

# Mcm10 Is Required for the Maintenance of Transcriptional Silencing in *Saccharomyces cerevisiae*

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## ABSTRACT

Mcm10 is an essential protein that participates in both the initiation and the elongation of DNA replication. In this study we demonstrate a role for Mcm10 in the maintenance of heterochromatic silencing at telomeres and *HM* loci of budding yeast. Two *mcm10* mutants drastically reduce silencing of both *URA3* and *ADE2* reporter genes integrated into these silent loci. When exposed to  $\alpha$ -factor, *mcm10* mutant cells display a “shmoo-cluster” phenotype associated with a defect in the maintenance of silencing. In addition, when combined with a defect in the establishment of silent chromatin, *mcm10* mutants demonstrate a synergistic defect in *HML* silencing. Consistent with a direct silencing function, Mcm10p shows a two-hybrid interaction with Sir2p and Sir3p that is destroyed by the *mcm10-1* mutation and dependent on the C-terminal 108 amino acids. Tethering *GBD-MCM10* to a defective *HMR-E* silencer is not sufficient to restore silencing. Furthermore, mutations in *MCM10* inhibit the ability of *GBD-SIR3* to restore silencing when tethered to a defective *HMR-E*. Suppressor mutations in *MCM2*, which suppress the temperature sensitivity of *mcm10-1*, fail to overcome the *mcm10-1* silencing defect, suggesting that *MCM10*'s role in transcriptional silencing may be separate from its essential functions in DNA replication.

**T**RANSSCRIPTIONAL silencing in the budding yeast *Saccharomyces cerevisiae* renders large regions of the genome inactive in a fashion similar to that in heterochromatic regions in higher eukaryotes (GREWAL and ELGIN 2002). Transcriptional silencing occurs at yeast telomeres and at silent mating-type (*HM*) loci, *HML* and *HMR* (reviewed in RUSCHE *et al.* 2003), which house silent endogenous copies of the mating-type genes (reviewed in HABER 1998). The *HM* loci are flanked by *cis*-acting regulatory elements called silencers (FELDMAN *et al.* 1984; BRAND *et al.* 1985, 1987). These elements consist of A, E, and B sites that are bound by the origin recognition complex (ORC) (BELL *et al.* 1993; MICKLEM *et al.* 1993; LOO *et al.* 1995), Rap1 (SHORE and NASMYTH 1987), and Abf1 (DIFFLEY and STILLMAN 1988), respectively. Once bound, these factors recruit the silent information regulator (Sir) proteins, Sir1–4. Although Orc1 directly interacts with Sir1 and Rap1 with Sir4 to establish a silencing complex that includes Sir2 and Sir3, it is unclear if each of these proteins can be recruited to the silencer independently of each other. A stepwise spreading of the Sir2/3/4 complex from the silencer leads to the silencing of nearby genes (HECHT *et al.* 1996; HOPPE *et al.* 2002; RUSCHE *et al.* 2002). Telomeric silencing is similar to silencing at *HM* loci, with the exception that the Sir2/3/4 complex is recruited by Rap1

bound to telomeric DNA (APARICIO *et al.* 1991; KYRION *et al.* 1993; MORETTI *et al.* 1994; RUSCHE *et al.* 2003). Silencing is thought to consist of two phases, establishment and maintenance (MILLER and NASMYTH 1984; PILLUS and RINE 1989). During the establishment phase, silent chromatin is formed on nascent DNA and in the maintenance phase it has to be sustained until the next establishment event. Mutations in genes such as *SIR1* and *RAP1* can lead to defects in the establishment but not maintenance of silent chromatin (PILLUS and RINE 1989; MAHONEY *et al.* 1991; SUSSEL and SHORE 1991), and mutations in genes such as *CAC1* and *SIR3* can cause maintenance but not establishment defects (ENOMOTO and BERMAN 1998; ENOMOTO *et al.* 2000).

A link between transcriptional silencing and DNA replication has been suggested by the observation that passage through S phase is required for the establishment of transcriptional silencing (MILLER and NASMYTH 1984). Another connection between silencing and DNA replication is evidenced by the fact that silencers can be used to initiate replication of plasmids (BRAND *et al.* 1987) and that some origins have silencer activity (GRUNWELLER and EHRENHOFER-MURRAY 2002). However, other studies have demonstrated that the S-phase requirement does not involve DNA replication (KIRCHMAIER and RINE 2001; LI *et al.* 2001). In *S. cerevisiae*, DNA replication is a tightly regulated process initiated in early S phase at specific loci called origins of replication or autonomously replicating sequences (ARs) (reviewed in DUTTA and BELL 1997). Budding yeast have ~400 such

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replication origins, but most have not been shown to play a role in silencing. Although the connection between DNA replication and transcriptional silencing is not completely understood, several DNA replication proteins have been implicated in silencing. These include *S. cerevisiae* ORC (BELL *et al.* 1993; MICKLEM *et al.* 1993), PCNA, RF-C, Cdc45 (EHRENHOFER-MURRAY *et al.* 1999; ZHANG *et al.* 2000), and Mcm5 (DZIAK *et al.* 2003). Similarly, DNA replication factors have also been implicated in the organization of chromatin structure in higher eukaryotes such as the fruit fly *Drosophila melanogaster* (PFLUMM and BOTCHAN 2001; CHRISTENSEN and TYE 2003) and *Schizosaccharomyces pombe* (BAILIS *et al.* 2003). One of these proteins, Mcm10, physically interacts with the heterochromatin factor HPI (CHRISTENSEN and TYE 2003; KELLUM 2003) in *D. melanogaster*, indicating a possible direct role in chromatin structure.

*MCM10* was originally identified in yeast screens that searched for mutants in DNA replication (DUMAS *et al.* 1982; SOLOMON *et al.* 1992) and minichromosome maintenance (MAINE *et al.* 1984). Mcm10 is an essential chromatin-associated protein (HOMESLEY *et al.* 2000; RICKE and BIELINSKY 2004; YANG *et al.* 2005). Temperature-sensitive *mcm10* mutants show sluggish passage through S phase (KAWASAKI *et al.* 2000), an inability to complete S phase (MERCHANT *et al.* 1997), and a replication fork pause phenotype at inactive origins of replication (MERCHANT *et al.* 1997). *MCM10* has also been implicated in the initiation of DNA replication (HOMESLEY *et al.* 2000; WOHLSCHEGEL *et al.* 2002; SAWYER *et al.* 2004). While its exact roles in DNA replication are not yet completely understood, Mcm10 shows physical and genetic interactions with subunits of ORC (MERCHANT *et al.* 1997; HOMESLEY *et al.* 2000; KAWASAKI *et al.* 2000; DOUGLAS 2003), which has been implicated in heterochromatin-induced silencing. Intriguingly, there are estimated to be ~40,000 copies of Mcm10p per cell (KAWASAKI *et al.* 2000), far exceeding in abundance the ~400 DNA replication origins in budding yeast. Although the reason for such abundance is not clear, it seems likely that Mcm10 may have other, previously unidentified functions.

In this study we demonstrate that Mcm10 plays a role in heterochromatin silencing in *S. cerevisiae*. Two mutant alleles of *MCM10* manifest significant defects in silencing at both the telomeric and *HMR* loci. Consistent with this observation, when *mcm10* mutant cells are exposed to  $\alpha$ -factor, they show a “shmoo-cluster” phenotype, signifying a defect in the maintenance of silent chromatin at the *HML* locus (ENOMOTO and BERMAN 1998). We also found two-hybrid interactions between Mcm10 and components of the silencing machinery and that these interactions are disrupted by the Mcm10-1 and Mcm10-43 mutant proteins. Interestingly, the temperature-sensitive phenotype of *mcm10* mutant cells can be genetically separated from the silencing defect by second-site mutations that suppress the temperature sensitivity but not the silencing defect. Together our findings demon-

strate a novel function for *MCM10* in the maintenance of silent chromatin that may be separate from its essential role in DNA replication.

