Yeast Recombination Enhancer Is Stimulated by Transcription Activation

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***Saccharomyces cerevisiae* mating type switching is a gene conversion event that exhibits donor preference. MAT α cells choose HML α for recombination, and MAT α cells choose HMRA. Donor preference is controlled by the recombination enhancer (RE), located between HML α and MAT α on the left arm of chromosome III. A number of α -cell specific noncoding RNAs are transcribed from the RE locus. Mcm1 and Fkh1 regulate RE activity in α cells. Here we show that Mcm1 binding is required for both the transcription of the noncoding RNAs and Fkh1 binding. This requirement can be bypassed by inserting another promoter into the RE. Moreover, the insertion of this promoter increases donor preference and opens the chromatin structure around the conserved domains of RE. Additionally, we determined that the level of Fkh1 binding positively correlates with the level of donor preference. We conclude that the role of Mcm1 in RE is to open chromatin around the conserved domains and activate transcription; this facilitates Fkh1 binding and the level of this binding determines the level of donor preference.**

Saccharomyces cerevisiae has two haploid mating types, α and α . Mating type of a cell is determined by regulatory proteins that are encoded from the mating-type locus (MAT), located near the centromere of chromosome III (Chr III) (reviewed in references 7 and 20). The same chromosome harbors two transcriptionally silent and heterochromatic mating type cassettes, HML and HMR, located at the far left and right arm, respectively. In most *S. cerevisiae* strains, these two cassettes contain the sequences for the α and α alleles and are thus often referred to as HML α and HMRA. Yeast cells of one mating type can switch to the other, as often as every generation. Mating type switching is a gene conversion event between MAT and one of the two mating type cassettes (reviewed in reference 7). Gene conversion starts with the introduction of a double-strand break at the MAT locus by the HO endonuclease. This double-strand break is repaired by replacing the allele present at the MAT locus with a copy of the allele taken from one of the two donor loci, HML α or HMRA. Mating type switching is controlled by a number of different mechanisms. First, only mother cells can switch their mating types (30). Second, switching starts at the G₁ phase of the cell cycle (3). Third, more than 85% of the switching attempts result in change from one mating type to the other (12).

The third mechanism that controls mating type switching is based on a directional recombination system. Thus, α cells use HML α and α cells use HMRA as the donor of gene conversion more than 85% of the time (12). This is called “donor preference.” Donor preference does not depend on the sequence of the recombining cassettes; rather, it depends on the location of

the cassettes on the chromosome (35). A small *cis*-acting sequence, the recombination enhancer (RE), controls donor preference (38). Deletion of the entire RE causes a dramatic change in the donor preference of α cells, where HML preference drops from ~85% to ~15%. RE is located within a ~2.5-kb intergenic region ~29 kb from the left arm of chromosome III, between HML and MAT.

The mechanism by which RE governs directional recombination is still unknown. One of the most remarkable features of RE is that it activates the whole left arm of chromosome III for homologous recombination in α cells (39). The same region is repressed for recombination in α cells. It has been shown that the differences in the primary chromatin structure of this region do not cause the differences in recombinational frequency between the two cell types (6). One mechanism by which RE may function is by regulating the localization of the mating type cassettes in the nucleus. The mobility of the left arm of chromosome III differs in α and α cells (4). However, it was also demonstrated that the proximity of the recombining cassettes may not play a big role in committing to recombination (13, 28).

While the mechanism of RE action is not clear, it is known that it is activated in α cells and repressed in α cells. In α cells, Mcm1 binds and activates RE, and in α cells Mcm1 binds along with α 2 and represses its activity (37). The chromatin structure of RE also differs according to cell type (36). In α cells, RE chromatin has an open structure with two protein footprints bordering an unusual nuclease-hypersensitive site. Recently, it was shown that in α cells, several transcription factors, Fkh1, Fkh2, and Ndd1, bind to RE (31). How these proteins affect RE function is not known. Nevertheless, among these, the deletion of Fkh1 protein reduces donor preference of α cells from ~85% to ~35% HML use. Interestingly, in α cells a number of noncoding RNAs are transcribed from the RE locus (33). In α cells, the noncoding RNAs are not transcribed. It is

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not known whether transcription from RE affects its activity. In addition, the relationship between transcription and the binding of other proteins to RE is unclear.

In this study, we present data on the mechanism of RE activation by a promoter, providing a transcription-associated function that enhances Fkh1 binding in α cells. We demonstrated that Mcm1 binding, which was shown to be required for RE function, is also required for the transcription of the α -specific noncoding RNAs. This requirement can be bypassed by inserting another promoter into the RE. The promoter insertion opens the chromatin structure around the conserved domains of RE and increases Fkh1 binding. Our results suggest that the role of Mcm1 at RE is to activate transcription and facilitate Fkh1 binding. Moreover, the level of Fkh1p binding positively correlates with the level of donor preference.

