Molecular Biology Reports (2005) 32: 197–204
DOI 10.1007/s11033-005-2312-x

Dual roles for Mcm10 in DNA replication initiation and silencing at the mating-type loci

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Accepted 10 August 2005

Key words: chromatin, minichromosome, replication, transcription, yeast

Abstract

Recent studies linking DNA replication proteins to transcriptional silencing suggest that some of the same mechanisms that facilitate the initiation of replication at origins might be involved in establishing repressed chromatin at silencer elements. Our ongoing studies of several mutants of the replication initiation factor Mcm10 of budding yeast revealed an associated defect in the production of mating type pheromones. This observation prompted us to look more directly at the effect of *MCM10* mutations on the expression of a reporter gene in the mating type locus and to assay for physical interactions between Mcm10 and known silencing factors. Our findings, that Mcm10 mutants disrupt mating loci silencing and that Mcm10 interacts with Sir2 and Sir3, suggest that Mcm10 also plays an essential, and separable role in transcriptional silencing.

Introduction

The recognition, unwinding and subsequent replication of DNA involves considerable rearrangement of nucleosomes and other DNA bound proteins. In addition to responding to changes in chromatin structure, several replication proteins play an active role in establishing, maintaining, and opening chromatin structure at origins as well as other chromosomal locations. Several replication initiation proteins interact with general chromatin remodeling factors to affect global chromatin structure [1, 2] while others have been found to have more direct influence on specific local nucleosomal positioning. The Origin Recognition Complex (ORC), for example, has been shown to be necessary for silencing of genes within the cryptic mating loci of yeast and its replication initiation and silencing functions are genetically separable [3]. ORC binds directly to the silencer elements flanking the mating-type loci and is responsible for the recruitment of the silent information regulator, Sir1, which, along with Rap1 and Abf1, subsequently recruits Sir3 and Sir4 and the histone de-acetylase, Sir2. The interactions of these proteins modify the local nucleosomes into a condensed array [Reviewed in 4 and 5]. The degree of transcriptional repression at the mating loci can also be influenced by defects in several replication proteins, such as members of ORC [6, 7], PCNA, RF-C, Cdc45 [8, 9].

Multiple studies of *Saccharomyces cerevisiae* Mcm10 (MiniChromosome Maintenance protein 10) have clearly established that it is involved in the initiation of DNA replication and that it may also participate in replication elongation [10–13]. Several aspects of Mcm10 suggested to us that it might also be involved in silencing

chromatin. Physical interactions with ORC [12, N. Douglas, unpubl.] implied that Mcm10 could also interact with ORC at the silent mating-type loci. The fact that Mcm10 is very abundant, far exceeding the number of replication origins in yeast, also suggested that it might bind sites other than origins [12]. Finally, as we present here, we found that several Mcm10 mutants have mating pheromone defects suggesting a connection with the silent mating-type locus. In this study we solidify this connection by demonstrating that Mcm10 is necessary for proper transcriptional silencing of a reporter gene at the silent mating loci and that its physically interactions with well established silencing proteins are important for this function.

Materials and methods

Plasmids and strains

Deletion construct C108 and site mutant STAA were created using the Promega Altered Sites II mutagenesis system. Mutagenic primers were annealed to phage produced single-stranded pALTER*MCM10*, the DNA elongated, ligated, amplified and subcloned into pRS315 containing *MCM10* and its promotor. Deletion construct N128, was constructed using the Invitrogen Gateway cloning system. PRS315 containing the *MCM10* promotor was converted into a destination vector by insertion of a cassette containing a chloramphenicol resistance and cytotoxic gene flanked by lambda recombination sites (courtesy of Tim Christensen). The Mcm10 mutants were PCR amplified to include recombination sequences that allowed insertion into pDONR201 and subsequent recombination into the destination vector. N128, C108, and STAA were subcloned into pRS405 then integrated along with the LEU2 gene into the *MCM10* locus of the null strain for further study and these strains were crossed with *mcm10-1* to produce both mating types.

