of Mcm1p in the intact RE region is required for the activation of RE because of its ability to remove highly positioned nucleosomes that lead to the repression of RE activity (Wu et al. 1998). Here we show that multimers of A, D, or E are each active by themselves and that the requirement for binding of Mcm1p in the wild-type RE can be bypassed. It is possible that the role of Mcm1p is to facilitate chromatin reorganization to provide access to the A, D, and E sites.

In *MATa* cells, the binding of Mata2/Mcm1p at the RE region was shown to be responsible for the repression of RE because of its ability to organize highly positioned nucleosomes that led to the repression of RE activity (Tanaka et al. 1984; Szeto and Broach 1997; Szeto et al. 1997; Weiss and Simpson 1997; Wu et al. 1998). Previously, we showed that mutations of the Mata2-binding site removed most of this repression (15% of *HML* usage in wild-type *MATa* vs. 55% in Mata2-binding site mutant) (Wu et al. 1998). In this study, we replaced the wild-type RE with tetramers of region A in *MATa* cells. Here, too, there was no repression caused by Mata2/Mcm1p. The use of *HML* increased from ≤10% without RE to 50% (data not shown). Thus, the repression normally seen in RE does not occur when there is no Mata2p-Mcm1p binding.

Region A contains a consensus binding site for forkhead transcription factors

We focused our attention on region A, where four tandem copies gave 65% *HML* usage in *MATa* cells. To determine the binding site for any potential trans-acting factors in this region, a series of deletions was created and then two such truncated region As were joined to C₅₅ and D to assess donor preference. As shown in Figure 5A, region A activity was apparently contained within a 15-bp region, which in this assay had as much activity as the intact A region. This region contains the 13-bp sequence 5'-GTAAATAAACAAA-3' conserved in *S. carlsbergensis*, *S. bayanus*, and *S. cerevisiae*. Deletions of either the left 8 bp or the right 5 bp of this 13-bp region abolished RE activity from ~50% to ~10% *HML* usage in *MATa* cells. We tested one TA to GC mutation within this sequence (5'-GTAAAGCAACAAA-3') and found that it also severely impaired *MATa*'s use of *HML* when two copies of the mutated A region (Am) were combined with single copies of C₅₅ and D (Fig. 5A, KS251). In contrast, three other 2-bp mutations did not affect *MATa*'s use of *HML* (Fig. 5A, see strains KS325, KS326, and KS327).

Having identified a mutation within region A that appears to abolish its function, we used MatInspector V2.2 (Quandt et al. 1995) to search the region A and the entire RE region for the binding sites of transcription factors. The 13-bp consensus A region was shown to contain a potential binding site (5'-GTAAATAAAA-3') for the forkhead family of transcription factors (Fig. 5B). Similar pu-

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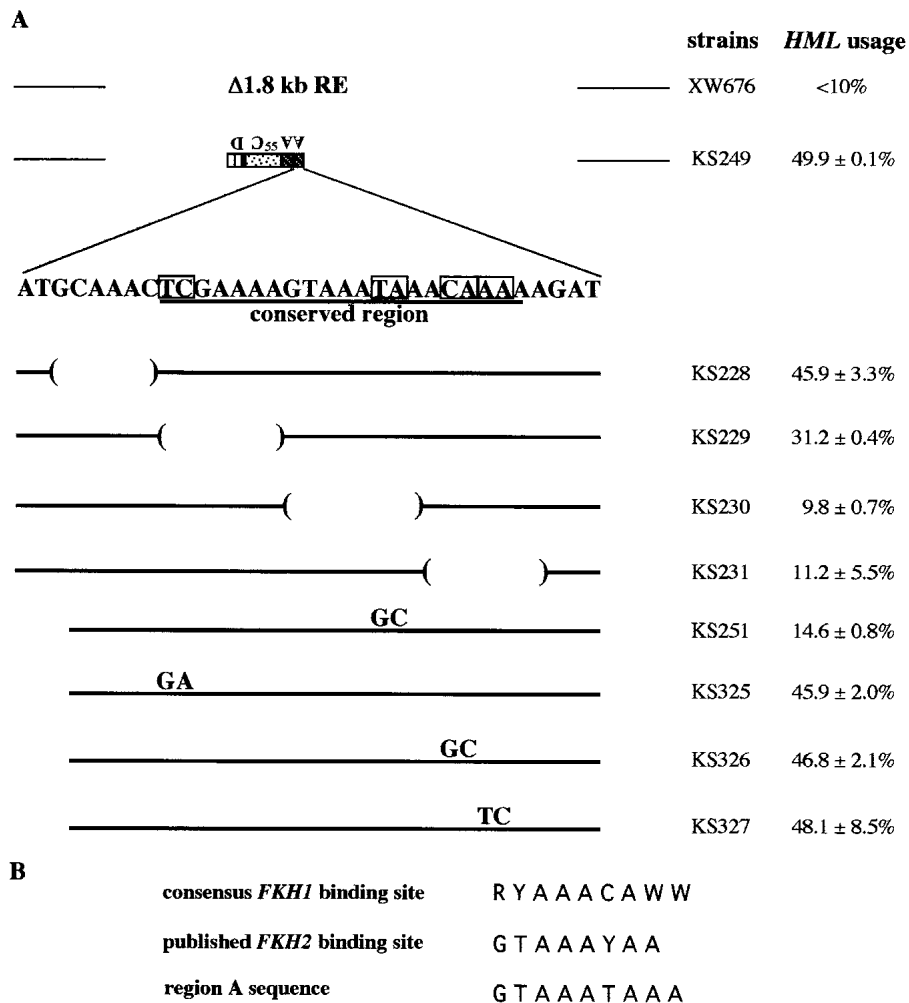