MATERIALS AND METHODS

Strains and cell growth. Standard yeast medium, yeast extract-peptone (YP), with an appropriate carbon source (2% dextrose, 2.5% lactose, 2% raffinose) was used. For cell cycle regulation of RE transcription, JKM161a (*ho HML α mata HMR α ade1-112 lys5 leu2-3 ura3-52 trp::hisG ade 3::GalHO*), provided by J. Haber, was used. For mating type switching, derivatives of CW157 (*HML α MAT α HMR α + BamHI ade1 ura3-52 leu2-3, 112 ade 3::GalHO*) (22), provided by J. Haber, were used (37). Insertion of *CUP1* promoter and other sequences into the genome was performed with pop-in/out with the use of YIp5 (24). A 660-bp RE was amplified from FYa for wild-type RE and from CW157 for RE containing an Mcm1 binding site mutation, from coordinates Chr III 28922 to 29580 and cloned into the BamHI site in YIp5 (YIp5-RE). The 165-bp *CUP1* promoter fragment, amplified from pYEX 4T-2 (Clontech), or a control sequence, amplified from the first 165 bp of the *STE6* gene, was inserted at the BstBI site of YIp5-RE. The *CUP1* promoter was inserted in both directions. To delete the two TATA boxes in the *CUP1* promoter, the TATA sequence was replaced by new sequences as previously described (27). The resulting plasmids (YIp5-RE+inserted fragment) were cut with SpeI and transformed into CW157 cells, and DNA fragments containing *URA3* markers were popped out with homologous recombination by growing the transformants on 5-fluoroorotic acid plates. The presence of the insertions and desired mutations in the genome was confirmed by PCR and sequencing. *FKH1* was deleted from the genome by one-step PCR replacement using the *TRP1* marker (2). The C-terminally myc-tagged Fkh1p was generated by using a PCR-directed method with a 13MYC-KanMx cassette in cells derived from CW157 (16).

Analysis of donor preference and switching. Cells were grown in YP-lactose (or raffinose) medium, and then galactose was added for 45 min. After induction, cells were collected by filtering and were transferred into prewarmed YP-dextrose medium containing 0.2 mM CuSO₄. The cells were harvested for DNA preparation after switching was completed. Donor preference analysis was performed as described previously (38, 39). HO-induced switching from *MAT α* to *MAT α* or *MAT α* +BamHI was monitored by Southern blot analysis (39). DNA isolated from switched cells was digested with HindIII and BamHI. A specific probe (Chr III 13044 to 13278) was used to give fragments that correspond to the two donor loci *HML α* and *HMR α* +BamHI (~5 kb) and the switch products, *MAT α* and *MAT α* +BamHI (4.4 kb and 3.1 kb, respectively). The bands corresponding to each donor were quantified by using ImageQuant version 5. The kinetics of mating type switching was determined by cutting DNA from different time points with StyI. Southern blot analysis was performed as previously described (5).

RNA analysis. For the analysis of transcription in cycling and switching cells, JKM161a cells were grown to an optical density at 600 nm (OD₆₀₀) of 0.2 in 900 ml of YP-lactose at 30°C. After synchronization by the addition of alpha-factor at a concentration of 10 μ g/ml, the culture was divided into two, and 50 ml of 20% (wt/vol) galactose was added to half of the cells for HO induction. After 45 min of induction, cells in both switching and cycling cultures were filtered, washed, and transferred into prewarmed YP-dextrose medium. Cells (20 ml) were collected at 10-min intervals for RNA preparation. RNA preparation from asynchronously growing cultures was done with cells that were grown in YP-dextrose medium up to an OD₆₀₀ of ~0.4 to 0.6; then 0.2 mM CuSO₄ was added for ~45 min before harvesting. For RNA and switching analysis of cells without copper induction, complete synthetic media lacking copper was used (101 BIO Systems).

Total RNA was prepared by glass bead cell disruption as described previously (40). For Northern blotting, 20 μ g of each RNA sample was subjected to electrophoresis in a formaldehyde-agarose gel, followed by transfer to a nylon membrane and hybridization to specific probes. Probes for Northern blot hybridization were prepared by amplification from genomic DNA by PCR. R1L&S probe is complementary to Chr III, 29712 to 30517; R2 probe is complementary to Chr III, 30817 to 31317; scR1 (for a small cytoplasmic RNA) probe is complementary to Chr V, 441736 to 442428; and HTA1 probe is complementary to Chr IV, 915527 to 9155910. Gel purification of the probes was followed by random primer labeling (Stratagene). Blots were stripped for reprobing by boiling in 0.1% sodium dodecyl sulfate (SDS).

ChIP assays. For chromatin immunoprecipitation (ChIP) analysis, cells were grown to an OD₆₀₀ of ~0.6 to 0.8 in 200 ml YP-dextrose medium, and then 0.2 mM CuSO₄ was added for ~45 min before formaldehyde cross-linking. ChIP assays were performed as described previously (8, 25). Briefly, after cross-linking with 1% formaldehyde for 15 min and quenching with 150 mM glycine for 5 min, cells were harvested and broken by glass beads for 45 min in FA (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin) buffer with 0.2% SDS. The chromatin was fragmented by sonication and extracts were prepared. From these extracts Fkh1-myc was immunoprecipitated in FA buffer by using antibody against myc tag (Roche) and then collected by protein A-Sepharose (Amersham Biosciences). Protein A beads were washed, and DNA was eluted with the elution buffer (25 mM Tris, pH 7.5, 2 mM EDTA, 0.2 M NaCl, 0.5% SDS) at 65°C for 30 min. Samples were treated with proteinase K, and cross-linking was reversed overnight at 65°C. DNA was prepared by phenol-chloroform extraction and ethanol precipitation with the addition of 50 μ g/ml glycogen. DNA was analyzed by PCR.

Nuclear DNA preparation and RE maps. Nuclei from 1 liter of cells of an OD₆₀₀ of ~1 were prepared as described (21). After preparation of nuclei, digestion was performed with 2.5 to 10 U/ml of MNase at 37°C, for 5 min. DNA was recovered after proteinase K and RNase A treatment with phenol-chloroform extraction. Naked DNA controls were generated by digesting 100 ng of PCR products (amplifying Chr III, 28125 to 29774) and 30 μ g of calf thymus DNA with 50 U/ml of MNase for 5 min at 30°C. Samples equal to 1 ng of PCR DNA were treated in parallel with the DNA purified from nuclei preparations. For the indirect end-labeling map, DNA was cut with ScaI and subjected to electrophoretic separation on 1.5% agarose gels, transferred to Hybond-NX membrane (Amersham), cross-linked with UV light, and hybridized to a specific probe. The probe was prepared by PCR amplification of a ~250-bp region specific to the distal end of the parental fragment including RE. Undigested control serves as a measure of the specificity of the probe.