The *SIR* and *RAP1* yeast two-hybrid plasmids described here were constructed using the Invitrogen Gateway cloning system. GAD2F and BTM116 plasmids [14] were converted into destination vectors by insertion of the recombination cassette described above into the *Bam*HI site. The full length GAD and BTM*MCM10* were described in [13].

Growth assay

The growth of strains containing each of the mutant constructs at the *MCM10* locus were compared to the wild-type on plates at 14, 25 and 37 °C and in liquid culture at 25 and 37 °C. The only growth defects observed were that both *mcm10-1* and *mcm10-43* grew slightly slower than the wild-type at 30 °C and growth of both mutants was arrested at 37 °C.

MCM assays

Single colonies from strains containing the ARS1 (YCp101) minichromosome grown on YPD were re-suspended and equal volumes plated onto YPD and selective media at 30 °C. Plasmid loss rates per generation were calculated using the equation $1-(\%f)^{1/N}$ where $\%f = (\#\text{ colonies on selective} \times \text{dilution factor})/(\#\text{ colonies on CM} \times \text{dilution factor})$ and $N = \ln(\#\text{ colonies on CM} \times \text{dilution factor})/\ln 2$. All values given here represent the average of at least three separate assays.

Halo assays

Both mating types of each strain were spotted onto YPD (Yeast Peptone Dextrose agar) and allowed to grow 24 h at 30 °C then overlaid with either α (sst2) or **a** (bar1) mating type tester strains. Halos were assessed and photographed after another 24 h at 30 °C.

Silencing assays

W303, *mcm10-1* and *mcm10-43* strains (W303 background) were crossed to the mating type silencing reporter strain, RS1295 [9], and ts, ADE+ spores selected for analysis by color assay. Mcm10 (WT), *mcm10-1*, and *mcm10-43*, carrying the *ADE2* reporter were plated onto Yeast Peptone Dextrose agar, grown for 3 days at 30 °C and photographed after another 3 days at 4 °C.

Yeast two-hybrid assays

GAD2F and BTM116 constructs were transformed into the two-hybrid strain EGY40 carrying

the pSH18-34 reporter plasmid [14] and plated on selective media at 30 °C. Ten colonies were re-suspending in 30 μl water and 10 μl spotted onto Xgal (Sigma) plates and grown overnight at 30 °C for photographs. Expression and stability of the fusion proteins was confirmed by western blots probed with anti-LexA antibodies from Invitrogen (cat# 460710) and Mouse anti-Gal4 antibodies from Santa Cruz (cat# C-10).

Results

Mating pheromone halo defects in Mcm10 mutants

The active mating-type locus, *MAT*, contains **a** or α specific genes whose expression leads to the production and release of the mating-type pheromones, **a**-factor or α-factor. Flanking the active *MAT* locus are two tightly repressed (silenced) mating-type loci, *HML*α and*HMR***a** that also contain α and **a** specific genes, respectively. Normally, in a haploid cell, the release of the mating pheromone arrests the growth of cells of the opposite mating-type, thus allowing them to mate and the information at the *HML*α and *HMR***a** loci needs to remain silent to ensure that haploid cells maintain their sexual identity [5].

Practically, these behaviors allow a relatively simple test for mating pheromone production, referred to as a halo assay. The strain to be tested is spotted onto a plate where the growing cells secrete mating pheromone into the surrounding media and then they are overlaid with a lawn of pheromone sensitive, **a** or α mating-type tester strains. If the spotted cells appropriately produce mating pheromones, then the tester lawn arrests in a ''halo'' around the spot.