## MATERIALS AND METHODS

**Strains and plasmids:** Strains used in this study are listed in Table 1. All strains were isogenic derivatives of W303-1A, unless otherwise indicated. All procedures were performed according to standard yeast methodology (SHERMAN 1991). Strains carrying silencing reporters were made by crossing strain YB541 or YB697 to the appropriate mutant strain and selecting desired segregants by their conditional phenotypes and/or auxotrophy. Genotypes were confirmed by PCR or by plasmid complementation where applicable. Plasmids used in this study are listed in Table 2. Plasmids used for the expression of tethering fusions were constructed by the Gateway system (Invitrogen, San Diego). To create pGBKgw, the *kan<sup>r</sup>* gene of plasmid pGBKT7 (CLONTECH, Palo Alto, CA) was replaced with an *amp<sup>r</sup>* gene and a Gateway (Invitrogen) cassette was inserted into the multiple-cloning sequence. Gateway cassettes were ligated into plasmids pBTM116 and pGAD2F, creating pBTMgw and pGADgw, respectively. Gateway cassette B was ligated into the *Sma*I site of pGBT9.C (BARTEL *et al.* 1996), creating plasmid pGBT9gw. pDONR201 entry clones containing *MCM10* and *SIR1* ready for N-terminal fusions were constructed according to Invitrogen instructions and sequenced. LR recombination reactions (Invitrogen) were set up between pGBT9gw, pGADgw, pBTMgw, or pGBKgw and each of the aforementioned entry clones. All yeast transformations were carried out using standard lithium acetate protocols.

**Silencing assays:** Strains bearing the *URA3* reporter were grown overnight in appropriate dropout media. Tenfold serial dilutions were set up in sterile 96-well plates and ~25  $\mu$ l of each dilution was spotted onto the appropriate plate using a multipipettor. 5-Fluoroorotic acid (5-FOA) was used at a concentration of 1 mg/ml unless otherwise stated. In cases where the *ADE2* reporter gene was used, cultures were diluted to ~300 cells/dropout plate. Colonies were grown for 3 days at 30° and incubated for 3 days at 4° for color development before being counted.

**$\alpha$ -Factor confrontation (shmooing) and mating assays:** For the shmooing assay, 50  $\mu$ l of a saturated *MAT $\alpha$*  culture was streaked along the middle of a YPD plate and incubated at 30° for 4–6 hr for the  $\alpha$ -factor to diffuse into the media. A small sample of *MAT $\alpha$*  culture being tested was then spotted near the edge of the plate and a dissecting microscope needle was used to line up individual unbudded *MAT $\alpha$*  cells approximately one-half of an optical field (at  $\times 160$  magnification) away from the *MAT $\alpha$*  streak. Cells were incubated at 30° for 16–18 hr and the resulting phenotypes of *MAT $\alpha$*  cells were scored. For patch-mating assays, the strains being tested were streaked in a parallel pattern on YPD plates, grown overnight at 30°, and then replica plated onto tester strains 6697-1 or 6697-3, of opposite mating type on a minimal media plate. Plates were then incubated for 2 days at 30°.

**Protein-protein interactions:** The pGADgw SIR and RAP1 two-hybrid plasmids were constructed using the Invitrogen Gateway cloning system. pGAD2F and pBTM116 plasmids (FIELDS and SONG 1989) were converted into destination vectors by insertion of the Gateway recombination cassette into the *Bam*HI site. The full-length pBTM.MCM10 was described in MERCHANT *et al.* (1997). pGAD2F and pBTM116 constructs were transformed into the two-hybrid strain EGY40 carrying the pSH18-34 reporter plasmid (FIELDS and SONG 1989). Interactions were assessed by the appearance of blue colonies on plates containing X-gal (Sigma, St. Louis). Ten

**TABLE 1**  
**Strains used in this study**

Strains	Genotype	Source
	Isogenic to W303	
W303-1A	<i>MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</i>	
W303-1B	<i>MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</i>	
BTY100	W303 <i>MATa mcm10-1</i>	This lab
BTY101	W303 <i>MATα mcm10-1</i>	This lab
BTY103	W303 <i>MATa mcm10-43</i>	This lab
BTY102	W303 <i>MATα mcm10-43</i>	This lab
YB541	<i>MATa adh4::URA3 Tel (VII-L)</i>	B. Stillman
YB697	<i>MATα hmr::ADE2</i>	B. Stillman
ILY171	W303 <i>MATa hmr::ADE2 adh4::URA3 Tel (VII-L)</i>	This study
ILY180	ILY171 <i>mcm10-1</i>	This study
ILY270	ILY171 <i>mcm10-43</i>	This study
GCY23	W303 <i>MATa RDN1::MET15 ΔAhmr::TRP1 adh4::URA3 Tel (VII-L) sir2Δ met15 ADE2</i>	D. Shore
ILY163	W303 <i>MATa adh4::ADE2 Tel (VII-L)</i>	This study
ILY165	ILY163 <i>mcm10-1</i>	This study
ILY167	ILY163 <i>mcm10-43</i>	This study
YJB1838	W303 <i>MATa cac1::LEU2</i>	J. Berman
YJB1940	W303 <i>MATa sir1::HIS3</i>	J. Berman
ILY153	W303 <i>MATα sir1::HIS3</i>	This study
ILY154	W303 <i>MATα cac1::LEU2</i>	This study
ILY155	YJB1940 <i>mcm10-1</i>	This study
ILY156	ILY153 <i>mcm10-1</i>	This study
ILY157	YJB1940 <i>mcm10-43</i>	This study
ILY158	ILY153 <i>mcm10-43</i>	This study
ILY159	YJB1838 <i>mcm10-1</i>	This study
ILY160	ILY154 <i>mcm10-1</i>	This study
ILY161	YJB1838 <i>mcm10-43</i>	This study
ILY162	ILY154 <i>mcm10-43</i>	This study
ILY155	YJB1940 <i>mcm10-1</i>	This study
ILY157	YJB1940 <i>mcm10-43</i>	This study
ILY159	YJB1838 <i>mcm10-1</i>	This study
ILY161	YJB1838 <i>mcm10-43</i>	This study
ILY212	W303 <i>MATa sir1::HIS3 cac1::LEU2</i>	This study
ILY235	YB541 <i>sir1::HIS3</i>	This study
ILY237	YB541 <i>cac1::LEU2</i>	This study
ILY239	ILY235 <i>mcm10-1</i>	This study
ILY241	ILY237 <i>mcm10-1</i>	This study
ILY220	ILY171 <i>Mcm2-S619Y</i>	This study
ILY221	ILY171 <i>Mcm2-S619F</i>	This study
ILY222	ILY171 <i>Mcm2-S619Y mcm10-1</i>	This study
ILY223	ILY171 <i>Mcm2-S619F mcm10-1</i>	This study
YEA80	<i>MATα Aeb::UASg hmr::URA3</i>	R. Sternglanz
YEA82	<i>MATα aeb::UASg hmr::URA3</i>	R. Sternglanz
ILY137	YEA80 <i>MATa mcm10-1</i>	This study
ILY139	YEA80 <i>MATa mcm10-43</i>	This study
	Other backgrounds	
EGY40[pSH18-34]	<i>MATa ura3-52 trp1-1 leu2-3,112</i> [pSH18-34]	E. Golemis
ILY267	EGY40[pSH18-34] <i>sir4::HIS3</i>	This study
ILY268	EGY40[pSH18-34] <i>sir3::HIS3</i>	This study
ILY269	EGY40[pSH18-34] <i>sir2::HIS3</i>	This study
PJ69-4a	<i>MATa ura3-52 his3-Δ200 leu2-3,112 trp1-901 gal4Δ gal80Δ</i>	P. James
6697-1	<i>LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-LacZ</i>	This lab
6697-3	<i>MATa met4</i>	This lab
	<i>MATα met4</i>	This lab

