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Cell Cycle-Dependent Regulation of *Saccharomyces cerevisiae* Donor Preference during Mating-Type Switching by SBF (Swi4/Swi6) and Fkh1

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***Saccharomyces* mating-type switching occurs through a double-strand break-initiated gene conversion event at *MAT*, using one of two donors located distantly on the same chromosome, *HML*α and *HMR*a. *MAT*a cells preferentially choose *HML*α, a decision that depends on the recombination enhancer (RE) that controls recombination along the left arm of chromosome III. We previously showed that an *fhk1*Δ mutation reduces *HML*α usage in *MAT*a cells, but not to the level seen when RE is deleted. We now report that donor preference also depends on binding of the Swi4/Swi6 (SBF) transcription factors to an evolutionarily conserved SCB site within RE. As at other SCB-containing promoters, SBF binds to RE in the G_1 phase. Surprisingly, Fkh1 binds to RE only in G_2, which contrasts with its cell cycle-independent binding to its other target promoters. SBF and Fkh1 define two independent RE activation pathways, as deletion of both Fkh1 and SCB results in nearly complete loss of *HML* usage in *MAT*a cells. These transcription factors create an epigenetic modification of RE in a fashion that apparently does not involve transcription. In addition, the putative helicase Chl1, previously involved in donor preference, functions in the SBF pathway.**

Mating-type switching in *Saccharomyces cerevisiae* is initiated by the expression of the HO endonuclease, which creates a double-strand break (DSB) at a specific site within the *MAT* locus. The DSB is repaired by a gene conversion event using one of two donor loci, *HML*α and *HMR***a**, located near the left and right subtelomeric regions, respectively (20, 21). These donors are maintained in a heterochromatic stage; they are not transcribed and are refractory to HO endonuclease cleavage (39, 47, 64).

Although *MAT* is able to use both *HML* and *HMR* to repair the break, there is a strong mating-type-dependent preference in the choice between the two donors. *MAT***a** cells preferentially recombine with *HML*, whereas *MAT*α selects *HMR* (33, 61, 67, 68). *HML* usage is mainly regulated, while *HMR* is used by default (67). Donor preference is independent of whether the donor carries **a** or α information; moreover, donor selection does not depend on any sequences that uniquely define *HML* or *HMR* or any sequences flanking or distal to *HML* or *HMR* (61, 67). Preferential selection of *HML* in *MAT***a** cells depends on an approximately 700-bp *cis*-acting element, the recombination enhancer (RE), which is located 17 kb centromere proximal to *HML* (66). Deletion of RE causes reversed donor preference in *MAT***a** cells: *HML* usage is reduced to 10% compared to 90% in the wild type (WT) (66). However, RE deletion does not affect donor preference in *MAT*α cells, showing that RE is simply turned off in these cells and is not responsible for the inhibition of *HML* usage.

In fact, RE regulates the entire left arm of chromosome III for recombination. In *MAT***a**, when *HML* is moved to other locations of the left arm of chromosome III it is still the preferred donor (66). In addition, RE activity is not limited to mating-type switching. The rate of spontaneous recombination between two different *leu2* alleles, one replacing *HML* and the other located near *MAT*, is ≥10 times higher in *MAT***a** versus *MAT*α strains (67). This difference is lost when RE is deleted (66).

Recently we showed that the two arms of chromosome III can be defined as two independent domains and that RE controls the accessibility between these domains (10), probably by increasing the movement and/or conformation of the left arm in the nucleus (4). Thus, when *MAT* is moved onto the left arm, it becomes the preferred donor in both *MAT***a** and MATα cells.

RE is well conserved in the *Saccharomyces* sensu stricto species (30; *Saccharomyces* genome database [www.yeastgenome.org]). Comparison of RE sequences from *S. cerevisiae*, *Saccharomyces carlsbergensis*, and *Saccharomyces bayanus* (53, 65), and also from *Saccharomyces mikatae* and *Saccharomyces kudriazvezii*, defines five highly conserved regions A, B, C, D, and E. Deletion studies show that each of these regions with the exception of B is necessary for complete RE activity (53, 68). Regions A, D, and E contain Fkh1 binding sites, and this transcription factor has been shown to play an essential role in *HML* activation in *MAT***a** cells (53). Fkh1 was first described as a component of the SFF complex, involved in the regulation of transcription of the *CLB2* cluster of genes at the transition between the G_2 phase and mitosis.

RE is regulated very similarly to **a**-specific genes (65). Binding of the Matα2-Mcm1 repressor complex to the region C inactivates RE in *MAT*α cells, and binding of Mcm1 activates RE in *MAT***a** cells (54, 55, 65, 68). As at **a**-specific genes, binding of the Matα2-Mcm1 complex to RE in *MAT*α cells is

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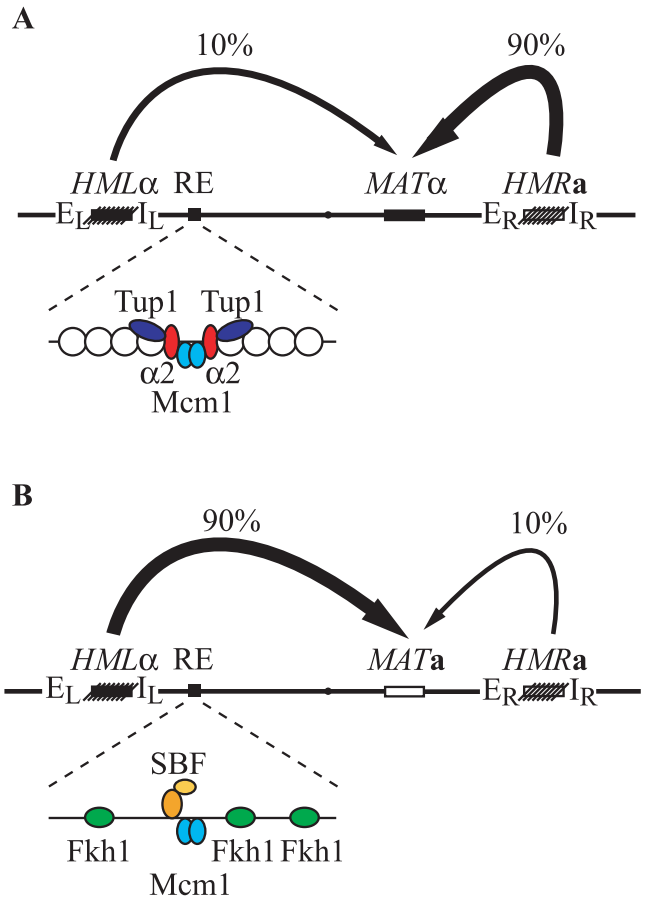

FIG. 1. RE regulation and donor preference during mating-type switching in *Saccharomyces cerevisiae*. (A) Donor preference in *MAT*α cells. A schematic representation of the mating-type locus (*MAT*), the two silent donor loci (*HML* and *HMR*), and the RE is shown. In *MAT*α cells, the Matα2-Mcm1 complex recruits the general repressor Tup1 to RE, inactivating RE by formation of highly organized nucleosomes, resulting in the preferred use of *HMR*. (B) Donor preference in *MAT***a** cells. Mcm1 binds to the RE and removes the positioned nucleosomes, allowing binding of *trans*-acting factors, including Fkh1 and SBF, leading to activation of RE and consequently to the favored usage of *HML*.

linked to the formation of a highly organized array of nucleosomes, covering the entire 2.5-kb intergenic region containing RE (63) (Fig. 1A). Formation of this chromatin structure is dependent on Tup1 but not Ssn6 (63, 65). This structure is absent in *MAT***a** cells when Mcm1 binds RE in the absence of Matα2 (Fig. 1B). Mcm1 function is limited to this opening, which allows *trans*-acting factors like Fkh1 to bind RE (14, 53). Recently, the DNA repair protein yKu80 has been shown to be involved in the activation of RE (48).