Figure 5. Identification of functional region A-binding site. (A) Identification of essential sequences within region A by introduction of the A + A + C₅₅ + D synthetic REs carrying different deletions or 2 bp mutations on each A into the chromosome III to replace a 1.8-kb sequence containing RE. The 2-bp changes are boxed. The conserved region is underlined. Donor preference is shown as the percentage of HML usage in *MATa* cells. (B) Comparison of consensus Fkh1p- and Fkh2p-binding sites reported previously (Zhu et al. 2000) with region A sequence. R: A or G; Y: T or C; W: A or T.

tative forkhead-binding sites were found in regions B, D, and E (Fig. 2).

The fkh1 fkh2 double mutant reduces donor preference in MATa cells

In *S. cerevisiae*, there are four forkhead homologs, *FKH1*, *FKH2*, *HCM1*, and *FHL1* (Zhu et al. 1993; Hermann-Le Denmat et al. 1994; Hollenhorst et al. 2000). Among the four members of the forkhead protein family, only Fkh1p and Fkh2p share significant sequence similarity (47% identity overall) between each other outside of the highly conserved forkhead DNA-binding domain. Fkh1p and Fkh2p were shown to activate transcription of the cell-cycle-dependent *CLB2* cluster and to affect *HMR* transcriptional silencing (Hollenhorst et al. 2000, 2001; Koranda et al. 2000; Kumar et al. 2000; Zhu et al. 2000). In addition, *FKH1* and *FKH2* also have redundant func-

tions in preventing pseudohyphal growth (Hollenhorst et al. 2000). On the basis of these observations, we made *fkh1Δ* and *fkh2Δ* single mutations and the *fkh1Δ fkh2Δ* double mutant in different strains containing either wild-type RE or synthetic REs and tested them for donor preference.

In a strain carrying wild-type RE, HML was used ~81% of the time (Fig. 6A, lane 1). A *fkh2Δ* derivative did not affect donor preference (Fig. 6A, lane 2). However a *fkh1Δ fkh2Δ* double or a *fkh1Δ* single mutant reduced HML usage to <40% (Fig. 6A, lanes 3,4).

In strains containing a synthetic RE, the wild-type RE was replaced with different fragments containing either C₅₅ + 3 × A (KS247 in Fig. 4B), 4 × A (KS338 in Fig. 4B), or 5 × E (KS357 in Fig. 4B). As shown in Figure 6, the strains carrying these synthetic REs used HML ~65% to 68% of the time (Fig. 6, lanes 4,7,9). There was no effect on HML donor preference in the *fkh2Δ* derivative (Fig. 6, lane 5),