**TABLE 2**  
**Plasmids used in this study**

Plasmid name	Description	Source
pRS315	YCP LEU2	New England Biolabs
pRS315MCM10	YCP LEU2 MCM10	This lab
pGAD2F	2 $\mu$ LEU2 GAD4-AD	S. Fields
pBTM116	2 $\mu$ TRP1 LEXA-DBD	S. Fields
pSH18-34	URA3 LacZ with LEXA binding sites	S. Fields
pGADgw	pGAD2F with Gateway cassette	This study
pBTMgw	pBTM116 with Gateway cassette	This study
pGBKgw	pGBKT7 with Gateway cassette amp <sup>r</sup>	This study
pBTMMCM10	pBTMgw MCM10	This study
pBTMmcm10-1	pBTMgw mcm10-1	This study
pBTMmcm10-43	pBTMgw mcm10-43	This study
pBTMMCM10-N128	pBTMgw MCM10 (128-571)	This study
pBTMMCM10-RIGHT	pBTMgw MCM10 (292-571)	This study
pBTMMCM10-C108	pBTMgw MCM10 (1-463)	This study
pBTMMCM10-LEFT	pBTMgw MCM10 (1-291)	This study
pBTMMCM10-MIN	pBTMgw MCM10 (128-463)	This study
pBTMSIR2	pBTMgw SIR2	This study
pBTMSIR3	pBTMgw SIR3	This study
pBTMSIR4	pBTMgw SIR4	This study
pGADSIR1	pGADgw SIR1	This study
pGADSIR2	pGADgw SIR2	This study
pGADSIR3	pGADgw SIR3	This study
pGADSIR4	pGADgw SIR4	This study
pGADRAP1	pGADgw RAP1	This study
pGADMCM10	pGADgw MCM10	This study
pGADmcm10-1	pGADgw mcm10-1	This study
pGBKMCM10	pGBKgw MCM10	This study
pGBKmcm10-1	pGBKgw mcm10-1	This study
pGBT9.C	YCP GBD cloning vector TRP1	P. Bartel
pGBT9gw	pGBT9.C with Gateway cassette	This study
pGBT9MCM10	pGBDgw MCM10	This study
pGBT9SIR1	pGBDgw SIR1	This study
pM165	GBD-Sir1p HIS3 2 $\mu$ m	D. Shore
pM2046	GBD-Sir2p HIS3 RAP1prom	D. Shore
pM1588	GBD-Sir4p HIS3 RAP1prom	D. Shore

microliters of a saturated culture started from 10 to 20 colonies per transformation was spotted onto X-gal plates for photographs.

**Microcolony growth rate assay:** Individual, unbudded YB541 cells from an overnight culture were lined up in a row parallel to a row of ILY100 cells, using a dissecting microscope. Cells were incubated on media plates as indicated and pictures were taken at  $\times 160$  magnification.

***mcm10-1* suppressor screen:** One-hundred-microliter samples of overnight cultures of a *mcm10-1* strain were plated on YPD plates and incubated for 2 days at 37°. Resulting colonies were screened for recessive cold sensitivity at 14°. Tetrad analysis was used to identify single-gene second-site suppressors. The mutations were cloned by transforming a YCp50 yeast genomic library (Rose *et al.* 1987) and screening for complementation of the cold-sensitive defect followed by sequencing.

## RESULTS

***mcm10* mutations affect silencing at telomeric and *HMR* loci:** To investigate the effects of *mcm10* mutants on silencing, we constructed a series of isogenic strains

containing recessive, temperature-sensitive *mcm10* mutations (*mcm10-1* or *mcm10-43*) as well as reporter genes (*URA3* and *ADE2*) integrated at the telomeric and *HMR* silent loci in the yeast genome. In the *URA3* constructs, silencing is assayed by measuring the sensitivity of cells to 5-FOA, a toxic precursor of uracil that kills cells expressing the *URA3* gene product. If *URA3* is silenced, more cells will survive on 5-FOA and if silencing is defective, more cells become inviable. In strains containing a *telVIII::URA3* reporter, *mcm10* mutant cells showed a sensitivity to 5-FOA that is approximately 4 orders of magnitude greater than that observed in wild-type cells (Figure 1A). In addition, a larger proportion of *mcm10* cells with the telomeric *URA3* reporter were able to form colonies on media lacking uracil than that observed in *MCM10* cells with the same reporter (data not shown). Derepression of the *URA3* reporter in these experiments indicates that the *mcm10* mutants are defective in telomeric silencing. To ensure that the



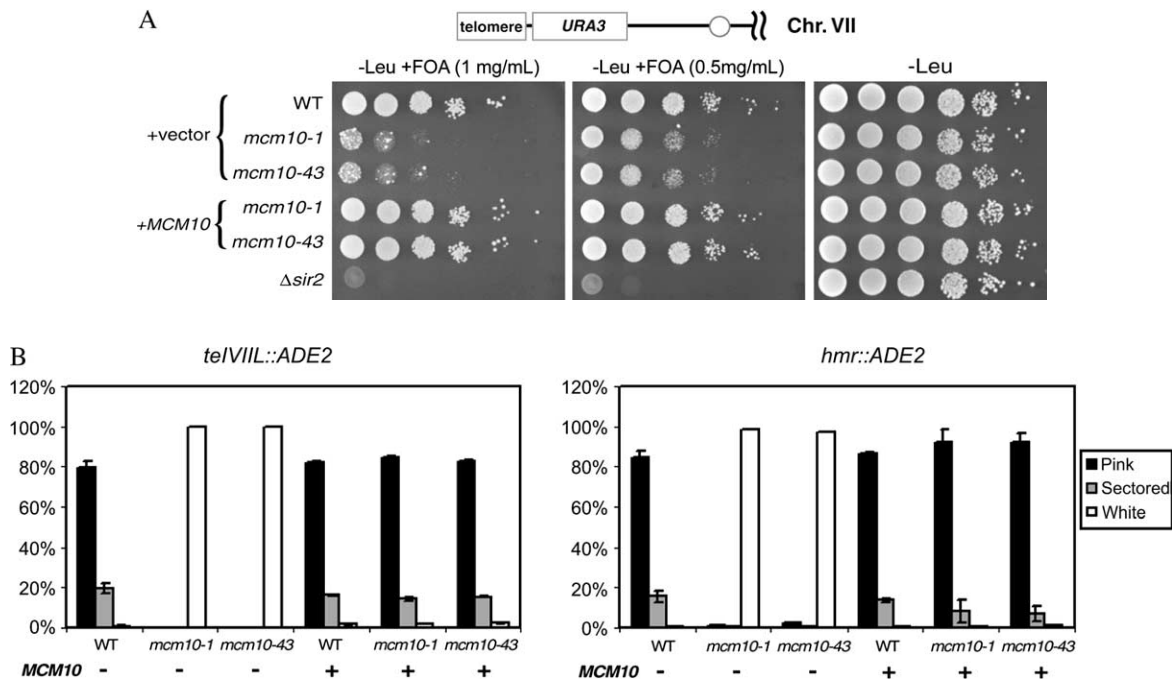


FIGURE 1.—*MCM10* is required for proper telomeric and *HMR* silencing. (A) Telomeric silencing assays with *mcm10* mutants. Strains ILY171, ILY180, ILY270, and GCY23, transformed with either pRS315 (top three rows and bottom row) or pRS315MCM10 (rows labeled “+MCM10”) are shown. Tenfold serial dilutions of overnight cultures were plated on CM-LEU media with or without the indicated concentration of 5-FOA. Plates were grown for 2 days at 30°. (B) Silencing assays of *mcm10* mutants using the *ADE2* reporter gene. Cultures were grown overnight in liquid selective media, diluted, and plated on CM-URA plates at ~300 cells/plate. Plates were grown for 3 days at 30° and then incubated at 4° for 3 days before colonies were scored for color. Strains ILY171, ILY180, and ILY270 were used for the left and ILY163, ILY165, and ILY167 for the right. All strains were transformed with pRS315 (–) or pRS315MCM10 (+). All data points are the averages of at least two trials. Error bars that appear to be missing are too small to be displayed.

silencing defect of the *mcm10* mutants is not promoter specific, we also assayed telomeric silencing by utilizing a *telVIII::ADE2* reporter (Figure 1B, left). If the *ADE2* gene is silenced, the resulting yeast cells will form pink colonies, whereas loss of *ADE2* silencing will give rise to white colonies. In wild-type cells, the vast majority of the colonies were either pink or white/pink sectored, but *mcm10* colonies were exclusively white. The results from this experiment showed that *mcm10* mutations engender a dramatic derepression of *ADE2* at the telomere.