Because *fkh1*Δ does not reduce *MAT***a** donor preference as much as deletion of RE, we looked for additional regulatory elements within RE. We report that an SCB (Swi4/Swi6 cell cycle box), present in region C, is important for RE activity. SCB is usually found in promoters of genes induced at the transition from G_1 to S phase. Activation of these genes depends on the SBF complex (Swi4/Swi6 cell cycle box binding factors) (3). SBF is necessary for complete RE activity, but its role in donor preference apparently does not involve its G_1/S transcription activity. We found that SBF binds RE in G_1/S, while Fkh1 binds in the G_2 phase. SBF and Fkh1 activate RE through two independent pathways. Our genetic analysis also shows that the putative ATP-dependent DNA helicase Chl1 (18, 19, 24), previously shown to be involved in donor preference (62), acts through the pathway that involves SBF. We propose that the activation of the left arm of chromosome III for recombination requires the establishment of signals in both G_1 and G_2 to create a specific organization of chromosome III in the nucleus that leads to the preferential choice of donors present on the left arm.

MATERIALS AND METHODS

Strains. The *swi4* strain was kindly provided by Brenda Andrews and is derived from BY263 (41). Donor preference in this strain was measured in parallel in a wild-type strain of the same background. All other strains used for donor preference experiments were derivatives of DBY745 (*ho MAT***a** *ade1-100 ura3-53 leu2-3,112*). Strains used to monitor *MAT***a** donor preference carry *HMR*α-BamHI, where Y**a** has been replaced by a Yα allele containing a single-base-pair mutation that creates the BamHI site (67), while strains used to monitor *MAT*α donor preference carry *MAT*α-BamHI. All strains have a galactose-inducible *HO* endonuclease gene integrated at the *ADE3* locus (49) or present on a *LEU2* centromeric plasmid (pJH727).

All yeast transformations were done by one-step transformation (7). *SWI6*, *FKH1*, *MBP1*, *ACE2*, *STE12*, *NDD1*, *KAR3*, *CTF4*, *CTF18*, *BIM1*, *NUP170*, *YKU70*, *YKU80*, and *YDR332W* deletions have been made by transformation of PCR-amplified fragments obtained from genomic DNA of the Research Genetics strain collection. The *chl1*Δ::*URA3* deletion was made using the split-vectors system (16) with the plasmids pUR-pl008 and pRA-pl008 provided by G.-F. Richard. The *cdc7-as3* mutant strain has been described before (26) and is suitable to measure donor preference. The G_1::*GAL*::*HO* construct (43) was introduced by a cross with JKM95 (42). *MAT*α*1* has been disrupted with a PCR fragment containing the *KanMX* gene flanked by 50-bp sequences identical to those flanking the *MAT*α*1* gene. This PCR has been performed using the pFA6 plasmid containing the *KanMX4* cassette (60) and mixed primers composed of *MAT*α*1* and pFA6 sequences (MATα1KanU, TATGAAATGTATCAACCATATAT AATAACTTAATAGACGACATTCACAATCAGCTGAAGCTTCGTACGC; and MATα1KanL, AGTCCCATATTCCGTGCTGCATTTTGTCCGCGTGCCATTCT TCAGCGAGCGCATAGGCCACTAGTGGATCTG). Y**a** has been deleted in the same way with the primers MatKanP1 (TAGGTAAATTACAGCAAATA GAAAAGAGCTTTTTATTTATGTCTAGTACAGCTGAAGCTTCGTACGC) and MatKanHO+L (AATCATTGAAAACGAATTTATTTAGATCTCATACGT TTATTTATGAACTAGCATAGGCCACTAGTGGATCTG), which conserved the integrity of the HO cut site.

All the modified RE sequences have been reintroduced in strains bearing a deletion of the recombination enhancer as previously described (65).

The strain bearing the Chl1-9xMYC fusion protein was made using the method of Longtine et al. (38) in the DBY745 background. Strains bearing Fkh1-3xHA have been described previously (53). Mcm1-13xMyc was kindly provided by B. Tye (6).

Analysis of donor preference. Quantification of donor preference on Southern blotting was previously described (65), using ImageQuant V1.2 (Molecular Dynamics). Differences among strains when *HML* usage is either >90% or <10% are not statistically significant.

Measurement of spontaneous recombination rates. Spontaneous formation of $Leu2^+$ recombinants was quantified by a fluctuation test based on a minimum of nine independent cultures of each strain, initiated from approximately 200 cells and grown to saturation (36).

ChIP. Chromatin immunoprecipitation (ChIP) analysis was performed as in reference 53, except for the use of an anti-MYC monoclonal antibody (Sigma) and protein A-agarose (Roche). ChIP of Swi4 and Swi6 used polyclonal antibodies provided by B. Andrews. The following PCR primers were used for amplification: for the RE region, SUN575 and Wu027; the *CLB2* promoter region, SUN842 and SUN843; the *ARG5,6* coding region, ARG5,6p1 and ARG5,6p2 (53); the *PCL1* promoter, prPCL1U (GCATTTGCTTACCAAACT GGC) and pRPCL1L (CAATCCCATTACCATGTAGGC), the *SUN4* promoter, SUN4pU (GGTTACCCGACATATATGCTGG) and SUN4pL (CATG CTGAAGGGAACGTGCG) and the *PHO3* locus, AW253-PHO35′RT (GGA GAGTTAGCCGATGTTGC) and AW254-PHO33′RT (TAGTCGCCAGGGA AAGAGAA).

Cdc7 inactivation, α-factor, and nocodazole arrest. Cdc7 was inactivated in *cdc7-as3* strains by adding the ATP analogue inhibitor 1-NMPP1 at 10 μM. *cdc7-as3* cells were usually arrested 1 doubling time after addition of the drug, and the *HO* gene was then induced by the addition of galactose to a final concentration of 2%. Cells were arrested in G_1 with 10 μg/ml α-factor. Hydroxyurea (HU)-arrested cells were first synchronized with 10 μg/ml α-factor, washed, and then released in the presence of 50 μg/ml pronase and 0.2 M HU. Cells were arrested in G_2/M with 20 μg/ml nocodazole.

RESULTS

Search for region C sequences necessary for RE activity. Because the conservation of region C sequences goes well beyond the Matα2-Mcm1 operator among *Saccharomyces* sensu stricto strains, we examined this region for potential protein binding domains that could be necessary for full RE activity. We took advantage of the newly available genome sequences from several *Saccharomyces* species (9, 30) to define a more precise consensus of this region. RE is conserved in *Saccharomyces* sensu stricto species, but not in the more evolutionarily distant *Saccharomyces* species (sensu lato). Analyzing the five *Saccharomyces* sensu stricto species available (Fig. 2A), we confirmed that the entire region C is very well conserved. Therefore, sequences other than the Matα2-Mcm1 operator could be important for RE function. We decided to conduct a mutational analysis of the whole region.