We tested the series of Mcm10 mutants drawn schematically in Figure 1 in the halo assay as well as the *mcm10-1*/*mcm7-1* double mutant. As can be seen in Figure 2, the temperature sensitive point mutants, *mcm10-1* and *mcm10-43*, the Mcm10 end deletions N128 and C108, and the *mcm10-1*/*mcm7-1* double mutant cause α-specific halo defects at 30 °C. Specifically, the α cells produced no detectible halo in the **a** tester lawn and a slight, aberrant, halo in the α lawn, suggesting that the mutants were not making α-factor, and that they may be inappropriately producing small amounts of **a**-factor. The **a** cells of all of the mutants, by contrast, had pheromone halos comparable to the wild-type on either tester lawn.

As Figure 1 indicates, we found that aberrant halos do not necessarily correlate with growth defects but that all of the single point and deletion mutants with halo defects also have replication initiation defects as measured by minichromosome maintenance assays. STAA was included as a control in our analysis because this alanine substitution of a potential Cdc28 phosphorylation site was known to have no effect on growth or replication and we found that it also did not show a mating pheromone halo defect. By contrast, the

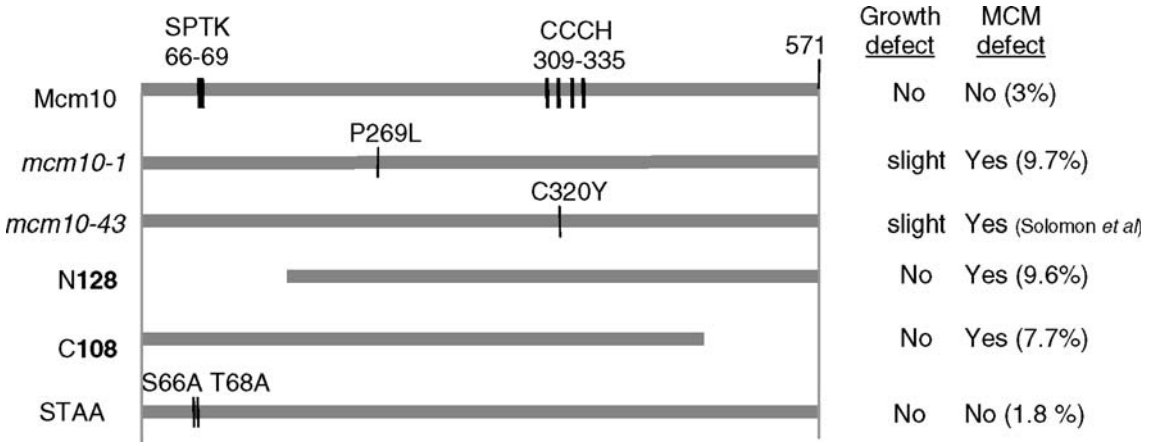

Figure 1. Schematic diagram of Mcm10 mutants. Full length Mcm10 is represented at the top with the positions of the potential phosphorylation site (SPTK) and zinc finger (CCCH). The number of amino acids deleted from the N or C terminus in each construct is indicated at the left. Whether or not each mutant affects growth or Minichromosome Maintenance (MCM) 30 °C is indicated to the right with the percent loss per generation of the ARS1 containing YCp101 plasmid in parentheses.