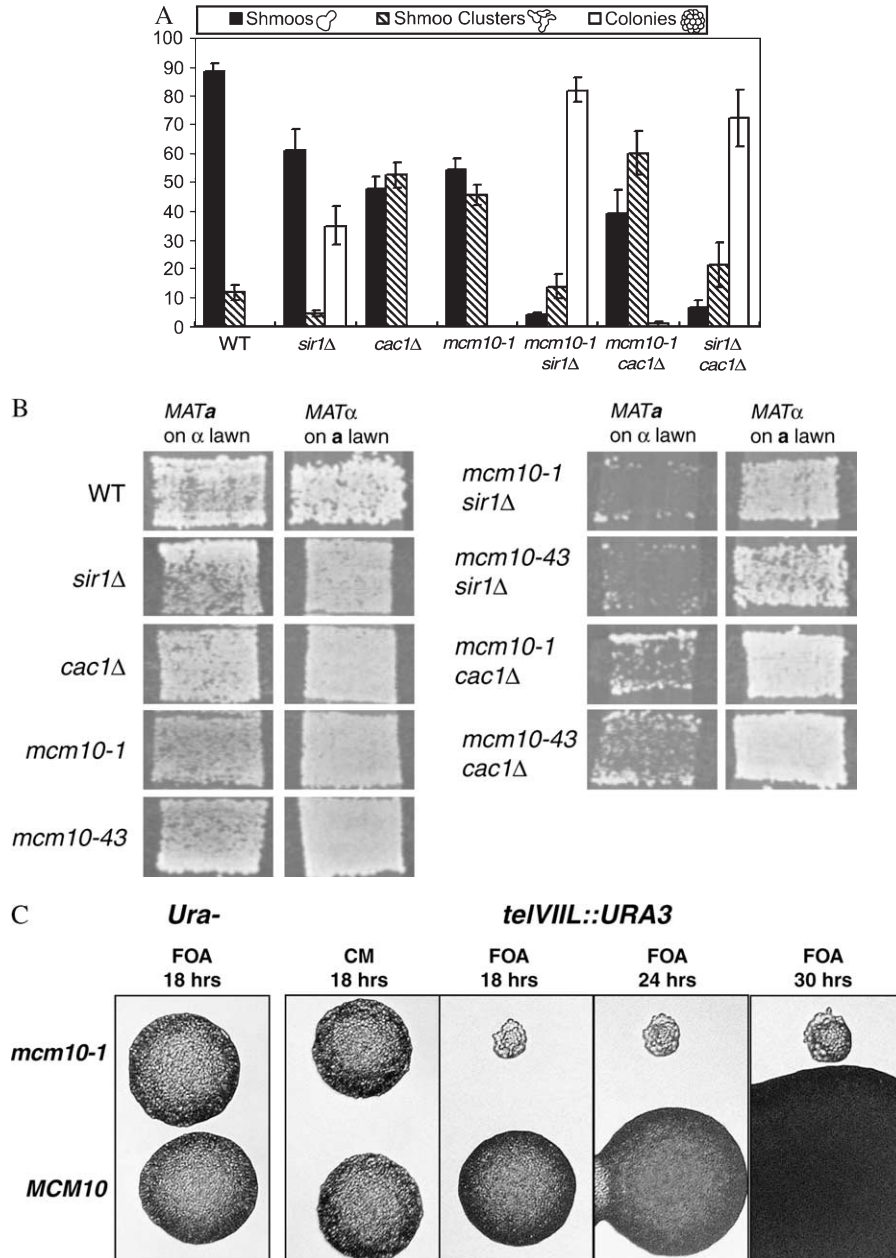
To investigate whether the silencing defect of the *mcm10* mutants also applies to other silent loci, we integrated *ADE2* into the *HMR* locus. Results from this experiment were similar to those observed at the telomeric locus; *mcm10* strains with a *hmr::ADE2* reporter gave rise to almost exclusively white colonies, whereas wild-type strains with the same reporter were predominantly pink (Figure 1B, right). These results indicate that the silencing defect of *mcm10* mutants affects both *HMR* and telomeric loci.

To investigate whether the silencing defects caused by these *mcm10* mutations are dominant or recessive, single-copy ARS/CEN plasmids with or without a wild-type copy of *MCM10* were transformed into each of the reporter strains. The presence of a *MCM10*-expressing plasmid restored silencing in all *mcm10* mutant strains to wild-type levels whereas empty vectors alone did not

(Figure 1, A and B). Together these findings show that the silencing defects caused by both *mcm10-1* and *mcm10-43* mutations are recessive and independent of reporter genes used and that they have a global effect on telomeric and *HMR* loci alike.

**Mcm10p plays a role in the maintenance but not the establishment of silent chromatin at *HML*:** To address the role of *MCM10* in *HML* silencing, we asked whether *MCM10* is required for proper silencing of the endogenous *MAT $\alpha$ 1* and *MAT $\alpha$ 2* genes present at the *HML* locus. A wild-type *MAT $\alpha$*  cell responds to a pheromone ( $\alpha$ -factor) produced by *MAT $\alpha$*  cells by arresting and forming a projection called a “shmoo.” A *MAT $\alpha$*  cell where the *HML $\alpha$*  is derepressed expresses information of both mating types and is no longer responsive to  $\alpha$ -factor (PILLUS and RINE 1989). Therefore, it is possible to measure the silencing of *HML $\alpha$*  in individual *MAT $\alpha$*  cells by assaying their response to  $\alpha$ -factor. Furthermore, by comparing how *mcm10* mutant cells respond to  $\alpha$ -factor to known silencing mutants it is possible to determine whether *mcm10* mutants are defective in the establishment or in the maintenance of *HML* silencing.

A *sir1* mutant, which has a defect in establishment, but not in maintenance of the silent state (PILLUS and RINE 1989; RUSCHE *et al.* 2002), formed shmooes and colonies representing the silenced and derepressed populations of cells, respectively (Figure 2A). In our



**FIGURE 2.**—Mcm10 is involved in the maintenance of *HML* silencing. (A) Shmoo phenotype analysis of *mcm10-1*. Individual, unbudded cells from strains W303-1A, YJB1940, YJB1838, BTY100, ILY155, ILY159, and ILY212 were exposed to  $\alpha$ -factor overnight on YPD plates before their shmooing phenotypes were scored. Approximately 150 cells of each strain were analyzed in three independent trials. (B) Patch-mating assays of various *mcm10* and silencing mutants. Cells of the indicated relevant genotype were mated to tester strains. *MAT $\alpha$*  strains were mated to tester strain 6697-1 ( $\alpha$ -lawn) and *MAT $a$*  strains were mated to 6697-3 ( $\alpha$ -lawn). Mated cells were allowed to grow on SD plates at 30° for 2 days. (C) *mcm10* mutants are defective in the maintenance/inheritance of telomeric silencing. The rates of colony size increase of mutant (top) and wild-type (bottom) strains were compared by lining up individual unbudded cells on synthetic complete media with or without 5-FOA. BTY100 (top, left), W303-1A (bottom, left), ILY180 (top, right four panels), and YB541 (bottom, right four panels) are shown. The three right panels show pictures of the same two colonies at different time intervals. At least 30 such cells were analyzed. Plates were incubated at 30°.