RESULTS

Transcription of the noncoding RNAs from RE is cell cycle regulated. Mating type switching is directional; α cells use *HML α* and α cells use *HMR α* as the donor of gene conversion more than 85% of the time (Fig. 1A). The donor preference is governed by the RE, which is active in α cells and turned off in α cells. There are two α 2/Mcm1 operators within the RE locus (Fig. 1B). In α cells, a number of noncoding RNAs, with sizes ranging from 0.3 to 1.3 kb, are transcribed downstream of these operators. The three major RNA species that are apparent in our Northern blot analysis are named R1L&S and R2. In α cells there is no transcription from the RE. The 750-bp region located around the first α 2/Mcm1 operator comprises most of the RE activity. This region includes the conserved domains of RE (shown in Fig. 1B) and 300 bp downstream of the E domain.

The role of the noncoding RNAs, the transcription, and their effect on RE activation are not known. Since mating type switching is a cell cycle-regulated event, we first wanted to monitor RE transcription during switching and the cell cycle. Normally, only mother cells undergo switching, because the expression of the HO endonuclease is confined to mother cells in the G₁ phase after cell division (30). In order to perform

FIG. 1. Donor preference during mating type switching and the RE locus. (A) The locations of the *MAT* locus, the two mating type cassettes, and RE are shown with the approximate distances from the left end of chromosome III. The two cassettes are on the left (*HML*) and the right (*HMR*) arms of the chromosome. RE is "on" in *a* cells and "off" in α cells. The centromere is depicted with a circle. The donor preference for each cell type is shown with a bracket from the *MAT* locus to the preferred cassette. (B) The two $\alpha 2$ /Mcm1 binding sites in RE are shown. Three major noncoding RNAs, named R1L, R1S, and R2 in this study, are transcribed from the RE locus in *a* cells. In the lower panel, RE domains are shown. TTT(G/A) repeats are indicated with vertical tick marks. This figure was adapted from Haber (7).

experiments with cells switching synchronously, others have developed an inducible HO system (5). This system uses *a* cells that express the HO endonuclease gene under galactose (*GAL*) control. The HO endonuclease is induced in these cultures by the addition of galactose to the medium. After induction, cells are transferred to glucose medium, which represses expression of *GAL* controlled *HO* gene. Cells then progress through a typical mating type interconversion.

In a previous study, we had determined the kinetics of switching in our experimental system (6). In order to monitor transcription during cell cycle and switching, cells were grown in lactose-containing medium and arrested at the G_1 phase of the cell cycle by alpha-factor. After the arrest, the culture was divided into two and galactose was added to half of the cells for *HO* induction (switching). After induction, cells in both switching and cycling cultures were released from arrest. Samples were collected at 10-min intervals for RNA preparation. Northern blotting was used to analyze transcription from the RE region. Our results suggested that the transcription of the noncoding RNAs is cell cycle regulated and that expression peaks around late G_1 and early S phase (Fig. 2). The timing was estimated by comparing the transcription of RE RNAs to that of *HTAI*, which encodes a histone protein (9, 10). The cycling cells showed another peak of RE and *HTAI* transcrip-

tion corresponding to the second G_1 /S. On the other hand, the second peak of RE RNAs in the switching cells did not appear because most of the cells changed from *a* to α within 1 to 1.5 h, consistent with the kinetics of switching. Note that a less defined second peak of *HTAI* transcript appeared in the switching cells, indicating that these cells continued cycling. At this resolution, although the transcription of R2 appeared to be 10 min earlier than that of R1L&S, the overall kinetics looked similar. Transcription started at late G_1 and continued through the S phase, coinciding with the timing of mating type switching, which is thought to start during G_1 phase and end before the S phase is completed for homothallic strains (18). However, whether transcription precedes or is coincidental with switching for each cell is not clear.

We repeated the kinetics of RE transcription in switching cells and also collected DNA from each time point. Northern blot analysis of RE transcription is shown in Fig. 2B. In order to visualize the kinetics of switching in the same gel, $\alpha 2$ expression is also shown. At 0 min, the $\alpha 2$ probe hybridized at background levels, possibly because of the presence of cells that were switched to α due to leaky expression of HO in lactose-containing medium. The expression of $\alpha 2$ started increasing above the background level at 40 min. We used a Southern blotting protocol to directly follow the time course of switching in *a* cells (Fig. 2B) (5). DNA fragments mark the *MAT* α locus, prior to or after cutting by HO endonuclease, the *MAT* α locus, and a loading control derived from common sequences to the right of *MAT* (Fig. 2B, Distal). Figure 2B documents the switching kinetics in our experiment. At 0 min HO has cut 91% of the cells at the *MAT* locus. Over the next 90 min, the bulk of the cells switched to α , signaled by the large *MAT* α fragment (Fig. 2B, arrowhead). A small fraction of cells switched by recombining with *HMR* α , leading to regeneration of the *MAT* α fragment. The disappearance of the HO fragment over the time course is shown below the Southern blot gel. Overall, in this experiment most of the cells switched with the right donor preference while transcribing the RE. However, one has to be cautious interpreting the timing of the events, since the HO induction is done artificially in all cells by galactose induction. This has been shown to increase the time required for switching in a population of cells (5). Therefore, we cannot resolve whether transcription occurs prior to or during switching in cells with wild-type HO expression. We also determined that the noncoding RNAs from RE are transcribed by RNA polymerase II and are polyadenylated (data not shown). Previously, the Broach laboratory reported that the transcription start site of the noncoding RNAs was ~250 bp downstream of the Mcm1/ $\alpha 2$ operator (33). We narrowed the region to within the E domain by performing primer extension analysis (data not shown).