A 35-bp deletion covering the entire Matα2-Mcm1 operator (Fig. 2B) confers partial RE activity (43% *HML* usage compared to 5 to 10% when RE is deleted). This partial activity allowed us to carry out a deletion analysis of the region C sequences adjacent to the Matα2-Mcm1 operator. Partial deletions were made in a 753-bp RE in vitro, and the different constructs were inserted in place of a 1.8-kb sequence containing RE and other intergenic sequences between *KAR4* and *SPB1*. A 53-bp deletion confers the same phenotype as the 35-bp deletion; however, 75- and 95-bp deletions show a reduction of *HML* usage to approximately 30%. Therefore, at least part of the 22-bp sequence between the 53- and 75-bp deletions is necessary for full RE activity. To identify more precisely the key DNA sequences, we made 2-bp mutations at positions perfectly conserved among the *Saccharomyces* sensu stricto species. Two such mutations were introduced in the 753-bp RE and inserted in place of the 1.8-kb sequence containing RE. A TC→GA mutation at positions 29181 to 29182 (ECY380) in the 22-bp sequences defined above reduced *HML* usage from 75% in the wild type to 27% in the mutant. We also used a 270-bp minimum RE lacking region E to introduce additional mutations in the chromosome (65). This minimum RE retains significant donor preference activity because strains carrying this insert use *HML* 45% of the time, whereas strains with the 1.8-kb deletion use *HML* 5% of the time. A CA→TG mutation in the sequence to the right of the Matα2-Mcm1 operator (Cwu182) does not show a strong reduction in *HML* usage, suggesting that this part of region C is not fundamental for RE activity. A TT→CC mutation in the 22-bp sequence defined above (Cwu181) does not affect *HML* usage, but the nearby TC→GA mutation strongly reduced *HML* usage, confirming our observation with 753-bp RE. These two results allowed us to define a new RE element within a 13-bp fragment. Using MatInspector V2.2 software (46) to look for consensus protein binding sites, we found a perfect match corresponding to the 8-bp reverse complement sequence (TTTTC GTG) of the well-defined Swi4,6-dependent cell cycle box (SCB). This result suggested that Swi4/Swi6 complex (SBF), which binds the SCB (3), could be involved in RE activation.

This SCB is perfectly conserved in *S. mikatae* and in *S. kudriazvezii* but somewhat degenerate in *S. carlbergensis* and in *S. bayanus* (Fig. 2A). However, genomic studies of the binding sites of SBF in *S. cerevisiae* show that 49% of the intergenic regions binding SBF do not contained a motif matching the defined SCB consensus (27). In addition, extensive study of the different Swi4 binding sites in the *S. cerevisiae* genome revealed that the TTTTCGCT sequence found in *S. bayanus* corresponds to one such site (8). We conclude that this element is probably conserved in these yeasts. The relative conservation of the SCB among the *Saccharomyces* sensu stricto species reinforces the idea that SBF is important for RE activation.

The Swi4/Swi6 complex is involved in RE activity. We measured *HML* usage in strains bearing a replacement of *HMR***a** by *HMR*α-BamHI (67), allowing the discrimination on Southern blots of *MAT*α and *MAT*α-BamHI products (Fig. 3A). *HML* usage is significantly reduced in strains with *SWI4* deleted (30%) compared to the WT (70%), showing that the SBF complex is involved in RE activation in *MAT***a** cells. The effect of the *swi4* deletion is similar to the *fkh1* deletion we observed previously (53) (Table 1). To confirm the importance of SBF, we tested the effect of *swi6*Δ on spontaneous intrachromosomal recombination between two *leu2* alleles. In this assay, *leu2-R* was inserted between *HML* and RE and *leu2-K* was placed near *MAT* (Fig. 3B). In wild-type cells, the rates measured in *MAT***a** cells are 10-fold greater than in *MAT*α, in an RE-dependent manner. We showed previously that deletion of *FKH1* decreases spontaneous *leu2* recombination in *MAT***a** cells (53). Here, we found that *swi6*Δ also reduces the formation of Leu^+ recombinants. Measurement of the spontaneous recombination rates by a fluctuation test (36) showed that *swi6*Δ caused a threefold reduction in the rate. Thus, the absence of the SBF complex leads to a reduction of *HML* usage and of *leu2* spontaneous recombination. We conclude SBF takes part in RE activation.

To rule out that any G_1/S-specific gene under the control of SBF is involved in donor preference, we measured *HML* usage (Fig. 3C) in a strain bearing both a deletion of *SWI6* and the TC→GA mutations in RE^{SCB} (Fig. 2B). The effect of the double mutant (45%) was similar to either single mutant: *swi6*Δ, 48%; and RE^{SCB}, 49%. Therefore, we conclude that SBF acts directly by binding to RE.

Binding of Swi5 to the promoter of the *HO* gene is necessary to recruit the chromatin remodeling factors Swi2/Snf2 and SAGA, which in turn allow SBF to bind (11). However, deleting *SWI5* does not affect *HML* usage (data not shown), indicating that SBF binding to RE is Swi5 independent. We also observed that ablation of the *WHI5* protein, which binds to SBF to repress G_1-specific transcription during early G_1 (12, 13), does not affect *HML* usage (data not shown).

Fkh1 and both SBF and Chl1 define two independent pathways involved in RE activation in *MAT*a cells. Previous studies provided evidence that Fkh1 (53) and Chl1 (62) are involved in *MAT***a** donor preference. To determine if these two proteins and SBF act together or in different pathways, we measured *HML* usage in single, double, and triple mutants derived from