study, ~30% of the *sir1Δ* cells formed colonies, consistent with previous results (PILLUS and RINE 1989). Cac1 is the largest subunit of the chromatin assembly factor 1 (CAF-1) complex that deposits histones H3/H4 onto newly replicated DNA (VERREAULT *et al.* 1996). A *cac1Δ* strain, which is defective in the maintenance of the silent state, but not in its establishment (ENOMOTO *et al.* 1997; ENOMOTO and BERMAN 1998) did not form any colonies, but formed an increased number of shmoo clusters (Figure 2A). This phenotype is indicative of cells that are able to respond to  $\alpha$ -factor, but are not able to maintain the arrested phenotype for an extended period of time and divide before arresting and becoming shmoos again. Both shmoos and shmoo clusters represent cells that can respond to  $\alpha$ -factor, indicating a stable establishment of silencing. The difference is that

the cells that form shmoos are able to stably maintain their silent state for a prolonged period of time, whereas cells that form shmoo clusters are not. Both *mcm10* mutants (only *mcm10-1* data shown) displayed a phenotype similar to that of the *cac1Δ* strain but not to that of the *sir1Δ* strain (Figure 2A). Since *mcm10* cells did not form colonies, it is likely that the establishment pathway in these mutants is still intact, since all cells are able to respond to  $\alpha$ -factor. These results suggest that Mcm10p plays a role in maintaining the *HML* silent chromatin, rather than establishing it.

To confirm our previous results, we tested the response of double mutants to  $\alpha$ -factor. *sir1Δ mcm10* cells showed a striking ablation of silencing, just like a *sir1Δ cac1Δ* strain (Figure 2A). In these strains, cells were almost completely unresponsive to  $\alpha$ -factor, with ~85%

of cells becoming colonies, whereas only ~5% of cells were able to form shmoo and ~10% formed shmoo clusters. This observation indicates a much greater derepression of the *HML $\alpha$*  locus in the double mutants than in either of the single mutants. In strains bearing both *cac1 $\Delta$*  and *mcm10* mutations there was a 10–20% increase in the number of shmoo clusters formed (Figure 2A), signifying a slightly greater defect in the maintenance of the silent state than in either of the two mutants alone. The absence of colonies formed by the *mcm10 cac1 $\Delta$*  double mutant supports the notion that both proteins play a role in maintenance, but not establishment of silencing at *HML*.

Another way to measure *HML* silencing is to measure the ability of *MAT $\alpha$*  cells bearing appropriate mutations to mate with *MAT $\alpha$*  cells in patch-mating assays (Figure 2B). If the *HML $\alpha$*  region is derepressed in *MAT $\alpha$*  cells, the cells will not mate efficiently. This experiment can also be done in reverse to measure silencing of the *HMR $\alpha$*  locus by assaying the ability of *MAT $\alpha$*  cells to mate. In our studies none of the single mutants of *MCM10*, *SIR1*, or *CAC1* showed mating defects for either mating type, whereas the double mutants did. *sir1 $\Delta$  mcm10* double mutants showed significant *MAT $\alpha$* -specific mating defects (Figure 2B), consistent with an abolishment of *HML $\alpha$*  silencing observed in Figure 2A. *cac1 $\Delta$  mcm10* double mutants showed a less dramatic *MAT $\alpha$* -specific mating defect. Similar effects have been observed before with other maintenance mutants (ENOMOTO *et al.* 2000). Together these findings are consistent with a role for Mcm10 in the maintenance of silencing.

***mcm10* mutants have a defect in the maintenance and/or inheritance of telomeric silencing:** PILLUS and RINE (1989) showed that *HM* chromatin in *sir1* mutant cells can stably exist in one of two epigenetic states—silent and active. These cells are able to propagate this state to their progeny for many generations. To stably propagate silencing to the next generation, a mother cell must also maintain the silent chromatin until it is inherited by the daughter cell. Inheritance of the silent state can be assayed by measuring how often a silenced mother cell gives rise to a derepressed daughter cell (MONSON *et al.* 1997). In the case where a telomeric *URA3* silencing reporter is used, when a cell divides, if the silencing is not properly inherited by the daughter cell, then it will be Ura<sup>+</sup> and will die in the presence of 5-FOA. Thus, a *telVIII::URA3* cell with a defect in the inheritance or maintenance of silencing will lead to a slower growing colony, since a proportion of its progeny will be dying on 5-FOA. By observing the rate of colony growth on 5-FOA it is possible to assay maintenance/inheritance of telomeric silencing in cells bearing a *URA3* reporter. However, this assay is not able to adequately distinguish between defects in the inheritance and the maintenance of silent chromatin, since the silencing may be lost due to a maintenance defect before the inheritance step takes place.

To investigate whether *mcm10* mutant cells properly propagate the telomeric silent state, we placed individual wild-type and mutant unbudded cells containing the *telVIII::URA3* reporter on media containing 5-FOA and compared growth rates by colony size (Figure 2C). *MCM10 ura3* cells bearing the telomeric *URA3* reporter proliferated at a rate comparable to that of isogenic cells without the reporter whether on 5-FOA or on regular complete media. Similarly, *mcm10-1 ura3* cells grew into colonies as fast as *MCM10 ura3* cells on 5-FOA. In contrast, although *mcm10-1* cells bearing the *URA3* reporter proliferated at a rate comparable to that of their wild-type counterparts on media without 5-FOA, 5-FOA significantly retarded their proliferation. Similar results were observed for *mcm10-43* cells (not shown). These results suggest that *mcm10* mutants are defective in the maintenance and/or inheritance of silent telomeric chromatin.

**Mcm10 interacts with Sir2 and Sir3:** If Mcm10 plays a direct role in silencing, it should interact with components of the silencing machinery. To test this possibility, we used yeast two-hybrid assays to investigate whether Mcm10 interacts with Sir1, Sir2, Sir3, Sir4, or Rap1. The combinations of *BTM-MCM10* and *GAD-SIR2* or *GAD-SIR3* showed activation of the *LacZ* reporter indicated by the blue color of the colonies formed on media containing X-gal (Figure 3A), signifying Mcm10 interactions with Sir2 and Sir3. These interactions are disrupted by two single-amino-acid substitution mutations, *mcm10-1* (P269L) and *mcm10-43* (C320Y). Western blot analysis of *BTM-mcm10-1* and *BTM-mcm10-43* indicated that these fusion proteins are stably expressed (Figure 3B). These results were largely confirmed by switching the bait/prey orientation in the two-hybrid experiment (Figure 3C). The lack of interaction between *BTM-SIR3* and *GAD-MCM10* is probably due to interference of the *BTM* tag with Sir3 function as previously observed (A. LUSTIG, personal communication). None of the other plasmid combinations showed an increase in *LacZ* activity (Figure 3A).

We also set up two-hybrid experiments in another system, using *GBK*-tagged proteins as bait and a *HIS3* gene as reporter (Figure 3D). In this system, *GBK-MCM10* showed interactions with *GAD-SIR2* and *GAD-SIR3* while *GBK-mcm10-1* disrupted these interactions, further confirming results observed in Figure 3A.