A promoter insertion into the RE can bypass the requirement for Mcm1 binding for correct donor preference of *a* cells. Mcm1 is a constitutively expressed protein that regulates transcription of a number of genes, including the *a*-specific genes. In α cells, $\alpha 2$ and Mcm1 bind to the $\alpha 2$ /Mcm1 operator and repress transcription of the downstream gene by several mechanisms, including organizing the chromatin structure into tightly positioned nucleosomes (21, 29). In *a* cells, Mcm1 binds to the operator and activates transcription. Previous studies showed that the first $\alpha 2$ /Mcm1 operator, located in the

FIG. 2. RE transcription during cell cycle in cycling and switching cells. (A) Northern blot analysis of transcription in *a* cells that were grown in lactose-containing medium and were arrested at the G₁ phase of the cell cycle with alpha-factor. In the left panel (Cycling) cells were released from alpha-factor arrest by filtration at 0 min. In the right panel (Switching) cells were induced for HO expression for 45 min with galactose and then released from arrest into fresh dextrose-containing medium at 0 min. RNA was prepared from aliquots of cells collected at 10-min intervals, and R1L, R1S, and R2 transcription was analyzed. *HTA1* encodes histone H2A, and *scR1*, a small cytoplasmic RNA transcribed by RNA polymerase III, was used as loading control. (B) Northern blot analysis of RE and α 2 transcription in switching cells (top). Southern blot analysis of switching in the same cells (bottom). DNA cut with StyI was blotted with *MAT*-specific probe. This probe detects a cell type-independent distal 2,220-bp fragment, a 1,880-bp fragment from *MAT α* (arrow), a 930-bp fragment from *MATa*, and a 723-bp fragment when *MAT* has been cut by the HO endonuclease (HO cut). The percentage of HO-cut DNA was calculated by using the following formula: [HO cut/(HO cut + *MATa* + *MAT α*)] $\times$ 100. The signal intensity from each band was normalized to the distal band.

~750-bp RE, is important for donor preference (33, 37). A mutation that prevents Mcm1 binding abolishes RE activity in *a* cells, where the donor preference reduces from ~85% to ~20% *HML* use. Although RE contains two α 2/Mcm1 operators, there are no open reading frames (ORFs) in this locus. On the other hand, the *a*-specific noncoding RNAs are transcribed downstream of these operators (33). Previous studies argued that these RNAs may not have a role in RE activity

because the deletion of most of the transcribed sequences did not cause drastic changes in donor preference (38). However, deletions toward the transcription start site (E region) gradually and significantly decrease donor preference (31).

Since the noncoding RNAs are a specific, we reasoned that Mcm1 may activate transcription. In order to determine whether a mutation that prevents Mcm1 binding to RE would reduce transcription of the noncoding RNAs, Northern blot analysis was performed. The noncoding RNAs, downstream of the first operator, were transcribed in cells with wild-type RE (W) (Fig. 3A). The transcription was abolished in cells containing the Mcm1 binding site mutation at this operator (M). This result showed that Mcm1 binding is required for RE transcription. Since Mcm1 activates both transcription and RE function, we wanted to test whether the Mcm1 protein itself or its function as a transcription activator was required. This was tested by inserting a well-defined promoter (the 165-bp fragment that contains UAS2 and the transcription start site of the *CUP1* gene) into an RE already containing the Mcm1 binding site mutation (MC, for Mcm1 with *CUP1* promoter insertion). The insertion site and other features of RE are schematically represented in Fig. 3A (left). Northern blot analysis showed that the *CUP1* promoter activated transcription in MC cells (Fig. 3A, right). Note that the MC cells exhibited longer RNAs than that of the wild type (indicated by arrows pointing to the RNA species that appeared after the insertion of the promoter), suggesting that transcription was governed by the *CUP1* promoter.

To determine the donor preference in these strains, a previously developed method was employed (schematically described in Fig. 3B, left) (38, 39). These strains contain *MATa*, *HMLα*, and a BamHI-marked *HMRα*, indicated as *HMRα*+BamHI. During switching, cells choose *HMLα* or *HMRα*+BamHI and give rise to *MATα* and *MATα*+BamHI, respectively. These two alleles can be distinguished by Southern blot analysis. Donor preference of a cells was determined by calculating the percent *HML* use, which can be used as a measure of RE activity. The donor preference of each strain was plotted with the standard deviations. The insertion of *CUP1* promoter into RE containing the Mcm1 binding site mutation increased the donor preference from ~25% to ~61% in M and MC cells, respectively (Fig. 3B). This result suggested that the requirement for Mcm1 binding can be bypassed by another promoter.

In order to test whether a control sequence that does not contain a promoter would also increase donor preference, a portion of the *STE6* ORF that is approximately the same size as the *CUP1* promoter was inserted into the RE containing the Mcm1 binding site mutation (MS, for Mcm1 with *STE6* ORF insertion). The control sequence failed to increase transcription (Fig. 3A, compare transcription of M and MS) as well as donor preference from 25% to 26% *HML* use in M and MS cells, respectively (Fig. 3B). This demonstrated that a function of the promoter may be required for RE activity. Reversing the orientation of the promoter reduced the donor preference only slightly, from 61% to 55% in MC and MR (for Mcm1 with the *CUP1* promoter insertion in reverse direction) cells, respectively (Fig. 3B). The transcription downstream of the reversed promoter is shown in Fig. 3A; the promoter induces transcription in reverse direction (data not shown). This result suggests

that the direction of transcription and the sequences that are transcribed do not influence donor preference significantly.