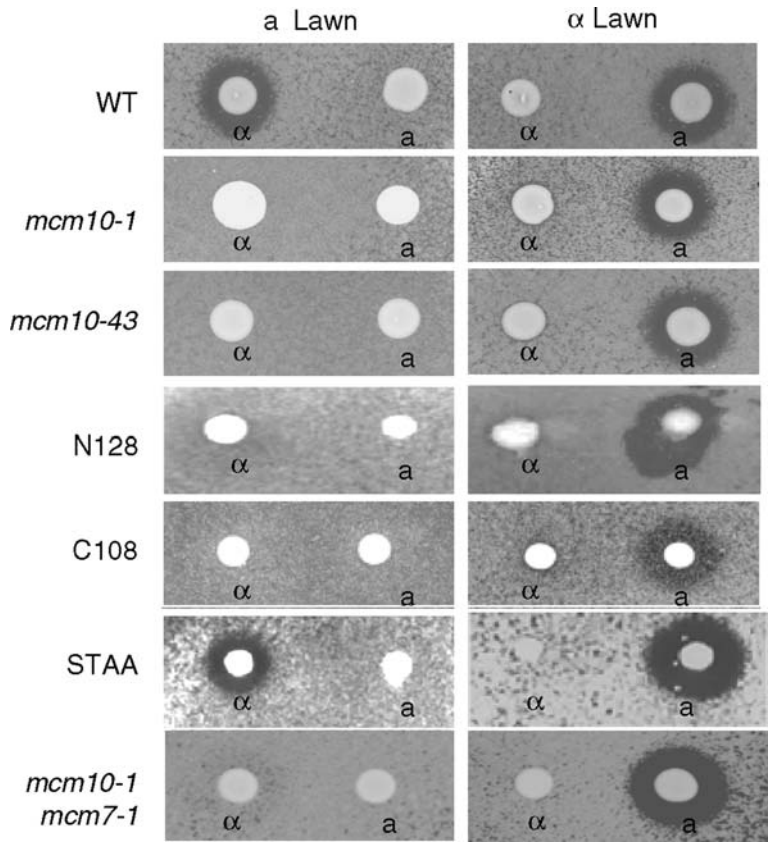

Figure 2. Mating pheromone defects associated with Mcm10 Mutants. The **a** and α versions of each of the indicated strains were spotted onto YPD at 30 °C, grown overnight and overlayed with lawns of phermone sensitive,**a** or α mating type tester strains. The plates were photographed after another 24 h at 30 °C. Clear regions (halos) around the spotted strains indicate arrest of the tester strain due to the presence of the mating type pheromones.

mcm10-1/*mcm7-1* double mutant has a halo defect despite the fact that the growth defect, minichromosome maintenance defect and protein interactions of the *mcm10-1* have been shown to be restored by *mcm7-1* [11]. We postulated that if the normally tightly repressed mating-loci *HML*α and/or *HMR***a** become de-repressed, their gene products will interfere with the production and release of the mating hormones. Thus, the halo assay could be an indirect measure of the integrity of silencing at the cryptic mating-type loci.

Expression of a reporter gene at the HMR locus

In order to look more directly at the degree of repression at the silent mating loci we utilized an integrated reporter gene. We crossed a strain containing *ADE2* in place of *HMR***a** [9] with *mcm10-1* and *mcm10-43* to obtain strains carrying the *MCM10* mutations with the *HMR* reporter. When the *ADE2* gene is properly repressed, the cells should turn pink/red and we saw appropriately pink colonies for the wild-type strain (Figure 3a). By contrast, the *mcm10-1* and *mcm10-43* colonies were white under the same conditions, indicating that both mutants caused significant de-repression at the *HMR* locus.

To confirm that the differences in color were actually due to the *MCM10* mutations, we transformed each of the strains with the high-copy, LexA fusion vector, BTM116, carrying *MCM10*, *mcm10-1* and *mcm10-43*. All three plasmids complement the *mcm10* null strain at 25 °C (data not shown). As Figure 3b shows, over-expression of full-length LexAMcm10 in both the mutant strains restored silencing of the *ADE2* reporter. Expression of more LexAmcm10-1 in the *mcm10-1* strain or more LexAmcm10-43 in the *mcm10-43* strain did not restore silencing. These results demonstrate that the silencing defect of *mcm10-1* and