To identify the Sir2- and Sir3-interacting domain of Mcm10, we carried out two-hybrid analysis with truncations of Mcm10 (Figure 3E). As shown above, full-length Mcm10 showed strong interaction with Sir2 and significant interaction with Sir3. Interactions with Sir2 and Sir3 were uncompromised by the N-terminal deletion of 128 amino acids from Mcm10 (128-571) and somewhat compromised by deletion from Mcm10 (292-571). In contrast, C-terminal deletion of 108 amino acids from Mcm10 (1-463) completely destroyed interactions with both Sir2 and Sir3. Western blot analysis indicated that



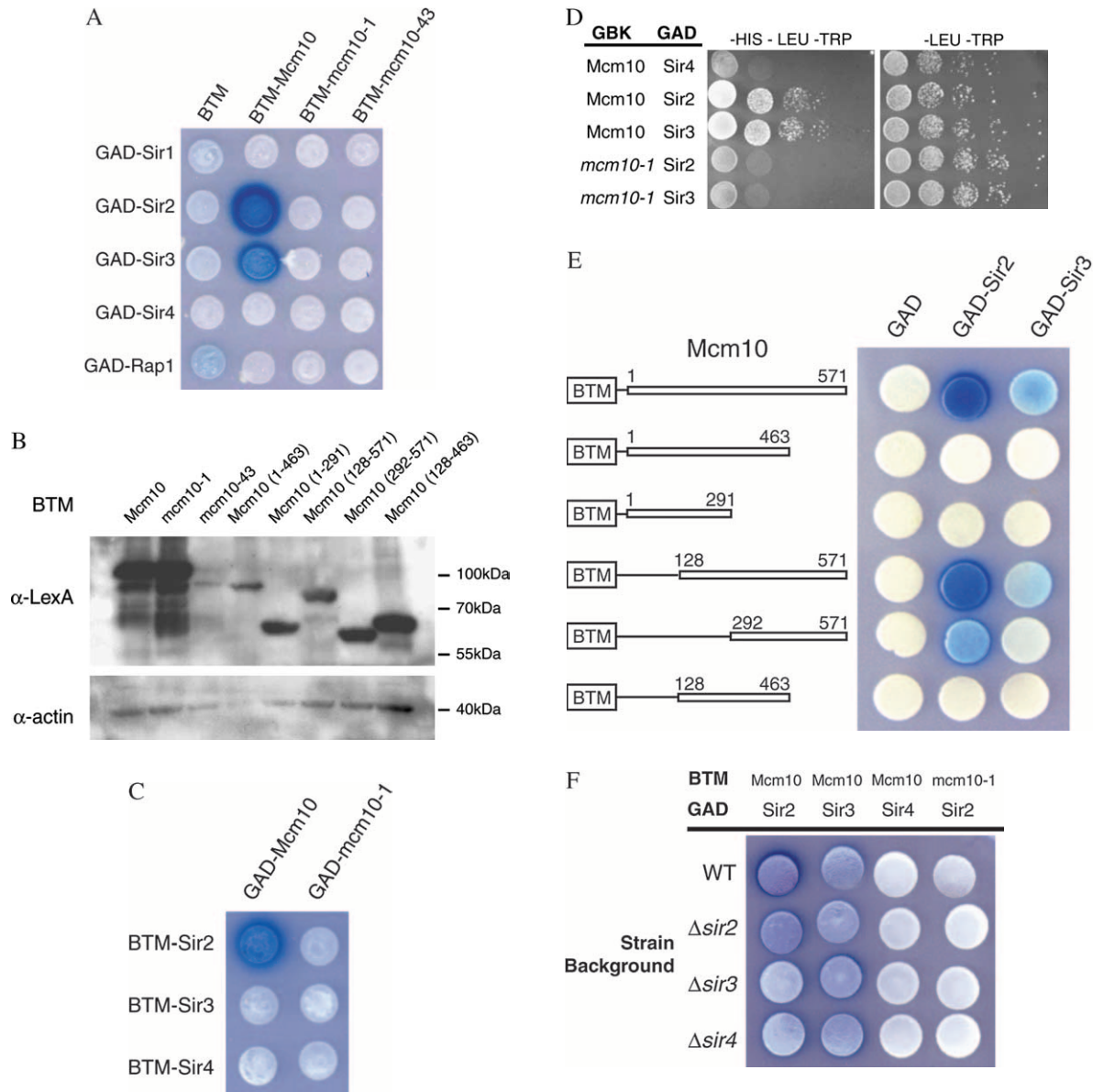


FIGURE 3.—The C terminus of Mcm10 interacts with silencing proteins. Yeast two-hybrid bait/prey constructs were transformed into reporter strains EGY40[pSH18-34] (A, B, D, and E), or PJ69-4a (C). (A, B, D, and E) Cultures were spotted onto selective X-gal plates. An interaction is indicated by blue color resulting from activation of the *LacZ* reporter. (B) Cultures of EGY40[pSH18-34] expressing BTM-Mcm10 fusions were grown overnight in CM-TRP media. Cells from 1-ml aliquots of these cultures were boiled and run on Western blots according to standard protocols. The blot was probed with rabbit anti-LexA antibody (a gift from Jeff Roberts), stripped, and re-probed with rabbit anti-actin antibody (a gift from Tim Huffaker). (C) Saturated cultures were serially diluted and spotted on selective plates. An interaction is indicated by growth on CM-HIS media caused by activation of the *HIS3* reporter. (E) Two-hybrid experiments were performed in  $\Delta sir2$ ,  $\Delta sir3$ , or  $\Delta sir4$  deletion strains ILY267, ILY268, and ILY269.

all of these truncation fusions are stably expressed (Figure 3B). These results indicate that the interacting domain of Mcm10 with Sir2 and Sir3 is located at the C-terminal region. We speculate that disruption of this interaction by *mcm10-1*(P269L) may be the result of an overall structural change in the Mcm10 protein that compromises the C-terminal interacting domain.

The finding that both Sir2 and Sir3 interact with Mcm10 through the C-terminal domain of Mcm10 could be the result of indirect interaction of one Sir protein mediated by another. To investigate if Sir2 is

mediated by Sir3 or vice versa in their interactions with Mcm10, we carried out the two-hybrid analysis in strains deleted in *SIR2*, *SIR3*, or *SIR4* (Figure 3F). We found that Sir2 and Sir3 interacted with Mcm10 independent of any of the Sir proteins.

Tethering Mcm10p to a defective *HMR-E* silencer is not sufficient to restore silencing. Directly recruiting one of the Sir proteins or Rap1 to a defective silencer can restore silencing (CHIEN *et al.* 1993; BUCK and SHORE 1995; LUSTIG *et al.* 1996; MARCAND *et al.* 1996). To investigate whether tethering Mcm10 is sufficient