We also wanted to determine whether the level of transcription was important for donor preference. Transcription was reduced by deleting the two TATA boxes in the *CUP1* promoter (MCΔT) (Fig. 3A). The deletion of the TATA boxes reduces transcription without preventing the activators' function at this promoter (26). When the level of transcription was reduced, donor preference was increased even more, from ~61% in MC cells to ~77% in MCΔT cells (Fig. 3B). Notably, these cells produced RNAs similar to that of the wild type, suggesting that the promoter lacking the TATA boxes is activating transcription from the native start site. The increase in donor preference in MCΔT cells, to 77% *HML* use, compared to the 61% *HML* use in MC cells may be a reflection of transcribing normal noncoding RNAs. It may also reflect the possibility that when started from the *CUP1* promoter, transcription passes through the entire E domain, hence preventing other regulatory proteins from binding to this region. We further reduced the transcription level in MC and MCΔT cells by growing them in complete synthetic medium lacking copper (Fig. 3C). Although the level of transcription was reduced, the donor preference of MC cells did not change significantly when cells were switched in medium lacking copper (59% without and 61% with copper) (Fig. 3D). Conversely, donor preference of MCΔT cells was lower when cells were grown in medium lacking copper 59%, compared to 77% in medium with copper (Fig. 3D). This may be a reflection of the binding of the copper-inducible activator to the *CUP1*. Our results suggested that while the promoter activity of *CUP1* is important for RE activation, the direction or the level of transcription may not affect its function. This also argued that the transcription activators, not the transcription itself, are necessary for RE function.

RE transcription does not require Fkh1. The transcription activators, Fkh1, Fkh2 and Ndd1, bind to RE in a cells. Among these, the deletion of *FKH1* significantly reduces the donor preference of a cells from ~85% to ~35% *HML* use (31). Since it was known that Fkh1 is a transcription activator of several genes, we wanted to know whether Fkh1 also activates RE transcription (14, 41). For this, RE transcription was analyzed in cells lacking Fkh1 ($\Delta fkh1$) by Northern blotting (Fig. 4A). The noncoding RNAs were transcribed in $\Delta fkh1$ cells. Since the donor preference in cells lacking Fkh1 is low, ~31%, while RE transcription occurs, we concluded that the transcription by itself is not sufficient for RE activity. We tested whether Fkh1 requirement could be bypassed by the *CUP1* promoter insertion at the RE, similar to its role in cells containing the Mcm1 binding site mutation. The donor preferences of a cells lacking Fkh1 with the *CUP1* promoter insertion ($\Delta fkh1$) or without this insertion ($\Delta fkh1$) were ~33% and ~31% *HML* use, respectively (Fig. 4B). Thus, *CUP1* promoter insertion failed to bypass the requirement of Fkh1 for donor preference, suggesting that a promoter cannot replace the function of this protein in donor preference.

We also tested whether the deletion of Fkh1 affected transcription in synchronously switching cells. Cells were grown in lactose-containing medium and arrested at the G₁ phase of the cell cycle by alpha-factor. Then, galactose was added for HO induction. After induction, cells were released from arrest (0

FIG. 3. RE transcription and donor preference of cells with *CUP1* promoter insertion. (A) The schematic representation of the *CUP1* promoter insertion site is shown at left. At right is a Northern blot analysis of transcription from **a** and **α** cells along with **a** cells containing wild-type RE, RE with an Mcm1 binding site mutation, and *CUP1* promoter, control sequence, *CUP1* promoter in reverse direction, and *CUP1* promoter lacking the TATA boxes inserted at the mutant RE. A key to the insertions is presented as a list in the figure. All cells were collected from asynchronously growing cultures, and 0.2 mM CuSO₄ was added to these cultures 45 min before harvesting. scR1 was used as loading control. (B) Donor preference analysis of the cells from panel A. The donor preference assay is schematically represented at left. The weight of the bracket represents the level of preference. HO-induced switching from *MATa* to *MATα* or *MATα*+BamHI was monitored by Southern blot analysis. DNA from cells that completed switching was digested with HindIII and BamHI. A specific probe was used to give fragments that correspond to the two donor loci (*HM*) (~5 kb) and the switch products, *MATα* and *MATα*+BamHI (4.4 kb and 3.1 kb, respectively). Donor preference was determined by densitometry and is shown as % *HML* use at the bottom of each lane. A graph was also plotted below the gel from three independent experiments with the standard deviations. (C) Northern blot analysis of RE transcription in cells grown in complete synthetic medium lacking copper or with the addition of 0.2 mM CuSO₄. (D) Donor preference of the cells grown and switched in complete synthetic medium lacking copper.

FIG. 4. RE transcription and switching in cells lacking Fkh1 ($\Delta fkh1$). (A) Northern blot analysis was performed as described in the legend of Fig. 3A. RNA from asynchronously growing $\Delta fkh1$ cells along with *a* cells is shown. (B) Donor preferences of $\Delta fkh1$ cells and cells lacking Fkh1 with the *CUP1* promoter insertion ($C\Delta fkh1$) were determined as described in the legend of Fig. 3B. (C) Northern blot analysis of switching in $\Delta fkh1$ cells. Cells were synchronized by alpha-factor and HO was induced. At 0 min cells were released from arrest into fresh dextrose-containing medium. Aliquots were taken every 10 min for RNA preparation. The analysis was performed as described in the legend of Fig. 2.

min). Samples were collected at 10-min intervals. Figure 4C presents the Northern blot analysis of RE and $\alpha 2$ transcription in $\Delta fkh1$ cells during switching. Although the peak for the RE transcription is broadened, most of the cells switch while RE is being transcribed, and the timing of transcription was not significantly affected. Combined with the observation that Fkh1 is not required for RE transcription, our results suggested that the function of Fkh1 protein in RE is different from its role in transcription activation.