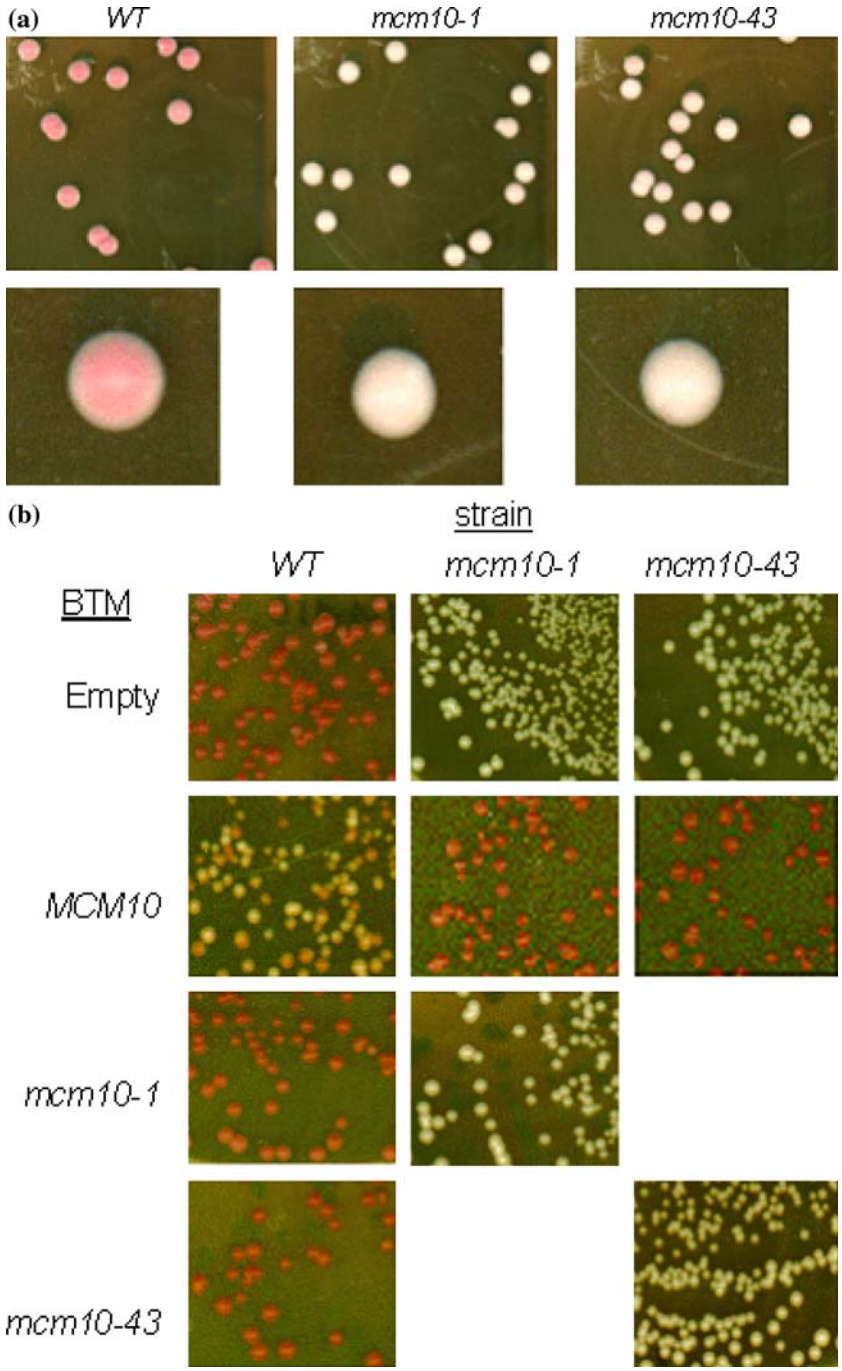

Figure 3. Loss of *HMR***a** repression in the *mcm10-1* and *mcm10-43* mutants. A qualitative color assay indicates the level of repression of *ADE2* inserted at *HMR***a**. (a) Mcm10 (WT), *mcm10-1*, and *mcm10-43*, carrying the *ADE2* reporter were plated onto Yeast Peptone Dextrose agar, grown for 3 days at 30 °C and photographed after another 3 days at 4 °C. Pink/red colonies appear dark in these photos and white colonies appear light. Single colonies were enlarged to better show the color differences between the strains. (b) The strains, shown in (a), were transformed with the expression vector BTM116 or BTM116 carrying *MCM10, mcm10-1* or *mcm10-43*. The transformants were plated on Complete Media – Tryptophan at 30 °C for 3 days then photographed after another 3 days at 4 °C.

mcm10-43, like their growth defects, are recessive. Unexpectedly, over-expression of full-length LexAMcm10 in the wild-type strain produced colonies that varied considerably in color, although the overall hue was still pink.