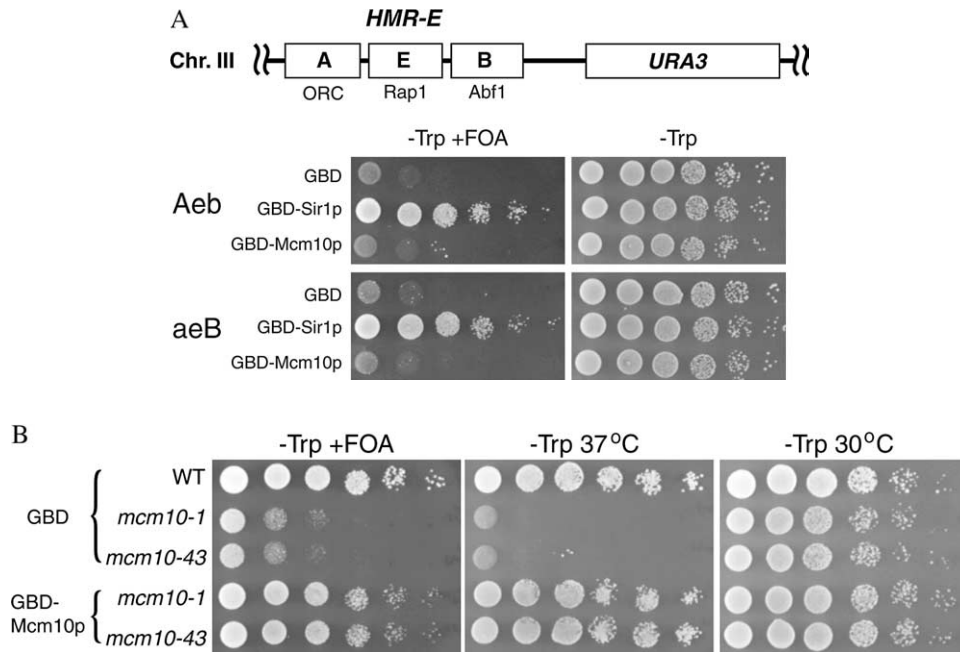


FIGURE 4.—Tethering Mcm10 to a defective silencer is not sufficient to restore silencing. (A) GBD-Mcm10 is not able to restore silencing at *HMR-E* (depicted in schematic). Strains YEA80 (top) or YEA82 (bottom) were transformed with pGBT9, pGBT9SIR1, or pGBT9MCM10. Tenfold serial dilutions were plated on the indicated media and grown at 30° for 2 days. (B) *GBD-MCM10* construct is able to restore both the temperature sensitivity and the silencing defect of *mcm10* strains. Strains YB541, ILY180, and ILY102 were transformed with either pGBT9 or pGBT9MCM10 (two bottom rows). Tenfold serial dilutions were plated on selective media with or without 5-FOA at 30° (permissive temperature) or on complete media at 37° (restrictive temperature).

to restore silencing, we used strains in which the silencer was disrupted and replaced by Gal4 binding sites (ANDRULIS *et al.* 2002). The mating-type gene cassette at the *HMR* was replaced with *URA3*, providing a way to measure silencing at this locus. We made a plasmid construct expressing a *GBD-MCM10* fusion. This hybrid gene is unable to restore silencing to an *HMR-E*, where either the Rap1 and Abf1 binding sites (Aeb, Figure 4A, top) or the ORC and Rap1 binding sites (aeB, Figure 4A, bottom) are deleted, while expression of *GBD-SIR1* was able to restore silencing in both cases (Figure 4A). Since it is possible that the fusion protein is unstable or otherwise defective, we checked its ability to complement *mcm10* mutations. *GBD-MCM10* was able to complement both the temperature sensitivity and the silencing defect of *mcm10-1* and *mcm10-43* (Figure 4B), suggesting that the hybrid protein retains its silencing function. Taken together, these data show that recruiting Mcm10 is not sufficient to restore silencing to a defective silencer.

**Mutations in *MCM10* inhibit the ability of *GBD-SIR3* to restore silencing:** Normally, tethering any of the Sirs to a defective silencer is sufficient to restore silencing (CHIEN *et al.* 1993; LUSTIG *et al.* 1996; MARCAND *et al.* 1996). If Mcm10 functions downstream of the Sirs, then a *mcm10* mutation may inhibit the ability of *GBD-SIR* constructs to restore silencing to a defective *HMR-E*. We constructed tethering yeast strains containing either *mcm10-1* or *mcm10-43* mutations as well as the defective silencer with GBD binding sites and *HMR* marked with *URA3*. As expected, tethering any of the Sirs in a *MCM10* strain restored silencing to approximately wild-type levels (Figure 5A, top). On the other hand, targeted silencing by *GBD-SIR3* was defective in both *mcm10* mutant strains (Figure 5A, bottom). We were able to

complement this defect by adding a plasmid containing a single copy of *MCM10* (Figure 5B). Interestingly, mutations in *MCM10* did not have an effect on targeted silencing by *GBD-SIR1*, *GBD-SIR2*, or *GBD-SIR4*, all of which would require the participation of Sir3 for silencing. Together these findings suggest that the silencing function of *MCM10* may lie downstream of the Sir proteins, with Mcm10 regulating the silencing activity of Sir3.

**The temperature sensitivity and silencing defects of *mcm10-1* can be genetically separated:** Since Mcm10 plays a role in DNA replication, it is possible that the silencing defect caused by *mcm10* mutations is a by-product of a replication defect or of an overall defective S phase. To address this point we asked if the temperature sensitivity (which is attributed to a defect in replication) and silencing defects of *mcm10-1* could be separated. In our studies of Mcm10 function we conducted a screen for second-site suppressors of the temperature-sensitive phenotype of *mcm10-1*. We screened for spontaneous reversion of the *mcm10-1* temperature sensitivity that also conferred a second phenotype (cold sensitivity) so that the mutation could be easily identified. We isolated two mutants, *MCM2-S619Y* and *MCM2-S619F*. These suppressors are capable of overcoming the temperature sensitivity of *mcm10-1* (Figure 6D) in a dominant fashion (data not shown). We constructed strains that contain the *HMR* and telomeric silencing reporters, *mcm10-1*, and/or the suppressor mutations to assay whether these mutations can also suppress the telomeric silencing defect conferred by *mcm10-1*. The mutations *Mcm2-S619Y* or *Mcm2-S619F* did not confer a silencing defect on their own (Figure 6, A and B). While these *mcm2* alleles were able to suppress the temperature-sensitive phenotype caused by the *mcm10-1* mutation,

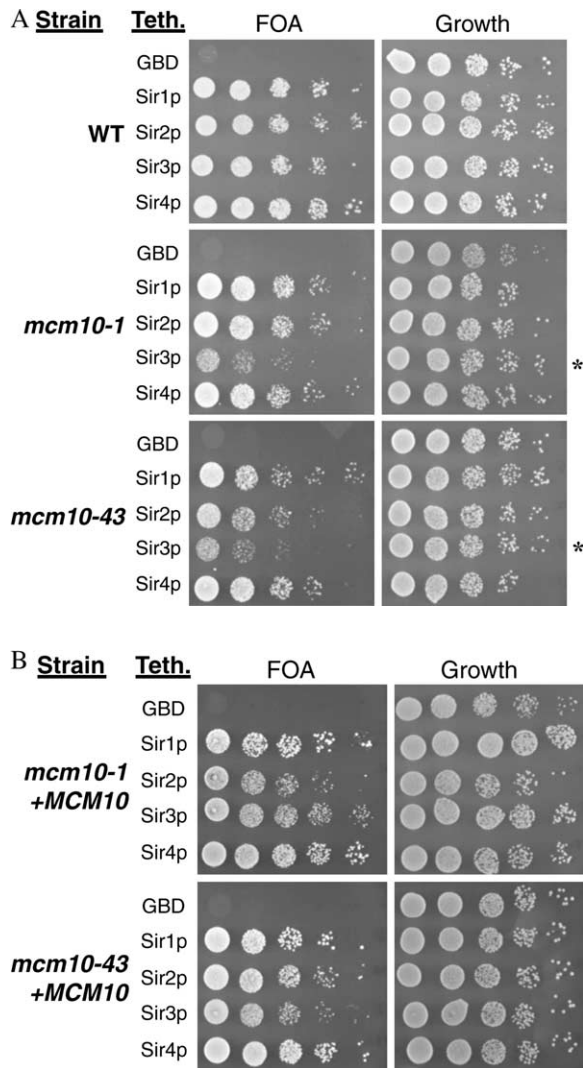


FIGURE 5.—Mutations in *MCM10* inhibit the ability of GBD-Sir3 to restore silencing when tethered to a defective silencer. (A) Tethering assays in the presence of *mcm10* mutations. Strains YEA80 (WT), ILY137 (*mcm10-1*), and ILY139 (*mcm10-43*) were transformed with pM165, pM2046, pM1587, and pM1588, respectively. Tenfold dilutions of overnight cultures were spotted on appropriate dropout media with or without 5-FOA. The strain background (Strain) and tethering plasmid used (Teth.) are indicated. All strains were also transformed with empty vector pRS315 (GBD) as controls. The strains that show a silencing defect are indicated by an asterisk. (B) *MCM10* restores silencing to strains from A by transforming these strains with pRS315MCM10. Silencing was assayed as in A.

they failed to restore proper telomeric (Figure 6, A and B) and *HMR* (data not shown) silencing to a *mcm10-1* strain. These results suggest that the temperature-sensitive phenotype of *mcm10-1* is not linked to the silencing defect.

