Saccharomyces forking 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. MATa cells recombine with HMLa ~90% of the time, whereas MATα cells choose HMRα 80%–90% of the time. HML preference in MATa 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 forking proteins Fkh1p and Fkh2p and their associated Ndd1p, although there are no adjacent open reading frames (ORFs). Deletion of FKH1 significantly reduces MATa's use of HML, as does mutation of the Fkh1/Fkh2-binding sites in a multimer of region A. We conclude that Fkh1p regulates MATa donor preference through direct interaction with RE.

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

Received April 4, 2002; revised version accepted June 18, 2002.

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-Ya or -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 Ya sequences [HMLα], whereas HMR carries Ya [HMRα]. One of the remarkable aspects of this process is the phenomenon of donor preference, in which MATa cells preferentially recombine with HMLa, ensuring that recombination will usually result in a change of mating type; similarly, MATα cells preferentially select HMRa [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 MATa cells will preferentially recombine with HMLα even versus HMRα [Weiler and Broach 1992]. Moreover, MATa'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]. MATa cells continue to choose a right-arm donor even if HMR is replaced by HML sequences, again regardless of whether the donor carries Ya or Yo [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 MATa 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-
cated that RE acted to make the left arm of chromosome III “hot” for recombination in MATa cells, whereas in MATa cells the left arm was made unusually inaccessible for recombination [thus allowing HML 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 \textit{S. cerevisiae} and \textit{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 \textit{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 1998). It is likely that an additional region, E \{Fig. 2\}, which shares strong sequence homology with D, especially in \textit{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 1998). 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 MATa donor preference is not yet understood; to date only one trans-acting mutation, a deletion of \textit{CHL1}, has been found that alters MATa donor preference, and in this case the effect is modest [Weiler et al. 1995]. We have a better idea how RE works to promote MATa donor preference. In the present study, we have further characterized the sequences that are critical for RE activity in MATa cells. We have refined the identity of these sequences by sequencing the RE region from \textit{S. bayanus}. We show that

Figure 1. Mating-type switching at the \textit{MATa} locus. \textit{(A)} Expression of HO endonuclease creates a double-strand break (DSB) at the Y/Z1 border of the \textit{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 MATa 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. \textit{(B)} HMRa is replaced by HMRa modified to carry a point mutation that creates a BamHI site, so that it is possible to distinguish which sequences have been gene converted into the \textit{MAT} locus. Donor preference is assayed in populations of cells induced to switch from MATa to either MATa or MATa-B [Wu and Haber 1995]. MATa cells recombine with HML \(-90\% of the time in the presence of RE, when RE is deleted, MATa cells prefer to choose HMR \(80\%-90\% of the time.\)
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 fkh1Δ/H9004 strain has a marked reduction in MATα 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 MATα’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...
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 (E113) also
contains TTT(G/A) repeats [bp29512–bp29532, designated E21, boxed in Fig. 2].

To investigate whether the other important cis-acting elements other than A, C, and D contribute to the func-
tion 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
HO
a
MAT
a, which can use either
YEP-galactose for 1.5 h to induce gene conversion of





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, CWa81, 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 chromo-
some 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 prefer-
ence is shown as the percentage of HML usage in MATa cells.
Important region E is from −29423 to −29535 bp as indicated
above.

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 C55
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 C55 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

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 con-
served 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 Mcm1p-
binding site (designated C55, 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

was inserted in the opposite orientation as the normal
RE, but previous studies had shown that RE is orienta-
tion 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.
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. 4A, 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.

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, E50, and E113 are indicated in Fig. 2. All other regions, A, C, and D, used here contain full length of consensus sequences.

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 consensus A region was shown to contain a potential binding site (5’-GTAAATAAA-3’) for the forkhead family of transcription factors (Fig. 5B). Similar pu-
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 functions 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).
but there was a significant decrease of HML donor preference in the fkh1 or the fkh1 fkh2 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 fkh1 fkh2 strain (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 RE 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 fkh1 fkh2 mutant is similar to that of the single fkh1 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 Leu2+ prototrophs. The wild-type MATa and MATa 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).