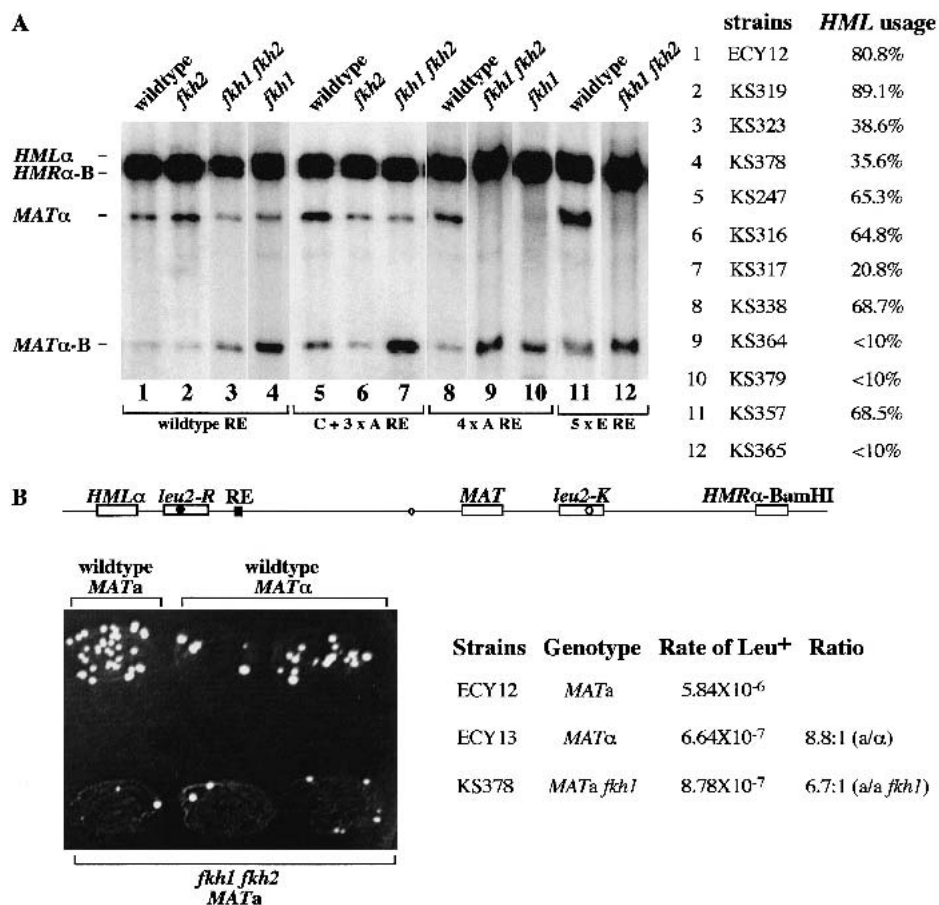


Figure 6. The effects of Fkh1p and Fkh2p on donor preference and *leu2* heteroallelic recombination in *MATa* cells. (A) Analysis of *HMLα* usage in different strains carrying single or double forkhead deletions in either wild-type RE or synthetic RE with C + 3 × A, 4 × A, and 5 × E. HO-induced switching of *MATa* to *MATα* (from *HMLα*) or *MATα*-Bam (from *HMRα*-Bam) can be assessed densitometrically on a Southern blot of DNA digested with *Bam*HI and *Hind*III restriction endonucleases. Yα-specific probe was used to give rise to four specific bands indicated above. Donor preference is shown as the percentage of *HML* usage in *MATa* cells. (B) Spontaneous recombination between *leu2-R* inserted at kb 22 of chromosome III and *leu2-K* located at kb 233 of the right arm of *MAT* produces Leu⁺ prototrophs. The wild-type *MATa* and *MATα* and mutant *fkh1 fkh2 MATa* were patched on a YEPD plate and then replica-plated to synthetic medium lacking leucine. The rate of *LEU2* prototroph formation was determined by a fluctuation test (Lea and Coulson 1949).

but there was a significant decrease of *HML* donor preference in the *fkh1Δ* or the *fkh1Δ fkh2Δ* double mutant strain, so that *HML* was only used <20% of the time instead of ~65% (Fig. 6, lanes 7,9,10,12). To make sure that the *fkh* mutations do not affect the efficiency of switching, we used the same genomic DNA made from *fkh1Δ fkh2Δ* (Fig. 6, lane 7) and confirmed that the efficiency of switching (i.e., the repair of the DSB) was very high (94.6%), and that (in this independent measurement of donor preference) the use of *HML* was 19.9% (data not shown), in excellent agreement with the measurement of 20.8% in Figure 6A. Thus, especially in the synthetic REs in which regions A, D, and E are not redundant with each other, the effect of deleting *FKH1* is profound. The effect of the double *fkh1Δ fkh2Δ* mutant is similar to that of the single *fkh1Δ* mutant, indicating that Fkh1p plays a more important role in regulating donor preference through the RE.

The *fkh1Δ fkh2Δ* mutant eliminates mating-type activation of heteroallelic *leu2* recombination

It has been shown that *fkh1Δ* weakly increases the expression of *a1* mRNA from the *HMR* locus when the adjacent I silencing region is deleted and the E silencer is simplified; however, this change was not seen in a *fkh1Δ fkh2Δ* double mutant (Hollenhorst et al. 2000). To be sure that the changes in donor preference we saw in *fkh1Δ* or *fkh1Δ fkh2Δ* strains were not attributable to some change in the heterochromatic structure of *HML* or *HMR*, we assayed the effect of the forkhead proteins on spontaneous intrachromosomal recombination between two *leu2* alleles to produce Leu⁺ recombinants. In this assay, neither *leu2* allele is silenced nor is recombination dependent on HO endonuclease cleavage. Previously, we showed that a *leu2-R* allele, replacing *HML*, recombined with a *leu2-K* allele, located near *MAT*, 25–30-fold more