Mcm10 interacts with known silencing proteins

Because both the halo assay and the *ADE2* reporter suggested that Mcm10 is involved in repression of the silent mating loci, we wanted to test for direct interactions between Mcm10 and proteins required for silencing. As described earlier, the Sir proteins not only participate in the chromatin structure of repressed regions, but they also directly interact with ORC and with each other. We tested the four Sir proteins and Rap1 for interactions with Mcm10 in a yeast two-hybrid assay and found that Sir2 and Sir3 gave strong positive signals (Figure 4). Rap1, Sir1 and Sir4, also essential for establishing silencing, did not interact with Mcm10 in this assay, although expression and stability of all of our two-hybrid fusion constructs were confirmed by western blot (data not shown). The Mcm10 mutant constructs varied in their ability to interact with Sir2 and Sir3 in the two-hybrid assay (Figure 4). Overall, the mutants lost or had diminished interactions with one (N128 with Sir3) or both of the Sir proteins (*mcm10-1*, *mcm10-43* and Deletion C108). By contrast, the STAA mutant maintained essentially wild-type interactions with both Sir2 and Sir3.

Discussion

The mating pheromone halo assay was our first indication that Mcm10 may have a functional role at the cryptic mating-type loci in yeast. Although we might expect that the two cryptic loci would be affected equally by Mcm10 defects, it appears that HMLα is not as sensitive as HMR**a**. Several studies have found significant architectural differences between the HMLα and HMR**a** loci and between the mechanism of regulation of α specific and **a** specific genes that could account for the mating-type specific phenotype that we observed [5, 15, 16]. In addition, there may be differences in the sensitivity of the overlain tester strains to the mating pheromones.

Our hypothesis that the halo defects we observed indicated a silencing defect was supported by our results looking at a reporter gene at one of the normally silent mating-type loci. We observed that over-expression of LexAMcm10 in the otherwise wild-type reporter strain resulted in

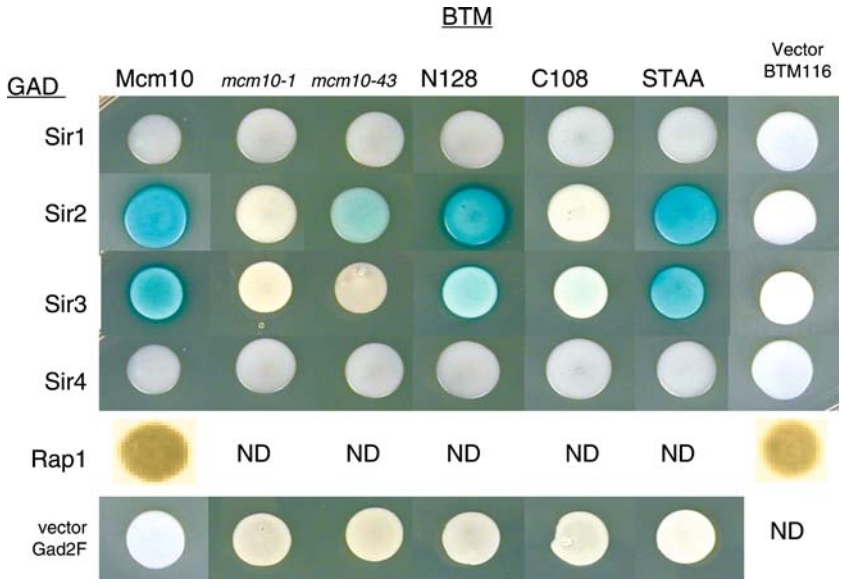

Figure 4. Two-hybrid interactions between Mcm10 and silencing proteins. BTM and GAD two-hybrid constructs were expressed in EGY40[pSH18-34]. Blue color of cells spotted onto plates containing XGAL and grown at 30 °C indicates activation of the pSH18-34 reporter. ND = not done.