Fkh1 binding is enhanced by *CUP1* promoter insertion. Since the deletion of *FKH1* did not affect RE transcription, we hypothesized that the activation of RE in *a* cells consists of a sequence of events that start with binding of Mcm1 in order to open chromatin and activate transcription, followed by Fkh1 binding. In order to test this hypothesis, we first wanted to determine whether Fkh1 binding requires Mcm1 binding at RE. Second, we wanted to know whether the *CUP1* promoter would increase Fkh1 binding independent of Mcm1. For this, the ChIP method was used. Fkh1 protein was precipitated by using an antibody against the myc tag that was attached to the C terminus of the protein. The functionality of the tagged protein was tested by assaying the donor preference (Fig. 5A). The donor preference of cells containing the myc-tagged Fkh1 was similar to that of the wild-type cells, 82% and 84%, respectively. For ChIP, cells were grown asynchronously in dextrose-containing medium, and 0.2 mM CuSO_4 was added to

the medium for 45 min before cross-linking with formaldehyde. The level of RE DNA enrichment was determined by PCR analysis of the immunoprecipitations from cells with wild-type RE, with the Mcm1 binding site mutation, *CUP1* promoter, or control sequence inserted into the mutant RE (Fig. 5B). The level of Fkh1 binding was expressed as the increase in enrichment (*n*-fold) of RE DNA in tagged versus untagged Fkh1-containing cells and plotted (Fig. 5B, right). The mutation that prevents Mcm1 binding also reduced the level of Fkh1 binding from about fivefold (in W cells) to no enrichment (in M cells) (Fig. 5B), suggesting that Fkh1 requires Mcm1 binding at the RE. The insertion of *CUP1* promoter at the mutant RE increased the level of Fkh1 binding from no enrichment to threefold enrichment (Fig. 5B, M and MC, respectively). This result suggested that *CUP1* promoter may activate RE in the absence of Mcm1 by enhancing Fkh1 binding. Moreover, the level of Fkh1 binding in these cells correlates with the level of donor preference.

Fkh1 was shown to be associated with RNA polymerase II transcription elongation (17). Fkh1 can bind both to the promoter and toward the coding region of the *CLB2* gene. We tested whether Fkh1 binding was higher toward the middle of the transcribed region. Figure 5C demonstrates that Fkh1 binding peaked around its binding sites, suggesting that the role of Fkh1 in RE may be different from its role in transcription elongation.

Fkh1 functions downstream of the chromatin remodeling in RE activation. In order to understand how Mcm1 and the *CUP1* promoter enhance Fkh1 binding, we tested whether they were required for the open chromatin structure of RE in *a* cells. For this, the overall chromatin structure was mapped in α cells, in *a* cells lacking Fkh1, and in *a* cells containing wild type, the Mcm1 binding site mutation with the *CUP1* promoter with or without TATA boxes, or the control sequence insertion. The map was performed at medium resolution with indirect end labeling by using MNase (Fig. 6 and 7).

The chromatin structure of RE has been mapped with DNase I at medium resolution and with MNase at higher resolution by primer extension in both cell types (6, 36). In *a* cells, RE chromatin was open and sensitive to nuclease digestion. In α cells, the nuclease digestion pattern suggested the presence of tightly positioned nucleosomes. Our MNase mapping of the region comprising the conserved domains of RE at medium resolution was consistent with these studies. In *a* cells (Fig. 6, W), the cutting pattern across the RE (lanes 2 and 3) was similar to that of the naked DNA (lane 4), indicating that this region was sensitive to nuclease digestion. In α cells, the sites that were apparent in the naked DNA digestion (lane 4) were mostly protected and the spacing of the MNase cutting sites suggested the presence of positioned nucleosomes (lanes 6 and 7). A scan of the indicated lanes corresponding to *a* (Fig. 6B, W) and α cells is shown in Fig. 6B. In α cells the $\alpha 2$ /Mcm1 operator positions nucleosomes, and an indication of this positioning is not only the protection of sites from nuclease digestion but also the presence of a strong nuclease sensitivity (Fig. 6B, asterisk) around the operator itself. Combined with periodical MNase digestion, this site is a signature for the closed chromatin structure. In *a* cells around the same region, additional sites were digested by MNase, and the sensitivity of the operator was reduced (Fig. 6B). High-resolution MNase

FIG. 5. Analysis of Fkh1 binding at the RE. (A) Donor preference in cells containing myc-tagged Fkh1 and an untagged strain was determined as described in the legend of Fig. 3. (B) ChIP analysis was performed in different strains. The set of primers used in this analysis amplified the RE domains A, B, C, and D. The increase in enrichment (*n*-fold) of RE DNA was calculated and plotted in the right panel with standard deviations from three independent experiments. Telomere primers (TEL) were used to amplify a nonspecific region of the genome and used as loading control (34). (C) Primer sets 1, 2, 3, and 4 were used to amplify the indicated regions in RE, shown schematically below. Fkh1 binding sites occur in domains A, B, D, and E (indicated with brackets). All samples were prepared from asynchronously growing cultures, and 0.2 mM CuSO₄ was added 45 min before cross-linking.