frequently in *MATa* cells than in *MAT α* (Wu and Haber 1995). In the present assay, *HML* is present and *leu2-R* is inserted at kb 22 of chromosome III, between *HML* and RE (Fig. 6B). In this strain, there is a >10-fold increase in the rate of spontaneous *Leu*⁺ recombinants in *MATa* cells versus *MAT α* . In the *leu2* recombination assay, we found a very similar effect as we did in the direct monitoring of the use of *HML* and *HMR* in the *fkh1 Δ* or *fkh1 Δ fkh2 Δ* mutant. As shown in Figure 6B, numbers of *Leu*⁺ papillae were much lower in the *fkh1 Δ fkh2 Δ* double mutant than in the wild-type *MATa* cells, and all the patches had the lower number of *Leu*⁺ papillae as seen in *MAT α* cells. We also measured the rate of spontaneous *Leu*⁺ recombinants by a fluctuation test based on a minimum of seven independent cultures of each strain, initiated from 100 cells and grown to saturation (Lea and Coulson 1949). As shown in Figure 6B, there is approximately a nine-fold difference in the rate of spontaneous *Leu*⁺ prototroph formation in *MATa* versus *MAT α* cells and approximately a seven-fold difference between wild-type *MATa* cells and *fkh1 Δ MATa* cells. Thus, we concluded that the changes in donor preference we saw in *fkh1 Δ* or *fkh1 Δ fkh2 Δ* strains were not attributable to some change in the heterochromatic structure of *HML* or *HMR*. The *fkh1 Δ* mutant has the same effect on both donor preference in HO-induced *MAT* switching and the mating-type dependent difference in spontaneous heteroallelic *leu2* recombination.

Ndd1p binds to region A

As an alternative way to screen for proteins that bind to region A, we did one-hybrid screening in which one assays the activation of transcription by a *GAL4* activation domain fused to another protein domain that can bind to a specific sequence upstream of two reporter genes. Four tandem copies of domain A were ligated together and subcloned upstream of the minimal promoter of both the pHis1 and pLacZi reporter plasmids and then integrated into the yeast genome of YM4271 (Materials and Methods). We screened 5×10^6 transformants and isolated 21 positive clones that were His⁺ on 3-AT containing plates lacking histidine and also showed high β -galactosidase activity. Among these 21 positive clones, 18 encoded a known transcription factor, Gln3p, which recognizes an AT-rich binding site (GATAAG) (Stanbrough and Magasanik 1996). Two were from the uncharacterized ORF *YDR317W*. However, neither the deletions of *GLN3* and *YDR317W* nor a double mutant had an effect on donor preference in mating switching (data not shown).

The last candidate was *NDD1*. *NDD1* is an essential gene whose function is required during mitosis (Loy et al. 1999). Recently, it has been shown that Ndd1p is associated with the *CLB2* and *SWI5* promoters in chromatin immunoprecipitation (ChIP) assays (Koranda et al. 2000). Its recruitment at these sites depends on Mcm1p and on both Fkh2p and Fkh1p. Because *NDD1* is essential, we could not assay directly its role in donor prefer-

ence, but we could obtain evidence of its participation by ChIP assays.

Fkh1p, Fkh2p, and Ndd1p bind to the RE region in vivo

If the consensus Fkh1p- and Fkh2p-binding sites represented bona fide Fkh-binding sites and Ndd1p can interact with region A in a one-hybrid assay, then the RE region should be enriched in a protein-DNA fraction prepared by ChIP with antibodies against Fkh1p, Fkh2p, or Ndd1p. Previous studies of enrichment of the *CLB2*-cluster promoter by ChIP experiments showed that both Fkh1p and Fkh2p bound to *CLB2*-cluster promoters, although generally Fkh2p bound more efficiently than Fkh1p (Koranda et al. 2000; Hollenhorst et al. 2001). Interestingly, some *CLB2*-promoters without Mcm1-binding sites or with a mutant Mcm1p-binding site were occupied equally well or more efficiently by Fkh1 (Hollenhorst et al. 2001). Moreover, Ndd1p was also associated with *CLB2* and *SWI5* promoters in ChIP assays (Koranda et al. 2000).

Yeast cells harboring integrated *FKH1-3xHA*, *FKH2-3xHA* or *NDD1-6xHA* genes (Materials and Methods) and wild-type cells containing untagged version of these genes were used in ChIP experiments with an antibody specific for the hemagglutinin epitope (α -HA). To test whether Ndd1, Fkh1p, and/or Fkh2p bound to the RE region in vivo, a 500-bp wild-type RE region was analyzed by ChIP. As shown in Figure 7A, Fkh1p, Fkh2p, and Ndd1p bound to the RE region, although to different extents. Unlike the *CLB2*-cluster promoter region (Hollenhorst et al. 2001), the signal generated by *FKH1-3xHA* was much stronger than the signal generated by *FKH2-3xHA*, although there is a Mcm1p-binding site inside the RE region. The assay did not allow a precise determination of the binding stoichiometry, but it indicated that Fkh1p may have a more important role than Fkh2p in controlling RE activity.