variation in color of individual colonies. Although we have observed that under some conditions, over-expression of Mcm10 in wild-type cells can be lethal or cause slow growth, we have expressed this LexAMcm10 construct in several wild-type strains without any detectible effect on growth (data not shown). The colony color variation, therefore, suggests that over-expression of LexAMcm10 affects the integrity of HMR silencing. A possible model to explain this variation is that LexAMcm10 titrates another silencing factor away from the already established repressed region. If silencing is not already established, as in the Mcm10 mutant strains, then there would be no change in the colony color – as we observed. We believe that the degree of "pinkness" in individual colony color reflects differences in the LexAMcm10 plasmid copy number from cell to cell and thus the degree of "titration". This conclusion is based on the observation that all colonies from the LexaMcm10 transformations became more pink and less variable when restreaked (data not shown) suggesting a reduction in the copy number of the plasmid over several generations.

Our two-hybrid results suggest that interactions between Mcm10 and Sir2 and 3 are necessary for proper transcriptional repression at the mating-type locus and that even slight loss of these interactions (as in the N128 mutant) can disrupt this region to an extent that is detectible by the halo assay. Because the loss of blue color in the N128/Sir3 assay was slight, it is possible that it is due to variation in the reporter plasmid copy number rather than a loss of physical interactions. If N128 is able to interact with Sir2 and Sir3 as well as the wild-type Mcm10, than it's silencing defect may be due to its loss of interactions with other proteins such as members of ORC (Data not shown) or diminished chromatin binding. In either case, the combined two-hybrid results of N128 and C108 suggest that the interactions between Mcm10 and Sir2 and Sir3 proteins require the intact C terminus of Mcm10. Our future studies will explore the functional dynamics of these protein interactions and how they relate to protein interaction occurring at replication origins.

The HMR reporter and two-hybrid interactions between Mcm10 and Sir2 and Sir3 imply a direct role for Mcm10 in mating-type silencing. Mutants of several Mcm2–7 members show the same α-specific halo defect as our Mcm10 mutants (J. Donato, unpubl.). If the halo defect reflects loss of silencing in the Mcm10 mutants, then we would argue that Mcm2–7 are also involved in silencing at the mating-type loci or that they indirectly affect silencing through Mcm10. In fact, the essential presence of the replication origins within the mating loci silencers suggests that all of the proteins that congregate at origins could have additional roles in silencing like has been demonstrated for ORC. Genome-wide distribution studies have placed Mcm2–7 proteins at the silent mating loci and the telomeres [17] and other replication proteins, such as PCNA, Polymerase epsilon, Rf-C and Cdc45, have been shown to affect HMR silencing [8, 9].

Several studies suggest that Mcm10, Mcm2–7 and ORC affect each others stability and function at replication origins in budding yeast [10–13]. We propose that these interactions also occur at the mating-type silencer elements and that they influence silencing of the region. If interactions between Mcm10 and Sirs facilitate silencing, then mutations in Mcm10 and proteins required for Mcm10 to function, such as Mcm2–7, might have similar silencing defects that result in similar halo defects. Study of the *mcm10-1*/*mcm7-1* double mutant further suggests that the interplay of Mcm10 and replication proteins such as Mcm7 may be different at origins than it is at silencer elements and that these two roles may be separable. It is possible that the double mutant restores interactions that are critical at the replication origin (Mcm10–Mcm7) but that it fails to restore independent interactions that are critical at the mating-type loci (Mcm10–Sirs).

Acknowledgments

These studies were conducted in the laboratory of Dr. Bik-Kwoon Tye and we thank her for critical reading of this manuscript. We thank the Stillman lab for providing the *ADE2* reporter strain. This work was supported by the NIH Biochemistry Molecular and Cell Biology predoctoral training grant and by NIH grant GM34190.

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