FIG. 6. The chromatin structure around the conserved domains of RE. (A) The chromatin structure was mapped by Southern blotting and indirect end labeling. An undigested sample with two levels of MNase digestion is shown for every sample. Naked DNA was used to monitor the sequence specificity of the enzyme. Wild-type **a** (W) and α cells along with **a** cells containing the Mcm1 binding site mutation (M), *CUP1* promoter (MC), or control sequence (MS) insertion are shown. The domains of RE are indicated to the left of each sample, and the insertions are indicated with brackets. (B) The scans of pixel intensities from lane 3, lane 6, and lane 9 are plotted. RE domains are labeled for α sample, and for other samples, lines indicating the domains are placed at corresponding regions for reference. Nucleosomes are shown as shaded ovals. The asterisk indicates the nuclease sensitivity at the $\alpha 2$ /Mcm1 operator. (C) Scans of lane 13 and lane 18.

maps have demonstrated that Mcm1 protein was required for open chromatin at the RE in **a** cells (37). We also observed that when Mcm1 binding was prevented (Fig. 6B, M), RE presented a more closed chromatin structure (lanes 9 and 10) compared to that of the **a** cells (lanes 2 and 3). The MNase cutting pattern in these cells indicated the presence of nucleosomes, and the $\alpha 2$ /Mcm1 operator showed strong sensitivity to nuclease digestion (Fig. 6A).

When *CUP1* promoter was inserted in cells containing the Mcm1 binding site mutation, RE presented a more open chromatin structure (Fig. 6B, lanes 13 and 14). Moreover, the cutting pattern around the region covering the A, B, C, and D domains in MC cells (lanes 13 and 14) was similar to that of the **a** cells (lanes 2 and 3). The chromatin was more open compared to the cells containing the Mcm1 binding site mutation only (M, lanes 9 and 10) or cells containing the control sequence insertion (MS, lanes 17 and 18). A comparison of the

scan of lanes with similar digestion levels for MC and MS cells is shown in Fig. 6C. While the MS cells showed the features of closed chromatin, the same region in MC cells presented open chromatin and lacked the strong sensitivity of the operator. This suggested that the insertion of *CUP1* promoter disrupts the repressive chromatin structure that is present at the RE when Mcm1 binding is prevented.

Since we observed that the Mcm1 binding site mutant cells with the *CUP1* promoter lacking the TATA boxes showed little transcription, yet high donor preference, we looked at the chromatin structure in these cells (Fig. 7A, MC Δ T). The overall chromatin structure in cells containing the *CUP1* promoter with or without TATA boxes was similar (lanes 2 and 3 and lanes 6 and 7, respectively). A scan of the similarly digested lanes comparing MC Δ T and MC cells is shown in Fig. 7C. In MC Δ T cells, the scan showed increased sensitivity to digestion around the E region, (Fig. 7C, arrowheads). Since MC Δ T cells

FIG. 7. The chromatin structure around the conserved domains of RE. (A) The map was generated as described in the legend of Fig. 6. **a** cells containing the Mcm1 binding site mutation with the insertion of *CUP1* promoter with (MC) or without (MCΔT) TATA boxes are shown. (B) Wild-type **a** cells and **a** cells lacking Fkh1 are shown. (C) The scan of lane 2 and lane 6 of panel A is plotted. The arrowheads highlight the differences in the intensity of cutting at two sites. (D) The scan of lane 2 and lane 6 of panel B.

have higher RE activity, these sites may correspond to the binding of regulatory proteins to the E region.

Unlike prevention of Mcm1 binding to RE, *Fkh1* deletion in **a** cells did not affect the overall chromatin structure (Fig. 7B). The cutting pattern in the mutant cells (lanes 6 and 7) was similar to that of the **a** cells (lane 2 and 3) as well as the naked DNA (lane 8). The scan of similarly digested lanes is shown in Fig. 7D. The overall chromatin structure in cells deleted of Fkh1 ($\Delta Fkh1$) was open like **a** cells, with a number of cutting sites around the conserved domains of RE, and lacked the periodical cutting pattern of closed chromatin structure and the strong sensitivity at the $\alpha 2$ /Mcm1 operator. However, two sites, although present, were less sensitive to digestion in $\Delta Fkh1$ cells, (Fig. 7D, arrowheads). These sites may correspond to regions where Fkh1 protein binds and changes the DNA structure to make it more accessible to MNase cutting. The presence of open chromatin structure without Fkh1 sug-

gests that Fkh1 protein is not required for opening chromatin within the RE region. Therefore, this protein may function downstream of the chromatin remodeling that is recruited to RE by Mcm1. This was supported by the data showing that in the absence of Mcm1, *CUP1* promoter also opens the chromatin structure and enhances Fkh1 binding.

DISCUSSION

Over the past 25 years, many details of yeast mating type switching have been revealed. Although mating type switching is commonly used as a model system to study homologous recombination in yeast, one aspect of switching remains a puzzle. This aspect is the directionality of recombination. During mating type switching, the donor preference is controlled by a small *cis*-acting element, the RE. A number of **a**-cell-specific noncoding RNAs are transcribed from the RE region

(33). However, the role of this transcription is not clear. In this study, we show that the transcription of the noncoding RNAs is cell cycle regulated. In **a** cells, RE transcription requires Mcm1 binding, and the role of Mcm1 protein in RE is to activate transcription and enhance Fkh1 binding. The level of this binding then determines the level of donor preference.

Role of Mcm1 in RE activation and transcription. $\alpha 2$ /Mcm1 operators are present at the promoter regions of **a**-specific genes. Although there are no ORFs in the RE locus, a number of **a**-specific noncoding RNAs are transcribed downstream of these operators (33). We showed that the transcription of these noncoding RNAs is cell cycle regulated. The nature and the significance of this regulation are not clear. Nevertheless, the timing of transcription coincides with mating type switching in a population of cells, suggesting that cell cycle regulation may precede or is partly a consequence of RE activity.