In *MAT α* cells, RE is covered with highly positioned nucleosomes that lead to the repression of RE activity; whereas the RE chromatin is less organized in *MATa* cells (Weiss and Simpson 1997). Our results clearly indicated that Fkh1p can be recruited to RE region in *MATa* cells. To ask whether Fkh1p can also be recruited to the RE in *MAT α* cells, *MATa* cells carrying *FKH1-3xHA* were switched to *MAT α* by introducing a plasmid containing *GAL-HO*. As shown in Figure 7B, unlike what we saw in *MATa* cells, Fkh1p was not recruited to the RE region in the wild-type *MAT α* cells. This result indicates that Fkh1p can only be recruited to the less-organized RE chromatin found in *MATa* cells.

To show that the recruitment of Fkh1p and Fkh2p to the RE region are the result of their direct interactions with the putative forkhead-binding sites and Ndd1p is recruited to the same regions as forkhead proteins are, we replaced wild-type RE with synthetic REs carrying $4 \times A$, $6 \times D$, or $5 \times E$ and then performed ChIP using the same antibody. As shown in Figure 7C and D, Fkh1p, Fkh2p, and Ndd1p can be recruited to the $4 \times A$, $6 \times D$,

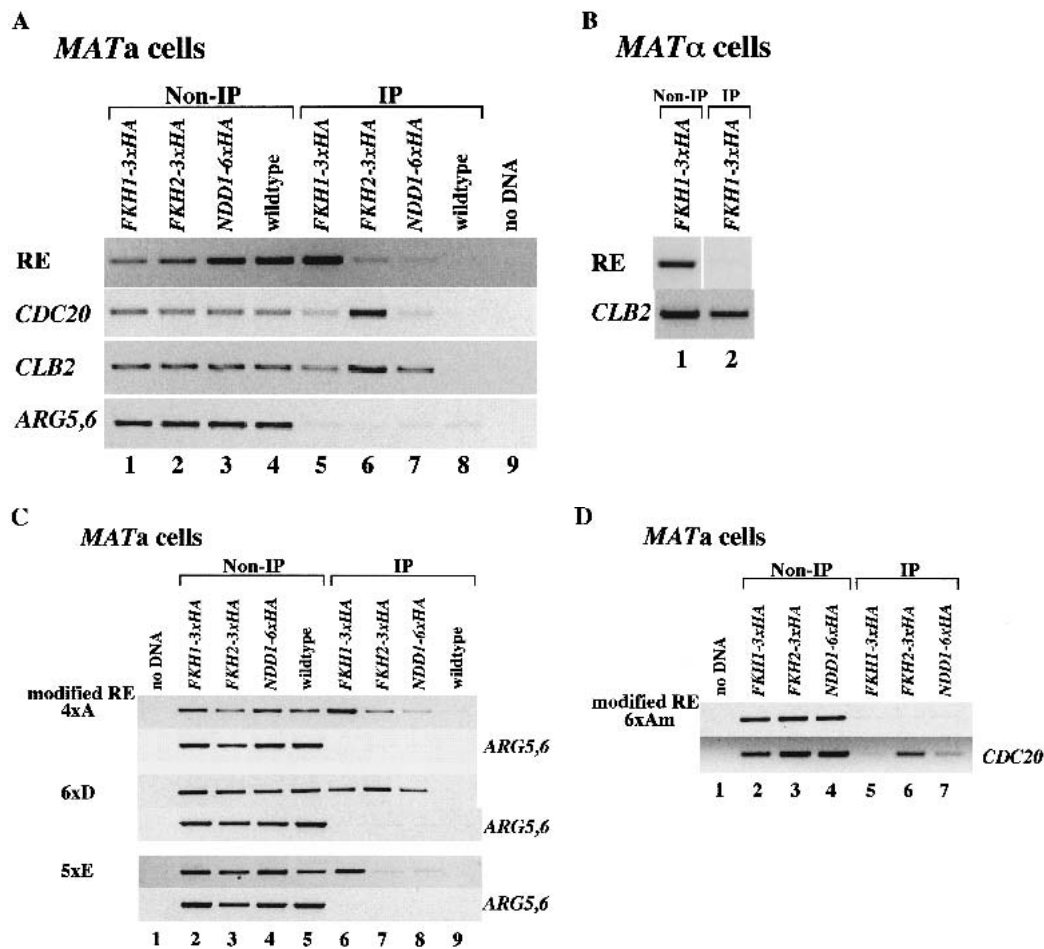


Figure 7. Fkh1p, Fkh2p, and Ndd1p are recruited to the RE in vivo. (A,B) Chromatin immunoprecipitation (ChIP) assays with a different pair of primers that can specifically amplify the wild-type RE region, the promoter regions of *CDC20* and *CLB2* (positive controls), and the coding region of *ARG5,6* (negative control). Strains used in this assay are CFY480 (A, lanes 1,5) and CFY854 (A, lanes 2,6) (Hollenhorst et al. 2000), 155 (A, lanes 3,7) (Koranda et al. 2000), W303 (A, lanes 4,8), and KS369 (*MATα*; B, lanes 1,2). (C,D) ChIP assays with pairs of primers that can specifically amplify either the modified RE region, the promoter of *CDC20* (positive controls), or the coding region of *ARG5,6* (negative control). The wild-type RE is replaced by either four copies of region A, six copies of D, five copies of E, or six copies of mutant region A, indicated as 4 × A, 6 × D, 5 × E, or 6 × Am at left. Controls that have fragments amplified from *ARG5,6* coding region and *CDC20* promoter are indicated at right. All strains used here are *MATa*. Strains used in this assay are KS358 (4 × A), KS359 (6 × D), and KS360 (5 × E) (C, lanes 2,6); KS370 (4 × A), KS371 (6 × D), and KS372 (5 × E) (C, lanes 3,7); KS374 (4 × A), KS375 (6 × D), and KS376 (5 × E) (C, lanes 4,8); KS361 (4 × A), 362 (6 × D), and 363 (5 × E) (C, lanes 5,9); KS365 (6 × Am) (D, lanes 2,5); KS373 (6 × Am) (D, lanes 3,6); and KS377 (6 × Am) (D, lanes 4,7).