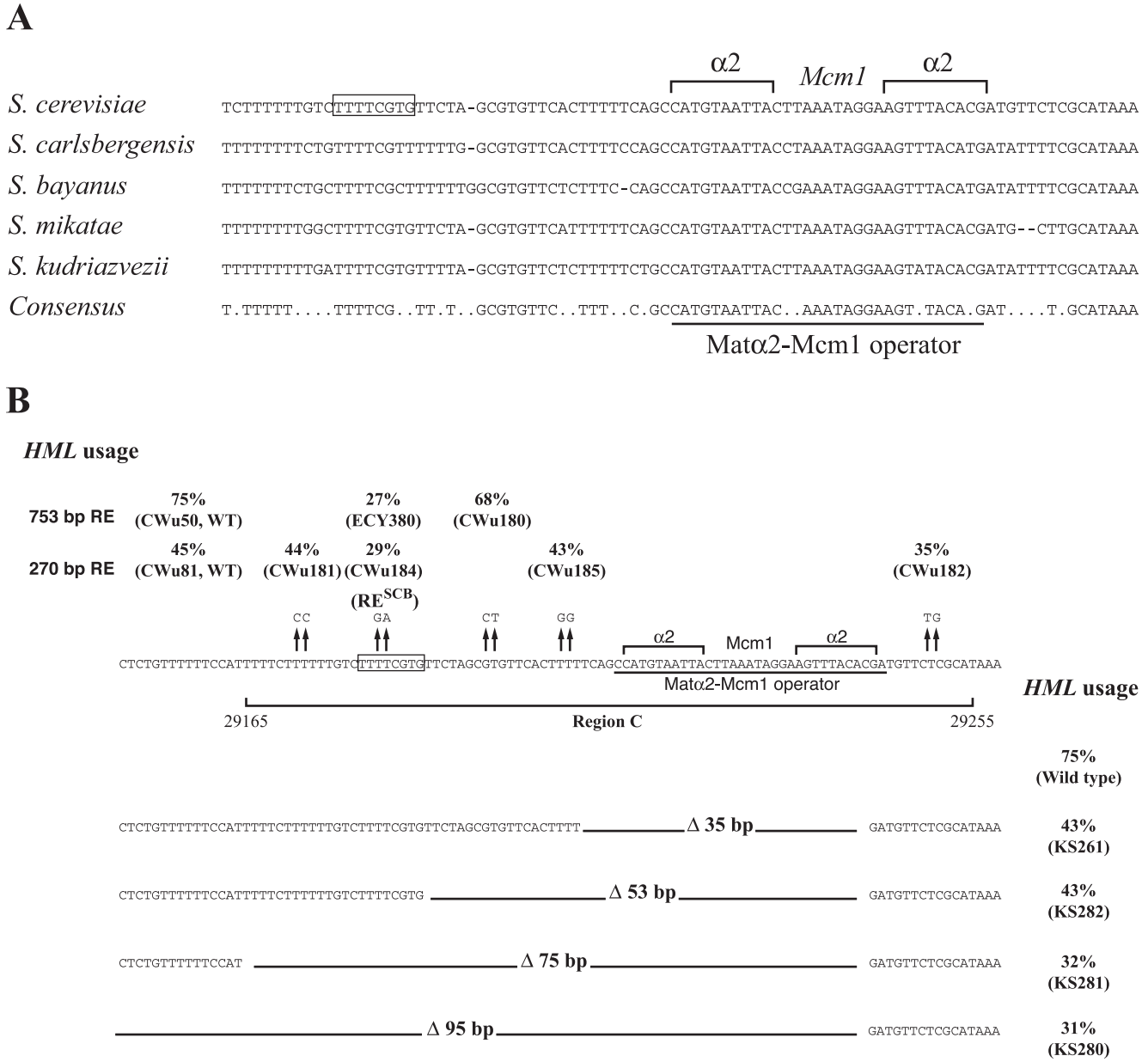

FIG. 2. Deletion and mutational analysis of region C shows that an SCB is necessary for complete RE activity. (A) Sequence comparison of region C among the S*accharomyces* sensu stricto species. The MATα2-Mcm1 operator is shown. Sequences enclosed by the box correspond to the SCB necessary for RE activity (reverse complement of the consensus). Only perfectly conserved residues between the five species are indicated in the consensus sequence. (B) (Top) *HML* usage in strains bearing different mutations in region C. Some of these mutations have been introduced in the 270-bp synthetic RE which lacks region E (lower lane). Others were introduced in the 753-bp minimal RE (upper lane). (Middle) Sequence of region C. The Matα2-Mcm1 operator is underlined, and the SCB is shown in the box. (Bottom) *HML* usage in strains bearing the 753-bp minimal RE with several deletions of region C sequences.

diploid strains heterozygous for *fkh1*Δ and *chl1*Δ and for the RE^{SCB} mutation. We confirmed the involvement of *CHL1* in *HML* activation in *MAT***a** cells (Table 1); the reduction in *HML* usage in the *chl1* mutant (to 50%) is less than that in the *fkh1* or RE^{SCB} mutant (approximately 30%). The difference in *HML* usage in the RE^{SCB} mutant in this experiment (30%) compared with the previous experiment (49%) is unexplained. *HML* quantification has been performed several times in both strains, and the difference is persistent. Each experiment was conducted with strains derived from two different crosses. The best explanation is that there is an unknown modifier that has arisen in these originally isogenic strains. As shown by reference 62, the involvement of *CHL1* in donor preference is specific for *MAT***a** since *chl1*Δ in *MAT*α cells does not affect donor preference (Table 1).

An RE^{SCB} *chl1*Δ double mutant shows the same *HML* usage as the RE^{SCB} single mutants (30%), suggesting that RE^{SCB} is epistatic to *chl1*Δ and that these two factors act in the same

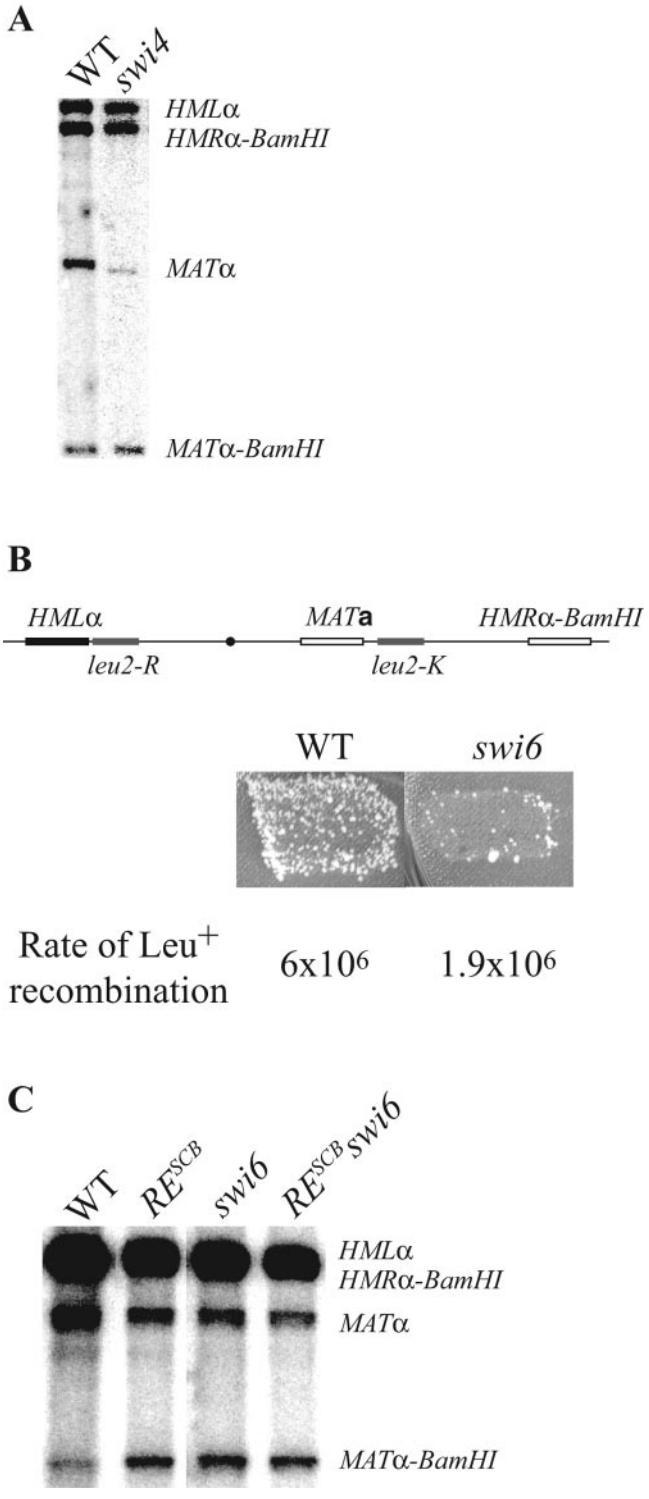

FIG. 3. Donor preference in *swi4* and *swi6* mutants. (A) Southern blot of genomic DNA extracted from wild-type (ECY384) and *swi4* (ECY411) strains after inducting *MAT* switching. Genomic DNA was cut with the BamHI and HindIII restriction enzymes, and the Southern blot was probed with Yα sequences. (B) Papillation test measuring recombination between *leu2* heteroalleles. In wild-type *MAT***a** cells, papillation is strongly reduced in a *swi6* mutant (ECY304). Fluctuation tests confirmed these observations. (C) Southern blot of genomic DNA from wild-type (ECY507), RE^{SCB} (ECY507), *swi6* (ECY508), and RE^{SCB} *swi6* (ECY509) strains analyzed under the same conditions as in panel A.

TABLE 1. Epistatic relationships between RE^{SCB}, *FKH1*, *CHL1*, and yKU80

Strain	Genotype	% *HML* usage[a]
ECY401	*MAT***a**	71.5 ± 1.8
XW676	*MAT***a** REΔ	<10
ECY400	*MAT***a** RE^{SCB}	33.1 ± 2.2
ECY399	*MAT***a** *fkh1*	34.0 ± 2.8
ECY398	*MAT***a** *chl1*	51.8 ± 2.5
ECY494	*MAT***a** y*ku80*	64.1 ± 1.1
ECY13	*MAT*α	<10
ECY111	*MAT*α *chl1*	<10
ECY397	*MAT***a** RE^{SCB} *fkh1*	17.7 ± 3.7
ECY396	*MAT***a** RE^{SCB} *chl1*	29.7 ± 3.9
ECY495	*MAT***a** RE^{SCB} *yku80*	34.8 ± 2.1
ECY496	*MAT***a** *chl1 yku80*	54.7 ± 4.6
ECY395	*MAT***a** *fkh1 chl1*	14.4 ± 2.2
ECY497	*MAT***a** *fkh1 yku80*	15.0 ± 1.4
ECY394	*MAT***a** RE^{SCB} *fkh1 chl1*	<10
ECY499	*MAT***a** RE^{SCB} *fkh1 yku80*	<10
ECY498	*MAT***a** RE^{SCB} *chl1 yku80*	17.8 ± 2.8
ECY500	*MAT***a** *fkh1 chl1 yku80*	14.3 ± 0.7
ECY501	*MAT***a** RE^{SCB} *fkh1 chl1 yku80*	<10
KS338	*MAT***a** 4A	65 ± 2
KS379	*MAT***a** 4A *fkh1*	<10
ECY177	*MAT***a** 4A *chl1*	54.4 ± 1.3