The relationship between RE transcription and Mcm1 protein was not understood. Our results indicate that when the Mcm1 binding site is mutated at RE, the transcription of the noncoding RNAs is abolished. The same mutation was shown to reduce donor preference (37). Additionally, a temperature-sensitive Mcm1 also affects donor preference. These results suggest that Mcm1 protein is required for both RE transcription and donor preference. Moreover, in **a** cells, the open chromatin structure in RE requires Mcm1 binding (37). However, it was not clear whether the Mcm1 protein itself or its requirement for open chromatin was necessary for RE function. In this study, we showed that the requirement for Mcm1 binding can be bypassed by inserting another promoter into RE. Therefore, Mcm1 activates RE by facilitating the formation of open chromatin structure and may not play a role in the downstream functions of RE. This is supported by data showing that Mcm1 is required for open chromatin at RE (37) and that the promoter insertion that increases donor preference also induces more open chromatin (Fig. 6).

We wanted to dissect the role of RNA synthesis and transcription activation in RE. However, this proved to be very difficult to address. In a number of experiments, we tried to distinguish whether the transcription itself or the activation of transcription, involving chromatin remodeling around the conserved domains, was required for RE function. Although we were able to show that a promoter was required, we could not separate the two possibilities. Since reversing the direction of transcription in cells containing the Mcm1 binding site mutation with the *CUP1* promoter inserted did not significantly reduce the donor preference, the sequence of the noncoding RNAs may not be important for RE function. We also found that the level of transcription is not important for RE function. The deletion of TATA boxes from the inserted *CUP1* promoter reduces the level of transcription but does not prevent the chromatin remodeling activity of the promoter (26, 27). The donor preference of these cells was high, suggesting that the chromatin remodeling activity of the promoter, rather than transcription itself, is important for RE function. However, these findings do not address the question directly. Since the transcription start site of the noncoding RNAs resides in a conserved domain that is known to affect donor preference, the deletion of this region would not distinguish whether transcription or these sequences are required for RE function. Due to the fact that the transcription of the noncoding RNAs dimin-

ishes at higher temperatures (data not shown), a temperature-sensitive mutant of the RNA polymerase II could not be used to test whether the polymerase itself is required for RE function.

Since the donor preference of **a** cells can be increased up to ~76% *HML* use without Mcm1 binding, we conclude that the role of Mcm1 in RE function is through transcription activation, involving the recruitment of chromatin remodeling and/or transcription complexes. Our results suggest that transcription activation and open chromatin provided by a promoter are necessary for RE function. We propose that this activation helps recruit the necessary proteins to govern long-range chromosomal interactions during mating type switching.

Fkh1 binding at the RE. Other groups have shown that several transcription factors, including Fkh1, bind to RE and regulate donor preference (31). The deletion of this protein causes a significant reduction in the donor preference of **a** cells. The transcription of the noncoding RNAs does not require Fkh1, suggesting that transcription by itself is not sufficient for donor preference. Additionally, the timing of the transcription and switching was not altered significantly by the deletion of Fkh1. Since Fkh1 is not required for the overall open chromatin structure of RE and its binding depends on Mcm1, Fkh1 may act downstream of the chromatin remodeling activity recruited to RE by Mcm1 or *CUP1* promoter. This was supported by our data showing that the insertion of the *CUP1* promoter enhances Fkh1 binding and also opens the chromatin structure around the conserved domains of RE. Additionally, the level of Fkh1 binding positively correlates with the level of donor preference. These results suggest that Mcm1 facilitates opening the chromatin around Fkh1 binding sites, and this enhances Fkh1 binding. The level of this binding, then, determines the level of donor preference.

The observations that Fkh1 is not required for RE transcription and that a promoter cannot bypass its function suggest that the role of this protein in RE is different from its role in transcription activation. Thus, we also wanted to know whether Fkh1 was involved in elongation of transcription in RE. In order to address this question, we determined the level of Fkh1 binding downstream of the transcription start site, similar to its binding at the *CLB2* gene (17). In the RE region, Fkh1 binding is higher around its binding sites. Combined with the results showing that Fkh1 is not required for RE transcription or open chromatin, this suggests that the function of Fkh1 in RE maybe different from its role in both transcription activation and elongation.

The results presented in this study suggest that the activation of RE in **a** cells consists of a sequence of events that starts with binding of Mcm1, which results in transcription activation and open chromatin structure. This is followed by Fkh1 binding, and the level of this binding positively correlates with the level of donor preference. After this step, how Fkh1 governs long-range chromosomal interactions is not known. Perhaps Fkh1 serves as a marker for the active RE, where recombination proteins would recognize and be directed to *HML*. This model would predict that Fkh1 may directly interact with one or more proteins that are associated with the double-strand break at the *MAT* locus.

The presence of transcription around active RE in **a** cells suggests a role for transcription in increasing the rate of ho-

mologous recombination. Another well-studied system that connects transcription to recombination involves V(D)J recombination in immune cells. This type of recombination is also directional, and the donor sequences that undergo recombination are marked by the presence of an active promoter upstream of the donor (reviewed in reference 19). The rate of homologous recombination can also be increased by transcribing the donor sequences (1, 23). The increase in the rate of recombination around *HML* by RE transcription might be due to a similar mechanism. However, the donor of recombination (*HML*) in mating type switching is silenced and heterochromatic (reviewed in references 7, 11, and 15). Moreover, the transcription happens ~16 kb away from the donor sequence. Therefore, we propose that RE might serve as an entry point for the recombination complex, scanning the region for homology. The advantage of having RE as an entry point would be twofold. First, RE activity may be modulated easily according to the cell type by having the $\alpha 2$ /Mcm1 operator to regulate transcription and the chromatin structure in a similar manner to **a**-specific genes. This is supported by the data presented in this study along with previous studies (32, 33, 36, 37). Second, if RE serves as an entry point, transcription and/or associated factors may increase the rate of recombination around the whole region, including *HML*. Increased recombination frequency around RE is consistent with the observations that the entire left arm of chromosome III is hot for recombination in **a** cells and that the location and distance of RE from *HML* influences donor preference (39).

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