and 5 × E, where the consensus forkhead protein-binding sites are present. In contrast, when we inserted six copies of mutant region Am (a TA to GC mutation within forkhead protein-binding site, see KS251 in Fig. 5A) into a 1.8-kb deletion that removed the native RE, Fkh1, Fkh2, and Ndd1 were not recruited to the 6 × Am region. Thus, the consensus Fkh1p- and Fkh2p-binding sites identified within the RE region represents bona fide Fkh-binding sites. Ndd1p can also be recruited to the same region as are forkhead proteins.

Discussion

We have investigated the manner in which the RE acts to control donor preference during mating-type switching

in *MATa* cells and made several important findings. First, the RE consists of several redundant domains whose exact spacing and orientation is surprisingly flexible. Second, multimers of region A, D, or E are sufficient to promote selective use of *HML*. Third, activation of *HML* donor preference does not require the presence of a Mcm1p-binding site. Finally, we have showed that the transcription regulator Fkh1p plays an important role in the activation of RE in *MATa* cells, in a region where there are no transcribed genes.

Our previous characterization of RE focused on the C domain in which there is a Mcm1p-Matα2p-binding site. It has been shown that Mcm1p-Matα2p is responsible for the repression of RE because of its ability to organize highly positioned nucleosomes that lead to the repres-

sion of RE activity in *MAT α* cells (Weiss and Simpson 1997; Wu et al. 1998). In *MATa* cells, the binding of Mcm1p, in the context of the complete RE, is required to activate donor preference (Wu et al. 1998). However, the C region is not essential for the activation of *HML* usage in synthetic REs, because multimers of 4 \times A regions, as well as 4 \times D or 4 \times E regions, lacking a Mcm1p-binding site are nearly as active as the entire RE region. We believe that the role of Mcm1 is to facilitate the removal of the highly positioned nucleosomes so that other factors can then be recruited to the RE and activate the RE. However, the possibility that Mcm1p could be recruited to the synthetic REs directly by binding to the Fkh2 protein (Hollenhorst et al. 2001) cannot be excluded, although *fkh2 Δ* did not affect donor preference.

The C region and its Mcm1p-Mat α 2p-binding site do seem to be essential for the repression of RE in *MAT α* cells. The absence of a Mcm1p-Mat α 2p site in the 4 \times A construct leads to constitutive RE activity in *MAT α* cells, although the lower usage of *HML* in *MAT α* cells (50%) versus *MATa* (66%) indicates the possibility of a *MATa*-specific gene product that may contribute to the full activation of RE in *MATa* cells. This difference between *MATa* and *MAT α* was previously seen in an intact RE in which the two Mat α 2-binding sites were mutated (Wu et al. 1998).