[a] *HML* usage was determined in *MAT***a** strains carrying *HML*α and *HMR*α-BamHI. Genomic DNA extracted from an entire population of cells in which mating-type switching has been induced was cut by BamHI and HindIII and probed with a Yα fragment.

pathway. On the other hand, *HML* usage decreases twofold in the RE^{SCB} *fkh1*Δ (18%) and *chl1*Δ *fkh1*Δ (14%) double mutants. This suggests than Fkh1 activates *HML* through a different pathway from SBF and Chl1. These results define two pathway of *HML* activation in *MAT***a** cells, one involving *FKH1* and another depending on SBF and *CHL1*. However, the RE^{SCB} *fkh1*Δ *chl1*Δ triple mutant shows a statistically significant reduction in *HML* usage (<10%), suggesting that SBF and Chl1 are not fully dependent on each other. *HML* usage in the triple mutant is similar to that observed in *MAT***a** cells with RE deleted (66), showing that *FKH1*, SBF, and *CHL1* act in the major *HML*-activating pathways in *MAT***a** cells.

Another way to study the relationship between these three factors is to compare the effects of deleting *FKH1* and *CHL1* in strains bearing four repeats of region A (4A) instead of RE. The 22-bp region A contains an Fkh1 binding site, and the relatively strong 4A activity (65% *HML* usage) depends completely on *FKH1* (53). This suggests that additional Fkh1 binding sites in tandem can bypass partially the role of SBF. The effect of deleting *CHL1* on *HML* usage in a strain bearing 4A (from 65% to 54%) is similar to the small decrease observed in the RE^{SCB} *fkh1*Δ *chl1*Δ triple mutant compared to the RE^{SCB} *fkh1*Δ double mutant. This result confirms that *CHL1* can act in an SCB-independent manner.

Recently Ruan et al. (48) reported that the DNA end-binding protein yKu80 played an important role in *MAT***a** donor preference. We confirmed that a *yku80*Δ strain reduced *MAT***a** donor preference (Table 1), but the roughly 10% reduction to 64% usage of *HML* was less dramatic than that reported by Ruan et al. (48). *yku80*Δ did not further reduce the effect of *chl1*Δ nor of RE^{SCB}, though—like *chl1*Δ—*yku80*Δ reduced the usage of *HML* in combination with *fkh1*Δ. These data suggest

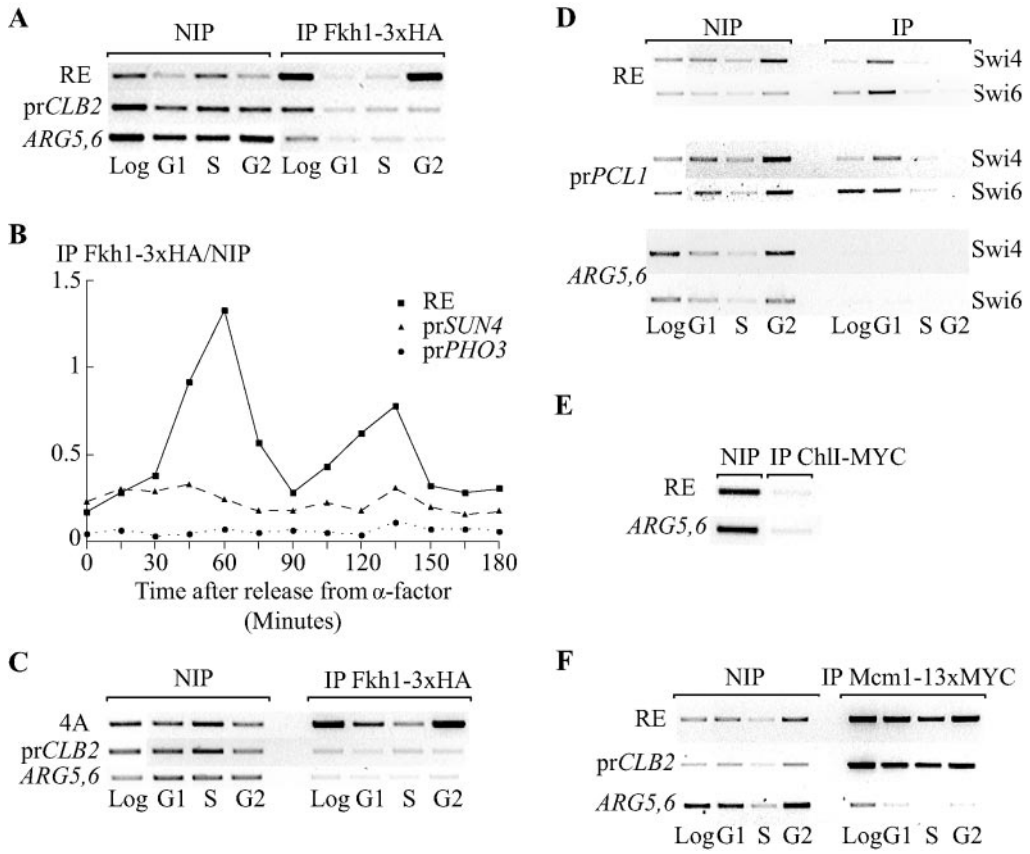

FIG. 4. ChIP analysis of Fkh1, SBF, Chl1, and Mcm1 binding to RE during the cell cycle. (A) Fkh1 binds RE in the G_2/M phase of the cell cycle. ChIP is shown for primers amplifying RE sequences, the *CLB2* promoter, and the unrelated *ARG5,6* coding region in a strain bearing the hemagglutinin (HA)-tagged Fkh1 at the natural *FKH1* locus (CFY480). ChIP was performed on cells in exponential phase (Log), arrested with α-factor (G_1), arrested with α-factor and released in the presence of HU (S), or arrested with nocodazole (G_2). Sequence enrichment was determined by PCR prior to (NIP) and after immunoprecipitation (IP). (B) Fkh1-3xHA binding to RE and to the *SUN4* and the *PHO3* promoters in synchronized cells released from α-factor arrest. The binding efficiency is expressed as the ratio between IP and non-IP values obtained from real-time PCR quantitation. (C) ChIP in a strain bearing four copies of region A instead of RE (KS358). (D) The Swi4/Swi6 complex binds RE in the G_1/S phase of the cell cycle. The *PCL1* promoter sequence was used as a positive control. (E) Chl1 does not bind RE. ChIP assay with primers amplifying RE sequences and the *ARG5,6* ORF on strain ECY266 containing an integrated *CHL1*-9x*MYC* gene at the natural *CHL1* locus. (F) Mcm1 binds RE all along the cell cycle. ChIP experiment with primers amplifying the RE region and the *ARG5,6* coding region in a strain bearing Mcm1 tagged with MYC at the *MCM1* locus (MJF190). All these experiments have been reproduced three times, except for that shown in panel B, which is supported by our data obtained with arrested cells.

that *yku80*Δ is in the same epistasis group as RE^{SCB} (and *swi4*Δ or *swi6*Δ) and *chl1*Δ.