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 MATa (from HMLα) or MATa-Bam (from HMRa-Bam) can be assessed densitometrically on a Southern blot of DNA digested with BamHI and HindIII 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 MATa 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).
frequently in MATa cells than in MATα [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 MATα. In the leu22 recombination assay, we found a very similar effect as we did in the direct monitoring of the use of HML and HMR in the fkh1Δ or fkh1Δ fkh2Δ mutant. As shown in Figure 6B, numbers of Leu+ papillae were much lower in the fkh1Δ fkh2Δ double mutant than in the wild-type MATa cells, and all the patches had the lower number of Leu+ papillae as seen in MATα 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 MATα cells and approximately a seven-fold difference between wild-type MATa cells and fkh1Δ MATa cells. Thus, we concluded 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. The fkh1Δ mutant has the same effect on both donor preference in HSU-1 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 assay says 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 pHI5-1 and pLacZi reporter plasmids and then integrated into the yeast genome of YM4271 [Materials and Methods]. We screened 5 × 106 transfectants 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 preference, 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 versions 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-lp 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 MATα 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 MATα cells. To ask whether Fkh1p can also be recruited to the RE in MATα cells, MATα cells carrying FKH1–3xHA were switched to MATα 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 MATα 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 × A, 6 × D, or 5 × 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 × A, 6 × D,
and $5 \times E$, 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-Matα/Hα2p-binding site. It has been shown that Mcm1p-Matα plays a role in the repression of RE because of its ability to organize highly positioned nucleosomes that lead to the repres-
sion of RE activity in MATα cells [Weiss and Simpson 1997; Wu et al. 1998]. In MATα 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 × A regions, as well as 4 × D or 4 × 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 Mcm1 could be recruited to the synthetic REs directly by binding to the Fkh2 protein [Hollenhorst et al. 2001] cannot be excluded, although fhk2Δ did not affect donor preference.

The C region and its Mcm1p-Mata2p-binding site do seem to be essential for the repression of RE in MATα cells. The absence of a Mcm1p-Mata2p site in the 4 × A construct leads to constitutive RE activity in MATα cells, although the lower usage of HML in MATα cells [50%] versus MATα [66%] indicates the possibility of a Mata-specific gene product that may contribute to the full activation of RE in MATα cells. This difference between MATα and MATα was previously seen in an intact RE in which the two Mata2-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 fhk1Δ single mutant or the fhk1Δ fhk2Δ double mutant show reduced HML usage in MATα cells. This is especially evident in the 4 × A or 5 × 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 region E, Szeto et al. did find a weak transcript including region E, Szeto et al. did find a weak transcript (Wu et al. 1998). 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 MATα 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 MATα cells in which RE is repressed and in MATα 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 MATα ade1–100 ura3–53 leu2–3,112]. Strains used to monitor MATα donor preference carry HMRα-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 ∆fhk1::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 SalI.
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 [MATa and MATa-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 as previously described 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, pHSi-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 pHSi-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) and not at all on SD (-histidine) + 15 mM 3-AT. Both strains also grew poorly on SD (-histidine) + 15 mM 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 µL of lysis buffer that contained 1 mM PMSE, 1 mM Benzmanidine, and 1 mg/mL of Bacintrin. 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 µL 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 µM each dNTP, and 500 nM or 1 µM of each primer. Typically, 25–30 cycles of amplification were performed. The following primers were used to amplify the RE region: SUN575, 5'-CCGCGATCCAAACTCGAAAAGTAAATA-3', and Wd027, 5'-ACGCTCGAGCGCCGGCTGCAAAATATTGT-3'; SUN540, 5'-CTTGTCGACATAAATCCTTGCTTAGC-3'; and P-MAB09, 5'-CAATTACCTTTTGAAACCATTTTCC-3'; CDC20 promoter region: SUN844, 5'-ACGTTATGTGAATCTTGGAAATCC-3', and SUN845, 5'-AAGTGTAATAATCCTTGCCAAG-3'; CLB2 promoter region: SUN842, 5'-GACAGATTGTATTCCCAATGCCG-3', and SUN 843, 5'-CGCTTTTCAAGAATATCC-3'; ARS5,6 coding region: ARS5,6 p1, 5'-CAAGGATGCAGGAAAATTTGGGAG-3', and ARS5,6 p2, 5'-GAAGGATCCAAATTGTGTAAGTGTGGGAACG-3'.

Acknowledgments

We are grateful to Catherine Fox and Gustav Ammerer for providing us strains, Philip James for providing the yeast genomic library, Ranian Sen for advice and critically reading the manuscript, Cherry Wu for her early work on mutations of region A, and Neal Sugawara for his invaluable technical advice. This work was supported by the National Institutes of Health [grant GM20056]. E.C. was supported in part by a grant from l’Association pour la Recherche sur le Cancer.

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Genes Dev. 2002, 16:
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