From the various arrangements of A, C, D, and E domains that activate *HML* usage, it is clear that the spacing and orientation of these regions are not critical for RE activity. What seems to be common to all of the different active arrangements of sequences is the presence of multiple sites where either Fkh1p or Fkh2p can bind (Fig. 2). Consistent with this observation is the fact that the *fkh1 Δ* single mutant or the *fkh1 Δ fkh2 Δ* double mutant show reduced *HML* usage in *MATa* cells. This is especially evident in the 4 \times A or 5 \times E constructs in which donor preference is reduced from ~65% to <10%. However, donor preference is not completely abolished when *FKH1* and *FKH2* are deleted in a strain carrying an unmodified RE (compare KS323 in Fig. 6A to the complete absence of RE, XW676, in Fig. 3: *HML* was used ~39% vs. 10%). The residual RE activity indicates that additional proteins contribute to RE function, probably interacting with other sequences.

The Fkh1p and Fkh2p proteins have been best understood in their role in activating transcription of the *CLB2*-cluster of cell cycle-regulated genes including *CLB2* and *SWI5* in the G2 phase of the cell cycle (Hollenhorst et al. 2000, 2001; Kumar et al. 2000; Pic et al. 2000; Zhu et al. 2000). A third protein, Ndd1p, is apparently recruited to these promoters by the Fkh proteins, but Ndd1p must have additional functions, as it is an essential protein, whereas even *fkh1 Δ fkh2 Δ* is viable.

We have shown by ChIP that RE and even simple multimers of A, D, or E regions bind Fkh1p, Fkh2p, and Ndd1p. This can occur even in the absence of a Mcm1p-binding site, as in the case of 4 \times A, whereas in *CLB2* cluster promoters, the recruitment of Fkh2p appears to require the binding of Mcm1p (Hollenhorst et al. 2001). We cannot rule out that Fkh2p is able to recruit Mcm1p

to promoters that lack Mcm1p-binding sites (Hollenhorst et al. 2001). In many other respects, the action of the Fkh proteins appears to be quite different from their role in *CLB2*-cluster promoters. First, RE activation does not seem to depend on transcription. In the region including region E, Szeto et al. did find a weak transcript that does not appear to encode a protein (Szeto et al. 1997); however, RE activity does not depend on these sequences because they were absent in the minimum enhancer (Wu et al. 1998). This is most evident in the case of the multimers such as 4 \times A, each unit of which is only 22 bp.

It should be noted that the role of Fkh proteins, in combination with Mcm1p and Ndd1p, is to activate the transcripts of *CLB2*-cluster in G2. Donor preference is not significantly different at different phases of the cell cycle (Wu et al. 1997); indeed, normal *MAT* switching occurs in G1 cells. Recently, it has been shown that Fkh1 and Fkh2 are also associated with genes expressed in G1 and S phase, whereas the binding of Mcm1 could not be detected (Simon et al. 2001). It seems clear that the way the Fkh proteins and Ndd1p act in donor preference is significantly different from their role at G2-regulated promoters.

Despite our progress in delineating the cis-acting domains and some of the key proteins that combine to create RE activity, we still do not know how RE acts to alter the ability of the entire left arm of chromosome III to recombine with the HO-cut *MAT* locus. Our previous results indicated that in *MAT α* cells, the entire left arm is inaccessible for recombination (Wu et al. 1996). We imagine that there must be additional sites along this chromosome arm that serve to alter (perhaps immobilize) the left arm to prevent its participation in recombination, but without impairing transcription, both in *MAT α* cells in which RE is repressed and in *MATa* cells when RE is deleted. The forkhead proteins and Ndd1p bound to RE may then interact with these repressive sites, thus liberating the left arm for recombination.

Materials and methods

Strains

All strains used for donor preference experiments were derivatives of DBY745 (*ho MATa ade1-100 ura3-53 leu2-3,112*). Strains used to monitor *MATa* donor preference carry *HMR α -B*, containing a single base-pair mutation that creates the *BamHI* site (Wu and Haber 1995). They also carry a galactose-inducible *HO* endonuclease gene integrated at the *ADE3* locus (Sandell and Zakian 1993).

All strains used in ChIP were derivatives of W303 provided by C. Fox and G. Ammerer.

All yeast transformations were performed by one-step transformation (Chen et al. 1992) or high-efficiency method (Gietz et al. 1995, 1997). All the disruptions were verified by Southern blot analysis. To disrupt *FKH1*, a PCR fragment was amplified from the strain that has $\Delta fkh1::KAN$ (Record #32290) obtained from ResGen. To introduce subfragments into a deletion of the RE, a plasmid pKS58 was constructed from pCWU155 (described previously; Wu et al. 1998) except two more unique sites, *BamHI* and *PmlI*, were introduced adjacent to *Sall*. De-

tails of other specific strain constructions are available on request. The selections of introduction of subfragments into a deletion of the RE were performed as described (Wu and Haber 1996).