Fkh1 and SBF bind RE at different stages of the cell cycle. SBF binds to SCB-containing promoters of G_1/S-regulated genes during G_1 (34). In contrast, Fkh1 binds to its targets all along the cell cycle (35). We examined the binding of these factors to RE during the cell cycle by ChIP. Fkh1 binding to RE was measured in a strain bearing a functional *FKH1-3xHA* gene integrated in the genome (53). ChIP was performed in extracts from logarithmic phase or from cells arrested in the presence of α-factor (G_1/S) or nocodazole (G_2/M). To study the binding of Fkh1 to RE in S phase, cells were first arrested with α-factor and then released in the presence of HU. The binding of Fkh1 to the *ARG5,6* gene was measured as a negative control. We confirmed the constant, but weak, binding of Fkh1 to the *CLB2* promoter all along the cell cycle (Fig. 4A) as previously described (35). The signal observed is two times stronger than the one obtained after amplification of the *ARG5,6* open reading frame (ORF). Surprisingly, Fkh1 binds RE mostly, if not exclusively, in the G_2/M phase. Fkh1 binding to RE is therefore regulated differently from its binding to the promoters of the *CLB2* cluster of genes. The signal generated by the binding of Fkh1 to RE is five times stronger than the signal generated by the binding to the *BCL2* promoter.

To exclude an effect of nocodazole arrest on the regulation of Fkh1 binding, we measured Fkh1 binding to RE in synchronized cells released from an α-factor arrest (Fig. 4B). Time points were taken every 15 min, the experiment covering approximately 2 generations. Binding of Fkh1 was also measured at the *SUN4* promoter, which is known to bind Fkh1 more efficiently than Fkh2 (22); *PHO3* was used as a negative control. A bud count analysis was performed at each time point to follow the cell cycle. Quantifying the formation of the PCR products with a real-time PCR apparatus, we observed constant binding of Fkh1 to the *SUN4* promoter through the cell cycle; however, the binding of Fkh1 to the RE is clearly cell

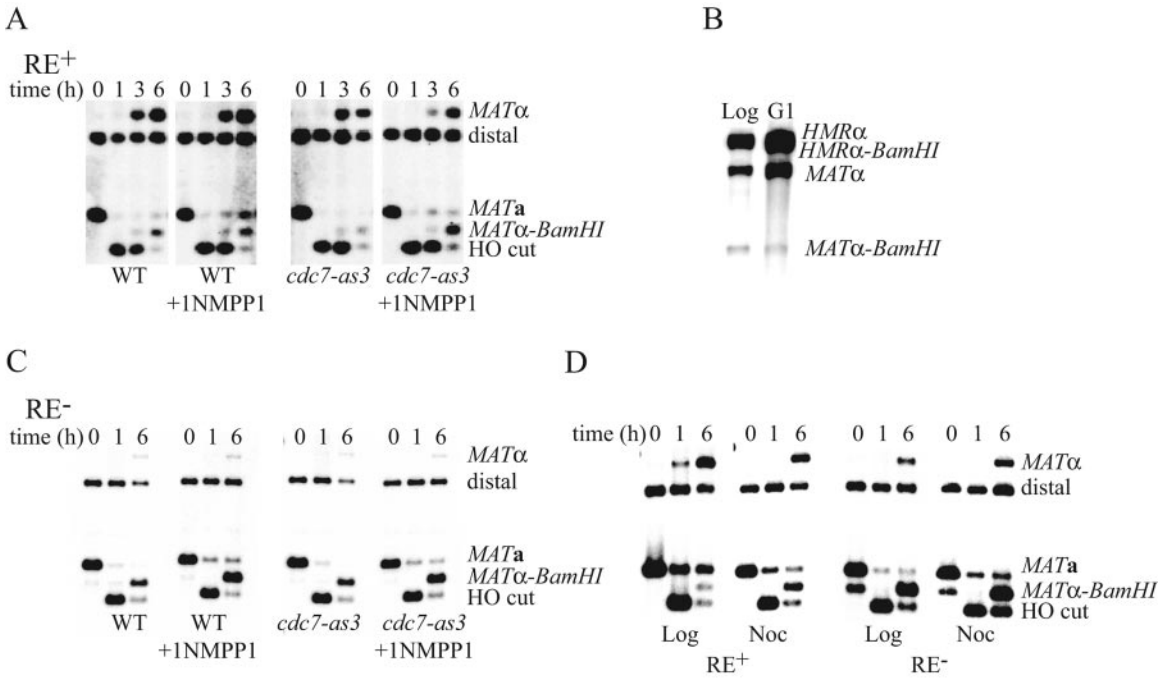

FIG. 5. Donor preference is affected by an arrest in G_1/S and in G_2/M in *MAT***a** cells but not in cells with RE deleted. (A) Time course experiment of *MAT* switching in *MAT***a** *CDC7* (XW652) and *MAT***a** *cdc7-as3* (GI560) cells arrested or not by the ATP analogue NMPP1. Genomic DNA cut with the restriction enzymes StyI and BamHI and probed with a *MAT* distal fragment reveals the parental fragment *MAT***a**, the fragment resulting from the HO cut, and the *MAT*α and *MAT*α-BamHI products. (B) Expression of HO under a Gal promoter specific for the G_1 phase does not affect donor preference in *MAT***a** cells. Genomic DNA of the WT strain used in panel A (XW652) and of a G_1-Gal-HO strain (ECY273) here digested with HindIII and BamHI and probed with Yα sequences shows the two products, *MAT*α and *MAT*α-BamHI. (C) The same experiment as in panel A but carried out in strains with RE deleted, thus mimicking *MAT*α cells (WT, XW676; *cdc7-as3*, ECY252). (D) *MAT***a** switching in strains with RE deleted or not (XW676 and XW652, respectively). *HO* is induced in exponential phase (Log) or when cells are arrested in the presence of nocodazole (Noc). Genomic DNA was cut with StyI and BamHI and probed with a *MAT* distal fragment.

cycle regulated, showing two peaks, 60 and 135 min after release (respectively, showing 18- and 15-fold increases over *PHO3*), where the cells are mostly in the G_2 phase. This confirms our observation made with cells arrested with nocodazole.

To test if regulated binding of Fkh1 to RE in the G_2 phase is linked to a modification of the chromatin structure of RE dictated by region C, we studied the binding of Fkh1 in a strain bearing four copies of region A instead of RE (Fig. 4C). We also observed a strong enrichment of the RE PCR signal in the population of cells arrested in G_2/M. Therefore, the binding of Fkh1 to RE does not fully depend on factors binding to the C domain, such as Mcm1 or SBF.

The same experiment was performed with antibodies directed against Swi4 and Swi6 proteins (Fig. 4D). As a control, we looked at the enrichment of the *PCL1* promoter sequence, a target of SBF (44). Both Swi4 and Swi6 bind RE mostly in the G_1 phase and to a lesser extent in S phase. This regulation of binding is exactly the same as for the binding at the *PCL1* promoter. Thus, we found that Fkh1 and SBF bound RE at two different stages of the cell cycle, which agrees well with the involvement of these two factors in two different pathways of RE activation.

We also studied binding of a Chl1-7xMYC hybrid protein to RE. Addition of the tag to the protein did not affect its function, as no deficiency was observed for donor preference or for chromosome loss, the original phenotype linked to the *chl1* mutation (19; data not shown). However, there was no significant binding of Chl1 to RE (Fig. 4E).

Finally, we looked at the binding of Mcm1 to RE and to the *BCL2* promoter in a strain bearing a MYC-tagged *MCM1* (Fig. 4F). As previously reported (1), Mcm1 occupies the *BCL2* promoter at every stage of the cell cycle. Similarly, we found that Mcm1 binds RE throughout the cell cycle. This binding probably could provide an open state of the RE chromatin in *MAT***a** cells, allowing SBF to bind in G_1/S and Fkh1 to bind in G_2/M.

***MAT*a cells arrested in G_1/S and in G_2/M show reduced donor preference, whereas *MAT*α cells do not.** Since SBF binds RE in G_1/S and Fkh1 in G_2/M, it seems that two independent events are necessary for full activation of RE. To test this idea, we quantified donor preference in cells arrested in G_1/S or G_2/M. It has been shown recently that HO-induced homologous recombination requires the activation of the cyclin-dependent kinase CDK1 (26); consequently, mating-type switching cannot occur in cells arrested before the "start" point, i.e., under α-factor arrest. However, cells lacking a functional Cdc7 protein kinase move beyond "start" but cannot initiate DNA replication; because CDK1 is activated, these cells perform DSB repair normally (26). Therefore, we quantified donor preference in cells expressing Cdc7-as3, a mutant sensitive to the ATP analogue inhibitor 1-NMPP1 (2, 26). As shown in Fig. 5A, neither the

drug nor *cdc7-as3* alone affects mating-type switching or donor preference. When HO is induced in mutant cells arrested in the presence of 1-NMPP1, repair efficiency is not affected, but 50% of the cells switched to *MAT*α-BamHI (from *HMR*), compared to 90% in the same strain without arrest, showing that *HML* use is reduced by the arrest.

An alternative way to restrict *MAT* switching to one part of the cell cycle is to use a strain in which HO is under the control of a hybrid promoter which restricts the transcription of the gene to the G_1 phase of the cell cycle in the presence of galactose (43). In this strain, *HML* usage is not affected (89% versus 94% in the wild type; Fig. 5B). Therefore, it is not the restriction of mating-type switching initiation in G_1 that causes the defect but rather the arrest itself. It is possible that the arrest modifies chromosome architecture in a way that reduces the accessibility of the left arm of chromosome III. Interestingly, the arrest conferred by the addition of 1-NMPP1 to *MAT*α *cdc7-as3* cells does not affect their preferential choice of *HMR*α-BamHI (Fig. 5C).

HO-induced *MAT***a** cells arrested in G_2/M in the presence of nocodazole show a similar defect to cells arrested in G_1/S (Fig. 5D); *HML* usage is reduced from 90% to 50%. However, *MAT*α cells arrested in G_2/M still use *HMR* 90% of the time to repair the break at *MAT*. Thus, the exclusion of *HML* in *MAT*α cells does not require any cell cycle-regulated mechanism.

We wondered if the defect in *HML* usage observed in arrested *MAT***a** cells was the direct consequence of the lack of Fkh1 binding in G_1/S-arrested cells and of SBF binding in G_2/M-arrested cells. If so, we would expect to observe a strong deficiency in *HML* usage in *MAT***a** RE^{SCB} cells arrested in G_1/S but still 50% of *HML* usage in G_2/M-arrested cells, since Fkh1 would activate RE normally. Reciprocally, *MAT***a** *fkh1*Δ cells arrested in G_1/S should show 50% of *HML* usage, since SBF can bind RE, but a complete deficiency when arrested in G_2/M. However, we observed approximately 30 to 40% of *HML* usage in *fkh1*Δ and RE^{SCB} mutants arrested either in G_1/S or in G_2/M (data not shown). Thus, even if their binding is regulated during the cell cycle, the roles of Fkh1 and SFF in RE activation can be perceived all along the cell cycle. The defect resulting from the arrest in G_1/S and in G_2/M in wild-type cells does not seem to be linked to the absence of binding of Fkh1 or SBF but rather to a modification of chromosome architecture, which antagonizes RE activation.

KAR3*, *CTF4*, *CTF18*, and *NUP170* genes, genetically related to *CHL1*, are not involved in donor preference.** To understand the role played by *CHL1* in donor preference, we monitored the effects of deletion of four genes synthetic lethal with *chl1*Δ: *KAR3*, *CTF18*, *BIM1*, and *YDR332W* (50, 57). We also tested deletions of *NUP170* and *CTF4*, which exhibit chromosome transmission fidelity deficiencies similar to *chl1*Δ and *ctf18*Δ (32). There was no modification of *HML* usage in *MATa** cells carrying deletions of any of these genes (data not shown).

Mbp1, Ace2, Ste12, and Ndd1 bind to the *KAR4-SPB1* intergenic region but are not involved in donor preference. A study of the association of many transcriptional regulators with intergenic regions across the genome confirmed that Mcm1, Fkh1, Fkh2, Ndd1, and SBF bind to RE (37). In addition, it was found that Mbp1, Ace2, and Ste12 bind the intergenic region containing RE. However, *HML* usage was not affected in strains carrying deletions of these genes (data not shown).

TABLE 2. α-specific genes are not required for *HML* repression as a donor in *MAT*α cells

Strain	Genotype	% *HML* usage[a]
Cwu150	*MAT***a**	75
Cwu128	*MAT***a** GT→TG AC→TA (Matα2 sites A and B)	75
KS338	*MAT***a** 4A	65
Cwu151	*MAT*α	15
Cwu134	*MAT*α GT→TG AC→TA (Matα2 sites A and B)	55
KS345	*MAT*α 4A	50
ECY119	*MATα1*::*KanMX4*	20
ECY120	*MATα1*::*KanMX4* GT→TG AC→TA (Matα2 sites A and B)	48
ECY121	*MATα1*::*KanMX4* 4A	47

[a] *HML* usage has been determined in *MAT***a** strains carrying *HML*α and *HMR*α-BamHI, in MATα-BamHI strains carrying *HML*α and *HMR***a**, or in *MATα1*::*KanMX4* strains carrying *HML*α and *HMR***a**. Genomic DNA extracted from an entire population of cells in which mating-type switching has been induced was cut by BamHI and StyI and probed with a *MAT* distal fragment.

We also measured *HML* usage in an *fkh2*Δ *ndd1*Δ double mutant strain, since *fkh2*Δ suppresses the lethality associated with the *ndd1* mutation (35). We know from previous studies that the *fkh2*Δ mutation does not affect donor preference in *MAT***a** cells (53). The double mutant also does not show any defect (data not shown), arguing that despite the fact that Ndd1 can undergo ChIP with RE, this protein is not involved in its activation.

Does donor preference depend on any a- or α-specific genes? As discussed above, when four repeats of region A (4A) replace RE, *HML* is used 65% in *MAT***a** but only 50% in *MAT*α (Table 2). Furthermore, in strains harboring mutations of the two Matα2 binding site in the Matα2-Mcm1 operator of region C (65), *HML* usage was 75% in *MAT***a** and only 55% in *MAT*α. Therefore, it appears that an **a**-specific activator or an α-specific repressor could regulate *HML* usage. The Matα2-Mcm1 complex represses expression of **a**-specific genes, whereas Matα1-Mcm1 activates transcription of α-specific genes. Deletion of *MATα1* in strains carrying 4A in place of RE or with mutations in the *MATα2* binding site of RE shows no increase in *HML* usage (data not shown). These results exclude involvement of an α-specific repressor in donor preference. Alternatively there could be an **α**-specific activator of *HML* as a donor in *MAT***a** cells. Deletion of the *MAT*-Y**a** sequences in a strain carrying the 4A RE does not affect donor preference (data not shown), ruling out the involvement of Mata1 and Mata2. It is possible that there is an undocumented small open reading frame that plays a role in the process (29).

DISCUSSION

We have found that an evolutionarily conserved SCB within RE plays an important role in donor preference and that the SBF complex, which binds this consensus sequence, is important for RE activity. We also described two independent pathways that govern donor preference: one depends on *FKH1*, and the other depends on SBF, *CHL1*, and *YKU80*.

Although two important transcription factors are involved in *MAT***a** donor preference, RE activation does not depend on transcription. Around region E, Szeto et al. (55) did find a weak transcript that does not appear to encode a protein.

However, RE activity does not depend on these sequences because they are absent in the 270-bp minimum enhancer (65) or when multimers of region A, D, or E were inserted in place of the 1.8-kb sequence containing RE. Therefore, the role of Fkh1 and SBF in donor preference is very different from their involvement as transcription factors in the G_2/M and G_1/S transition, respectively.