Identification of the RE in *S. bayanus*

An *S. bayanus* clone containing genes homologous to *KAR4* and *SPB1* of *S. cerevisiae* was obtained as part of the study of the evolution of hemiascomycetes (Bon et al. 2000). The *S. bayanus* DNA was inserted into plasmid pBAM3 in *Escherichia coli* strain DH10B (as described in Bon et al. 2000). The intervening region was sequenced and aligned with RE regions from *S. cerevisiae* and *S. carlsbergensis* (Wu et al. 1998). A 3.1-kb fragment containing the conserved *S. bayanus* sequences was inserted in place of the 1.8-kb deletion of *S. cerevisiae* RE, as described previously for testing the *S. carlsbergensis* RE (Wu et al. 1998). The *S. bayanus* sequence has the GenBank accession no. AY123283.

Analysis of donor preference

The measurement of donor preference was described previously (Wu and Haber 1995, 1996). The bands (*MAT* α and *MAT* α -BamHI) corresponding to the use of *HML* and *HMR* were quantified using ImageQuant V1.2 (Molecular Dynamics). In some experiments, YEP- raffinose was used instead of YEP-lactate medium.

One-hybrid screen

The yeast MATCHMAKER one-hybrid system was purchased from CLONTECH. The screening was essentially performed as recommended by the manufacturer. The *S. cerevisiae* genomic library is provided by Philip James (James et al. 1996). Four tandem copies of 22 bp of wild-type region A were inserted into the polylinker of two plasmids, pHISi-1 and placZi, just upstream of the minimal promoter of *HIS3* and *lacZ* genes to generate pKS133b and pKS132-2. Four tandem copies of 22 bp of nonbinding mutant region A (TA-GC, see KS251 in Fig. 5A) were also inserted into the polylinker of pHISi-1 at the same location to generate pKS165. A wild-type dual reporter yeast strain (KS257) was constructed by integrating pKS133b and pKS132-2 into the genome, and a nonbinding mutant reporter strain (KS259) was also constructed by integrating pKS165 into the genome. Both strains grew poorly on SD (-histidine) + 15 mM 3-aminotriazole (3-AT) and not at all on SD (-histidine) + 45 mM 3-AT plates. The yeast transformation was performed as described by Gietz et al. (1997).

ChIP and analysis of immunoprecipitated DNA

ChIPs were performed as described previously (Strahl-Bolsinger et al. 1997) with some modifications. The cross-linking time was 10 min instead of 15 min. Breakage was performed in 500 μ L of lysis buffer that contained 1 mM PMSF, 1 mM Benzamidine, and 1 mg/mL of Bacitracin. The extract was sonicated for a total of 60 sec (resulting in an average DNA size of 500 bp-1 kb), clarified by centrifugation, and subjected to immunoprecipitation with anti-HA monoclonal antibody (12CA5, Roche) and protein G-Agarose beads (Roche).

PCR reactions were performed in 50 μ L volume with one fifth of the immunoprecipitated material or one fiftieth of the non-

immunoprecipitated material and serial two-fold dilutions thereof as templates. Hotstar Taq polymerase (Qiagen) was used in 1 X manufacturer's buffer supplemented with 2.5 mM MgCl₂, 200 μ M each dNTP, and 500 nM or 1 μ M of each primer. Typically, 25-30 cycles of amplification were performed. The following primers were used to amplify the RE region: SUN575, 5'-CGCGGATCCAAACTCGAAAAGTAAATA-3', and Wu027, 5'-ACGCTCGAGCCCGGGCTTGCAAATATTGT-3'; SUN540, 5'-CTTGTCGACATAAAATCCTTGCTTAGC-3', and P-MAB09, 5'-CAATACCTTCTTGAACCATTTCCC-3'; CDC20 promoter region: SUN844, 5'-ACGTTAGTTGAACCTGAATTCCG-3', and SUN845, 5'-AAGGTGATAAAATCTTTGCCTGC-3'; CLB2 promoter region: SUN842, 5'-GACAGATTTTATTCCAAATGCGG-3', and SUN 843, 5'-CGCTTTTCAGAAGTATCAA TTCCG-3'; ARG5,6 coding region: ARG5,6 p1, 5'-CAAGGATC CAGCAAAGTTGGGTGAAGTATGTA-3', and ARG5,6 p2, 5'-GAAGGATCCAAATTTGTCTAGTGTGGGAACG-3'.

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***Saccharomyces* forkhead protein Fkh1 regulates donor preference during mating-type switching through the recombination enhancer**

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An advertisement banner for Dharmacon Reagents and Horizon. On the left, it says "Dharmacon™ Reagents" with the tagline "Custom synthesis, RNAi, and CRISPR solutions". In the center, the text "Infinite Reliability" is displayed in large white font. To the right, the "horizon" logo is shown in white, with "a PerkinElmer company" underneath. A "More" button is visible in the bottom right corner of the banner. The background features a colorful, abstract representation of DNA or protein structures in shades of purple, blue, and green.