It is also possible that the binding of SBF and Fkh1 to RE provokes a change in the chromatin structure of the left arm leading in some way to a greater accessibility of the resident donor; however, a recent study of the global chromatin structure of 45 kb of the left arm of chromosome III covering *HML* and RE did not shown any differences between *MAT***a** and *MAT*α cells except for the RE region (15). We propose that the binding of Fkh1 and SBF to RE could reorganize the architecture and the nuclear localization of the left arm of chromosome III. In mammalian cells, the position of a locus can move relative to a heterochromatin domain, in response to a change in its transcriptional state (for review, see references 52 and 56). Also, massive decondensation can occur in the absence of transcription per se (5, 59). We suggest that binding of SBF and Fkh1 to RE could control the nuclear organization of the left arm of chromosome III in a similar way. SBF and Fkh1 could counteract a compact organization of the left arm, making *HML* more mobile in the nucleus.

Recently, we showed that *HML* motion is strongly constrained in both *MAT*α and RE-deleted *MAT***a** strains, compared with *MAT***a** (4). Additionally, the three-dimensional configuration of *MAT*, *HML*, and *HMR* is mating-type dependent, the distance between *HML* and the other cassettes being greater in *MAT***a** cells (4). These data suggest there is constitutive tethering of *HML*, which is relieved in *MAT***a** cells through the binding of SBF and Fkh1 to the RE.

The consequences of cell cycle-dependent regulation of SBF and Fkh1 binding to RE are still unclear. It is possible that the establishment of chromosome III architecture needed for the activation of *HML* usage relies on two independent events, taking place in G_1 through SBF binding to RE and in G_2 through Fkh1 binding. Even if HO breaks the DNA at the *MAT* locus in G_1, the absence of binding of Fkh1 in G_2 would compromise the accessibility of *HML*, because the conformation of the left arm has not been properly established. This hypothesis is in agreement with the fact that *fkh1*Δ and RE^{SCB} mutants show the same *HML* usage defects in both G_1/S- and G_2/M-arrested cells. It seems that the recruitment of these factors to RE creates an epigenetic state necessary for the organization of the left arm in the nucleus in the subsequent phases of the cell cycle. The defect encountered in the G_1 phase in RE^{SCB} mutants could lead to the disorganization of chromosome III structure in the subsequent S and G_2 phases, explaining the defect also observed in the G_2-arrested phase in this mutant. The same idea could apply to the *fkh1* mutant. In contrast, arrest in both G_1/S and G_2/M does not affect donor preference in *MAT*α cells.

Transcriptional activation at G_1/S promoters follows a complex ordered series of events first delineated for the developmental and cell cycle-regulated *HO* promoter which depend on Swi5, SBF, and Whi5 (11–13). At RE, there seems to be no involvement of either Whi5 or Swi5. In addition, the study of the association of Swi5 with intergenic regions across the genome (37) does not show any binding to the RE-containing intergenic region. Finally, we show that the MBF complex, involved in the transcription regulation of another set of genes at the G_1/S transition is not involved in RE activation, underlining the specific role of SBF in donor preference.

We have previously suggested that the role of Mcm1 is to open the chromatin structure at RE to allow effector proteins to bind (53, 65). We show here that this protein binds all along the cell cycle. It is therefore possible that the role of Swi5 to recruit SBF at the *HO* promoter is fulfilled by Mcm1 at RE.

Fkh1 has been shown to bind the promoters of the *CLB2* cluster all along the cell cycle (35), an observation that we confirmed. However, binding of Fkh1 to RE is restricted to the G_2/M phase of the cell cycle. This property is also observed for the binding to the 4A synthetic RE. This result could mean that the binding of Fkh1 to RE is regulated by a direct modification of the protein. However, as Fkh1 binds the *CLB2* cluster promoters all along the cell cycle, it seems that this regulation does not affect directly the DNA binding properties of the protein, but rather its capacity to interact with other proteins binding RE. The cycling properties of Fkh1 binding are not directly linked to Mcm1 or SBF, since these proteins do not have a binding site in the 4A synthetic RE. It is possible that unidentified factors that are involved in Fkh1 regulation bind RE in domains A, D, and E. We are currently working on identifying these factors.

Fkh1 may be more important for RE function than in the regulation of the transcription of the *CLB2* cluster. Fkh1 ChIP signals are stronger for RE than for the *CLB2* or *SUN4* promoters in G_2-arrested cells, in logarithmic phase, and in synchronized cells (Fig. 4). The Fkh1 ChIP signal at RE is stronger than the one of Fkh2 or Ndd1 (53). Reciprocally, Fkh2 and Ndd1 ChIP signals are much stronger at the *BCL2* cluster promoters than Fkh1 (23, 35, 53). Fkh1 activity can clearly substitute for the one of Fkh2 in the control of the *CLB2* cluster expression (35), but Fkh2 cannot replace Fkh1 for RE activation.

We also found that the mutated SCB is epistatic to *chl1*Δ and to *yku80*Δ, showing that SBF, *CHL1*, and *YKU80* act in the same pathway of RE activation. Since the deletion of RE reduces the usage of *HML* in *MAT***a** cells to the level observed in *MAT*α cells (66), we know that deleting the SCB element is epistatic to both *chl1*Δ and *yku80*Δ. Therefore, as Chl1 does not bind RE, we suggest that SBF bound to RE needs Chl1 to perform its function. Although Chl1 is involved in the establishment of sister chromatid cohesion during S phase (40, 45, 50), none of the other components that genetically interact with Chl1 were found to affect donor preference, including *CTF4*, *CTF18*, or *KAR3* (31, 40, 51, 58). It is therefore possible that the role of *CHL1* in donor preference is not related to its activity in sister chromatid cohesion. It is important to keep in mind that Chl1 acts also in an SBF-independent manner, since the RE^{SCB} *fkh1 chl1* triple mutant shows a slightly stronger reduction of *HML* usage than the RE^{SCB} *fkh1* double mutant.

How Yku80—primarily implicated in DNA end-joining and telomere silencing—acts at RE or in facilitating recombination at *HML* is unclear, especially given that *yku70*Δ deletions have little effect (48; our data not shown). Whether it associates directly with RE or binds through Swi4/Swi6 or Fkh1 remains to be established.

Finally, we have ruled out the involvement of other **a-** and α-specific genes in donor preference. Among the five **a**-specific genes that have been identified (17), all of them are involved in conjugation and both their cellular localization and their enzymatic properties do not fit with a role in donor preference (25). We cannot fully rule out the involvement of an **a**-specific sterile RNA or of the product of an unidentified small ORF (29) to explain why *HML* is slightly less used in *MAT*α cells when the natural RE is replaced by 4A, for example. We cannot also rule out that *HMR* usage can be slightly increased in *MAT*α cells by an RE-like activity (67, 69).

We still don't know how RE works, but regulation of its activity is very complex. The involvement of SBF and Fkh1 make RE resemble a hybrid-regulated promoter, which recruits SBF in a Swi5-independent manner and Fkh1 in a G_2-specific way. Given that RE does not cause modification of chromatin around *HML*, we speculate that the recruitment of transcription factors can create an epigenetic modification of RE which leads to a change in the regional conformation of the left arm of chromosome III in the nucleus, making it more accessible for recombination, possibly by blocking the tethering of the chromosome arm at several as yet unidentified sites. This hypothesis is supported by the increased mobility of the left arm in *MAT***a** cells (4). In *Schizosaccharomyces pombe*, recent studies have shown that donor preference is dictated by a Swi2-dependent recombination enhancer that controls the choice of the donor by controlling the spreading of a recombination-promoting complex on the donors in a heterochromatin-dependent manner (28). The function of RE in *S. cerevisiae* appears therefore to be radically different.

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