## DISCUSSION

Involvement of numerous DNA replication proteins such as the ORC, PCNA, RF-C, Cdc45, and Mcm5 in transcriptional silencing has been implicated in *S. cerevisiae*.

Recently, involvement of silencing proteins such as Sir2 and Sir3 in the regulation of replication initiation has also been reported (PAPPAS *et al.* 2004). The relationship between these two multistep processes and their participating components remains an enigma. Mcm10 is a DNA replication factor involved in both the initiation and elongation steps of DNA replication (HOMESLEY *et al.* 2000; WOHLSCHEGEL *et al.* 2002; LEE *et al.* 2003; FIEN *et al.* 2004; SAWYER *et al.* 2004). Although Mcm10 has previously been shown to interact with DNA replication proteins implicated in silencing (HOMESLEY *et al.* 2000; KAWASAKI *et al.* 2000), a direct silencing function has never been described. The results presented in this study suggest that Mcm10 interacts with Sir2 and Sir3 in the maintenance of transcriptional silencing and that this function appears to be separable from its functions essential for DNA replication.

We have demonstrated that two recessive alleles of *MCM10* were able to derepress *URA3* and *ADE2* reporter genes at telomeric and *HMR* loci (Figure 1). In addition, these mutations were able to derepress endogenous genes at *HML* (Figure 2, A and B). Thus, Mcm10 has a rather ubiquitous role in silencing, in contrast to ORC, which plays a role only in *HM* silencing. Although silencing in *S. cerevisiae* also occurs in ribosomal DNA loci, the effects of Mcm10 on *rDNA* silencing were not examined in this study. Using reporter genes at *HMR*, *mcm10* mutants clearly showed derepression of *HMR* (Figure 1B). However, mating assays in the *mcm10 sir1Δ* double mutants showed a *MATA*-specific mating defect supporting the notion that these mutants derepress *HML* more efficiently than *HMR* (Figure 2B). This phenomenon has been observed before with other maintenance mutants (ENOMOTO *et al.* 2000) and can most likely be attributed to the fact that silencing is stronger at the *HMR* than at the *HML*.

The possibility that Mcm10 may have a separate silencing function is supported by the observation that dominant extragenic suppressors (*MCM2-S619Y* and *MCM2-S619F*) of the temperature sensitivity of *mcm10-1* are not able to suppress the silencing defect (Figure 6). In fact, it is possible that Mcm10 may execute its silencing function at the replication fork and yet its silencing function could be uncoupled from its replication activity. However, we cannot rule out the unlikely possibility that these two apparently independent phenotypes are a manifestation of the same defect at different levels of severity. Future experiments with the aid of separation-of-function mutations in *MCM10* should clarify this point.

Tethering silencing proteins to a defective silencer can restore silencing. However, tethering GBD-Mcm10 to a defective silencer could not restore silencing (Figure 5). This finding suggests that Mcm10 functions downstream of the establishment of silent chromatin in the propagation or maintenance of silent chromatin. This hypothesis is supported by epistasis analysis with

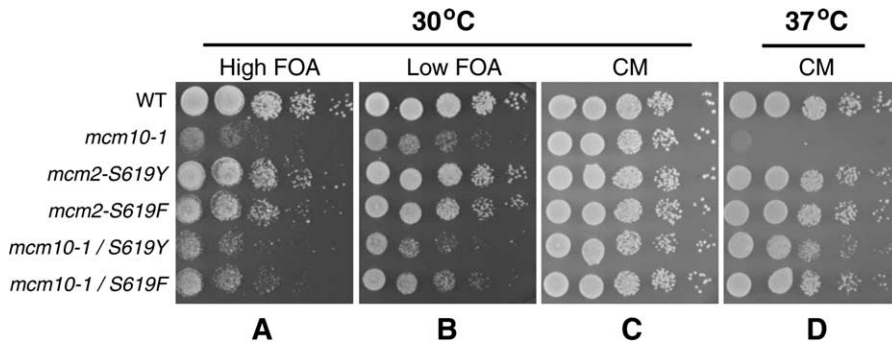


FIGURE 6.—The temperature sensitivity of *mcm10-1* can be separated from its silencing defect. *MCM2-S619Y* and *MCM2-S619F* suppress the temperature sensitivity of *mcm10-1*, but not the silencing defect. Tenfold serial dilutions of overnight cultures of strains ILY171, ILY180, ILY220, ILY221, ILY222, and ILY223 were plated on complete media plates at 37° (D) or 30° (A–C). A and B show growth on synthetic complete media containing 0.5 mg/ml (low FOA) or 1 mg/ml (high FOA). Plates were grown for ~2 days.

*sir1* and *cac1* in the shmoo assay and the reporter silencing assays (Figure 2, A and B). *MCM10* is in the same epistasis group as *CAC1*, which encodes a subunit of the histone chaperone CAF-1 known to be involved in the maintenance of heterochromatin. It is in a different epistasis group from *SIR1*, whose role in silencing is restricted to establishment. Previous tethering experiments with Sir proteins showed that recruiting any of the four Sirs to a defective silencer can restore silencing to wild-type levels (CHIEN *et al.* 1993; MARCAND *et al.* 1996; TRIOLO and STERNGLANZ 1996). When we conducted similar experiments in *mcm10* strains, we noted that GBD-Sir3 constructs lose their ability to properly restore silencing (Figure 5). This result suggests that Mcm10 may modulate Sir3 activity and is consistent with the two-hybrid interaction between Mcm10 and Sir3 (Figure 3). However, although Mcm10 also interacts with Sir2, silencing by GBD-Sir2 is not inhibited. One explanation is that the GBD fusion may be altering the structure of Sir3 in such a way to make it hypersensitive to mutations in *MCM10*. Another explanation is that Sir2 and Mcm10 interact indirectly, through Sir3 or another yet unidentified factor. However, deletion of *SIR3* had no effect on the two-hybrid interaction of Sir2 and Mcm10, suggesting that Sir3 cannot be the mediator. Taken together, these data indicate that the nature of the Mcm10-Sir2 interaction may be different from that of the Mcm10-Sir3 interaction. Sir3 has been shown to have homology to Orc1, a member of the origin recognition complex (ZHANG *et al.* 2002). Mcm10 also interacts with members of the ORC including Orc1 (KAWASAKI *et al.* 2000), Orc2 (KAWASAKI *et al.* 2000; CHRISTENSEN and TYE 2003), Orc3, Orc5, and Orc6 (DOUGLAS 2003). It is possible that functional and/or structural similarities between silencing and replication proteins may mediate the Mcm10-Sir3 interaction.

The fact that Mcm10's function lies in the maintenance of silent chromatin and yet tethering Mcm10 does not restore silencing suggests that Mcm10 may act to stabilize silent chromatin structures, such as Sir2-4 complexes, after they have been recruited. Stabilization may be accomplished by Mcm10 binding to assembled Sir2-4 complexes on chromatin (Figure 7A) or escorting soluble Sir2-4 to chromatin (Figure 7B), consistent with

the interaction of Mcm10 with both Sir2 and Sir3. Deposition of Mcm10 on propagating heterochromatin may be mediated by the elongation machinery (Figure 7A) or drawn directly from soluble Mcm10 (Figure 7, A and B). However, if the function of Mcm10 is to simply stabilize established silent chromatin, it should not matter which *GBD-SIR* fusion is used to initiate silencing in our tethering experiments. Our data suggest that in the absence of wild-type Mcm10, GBD-Sir3 has difficulty nucleating the spread of silent chromatin. The fact that only GBD-Sir3-mediated silencing is affected in *mcm10* strains may indicate that this defect occurs during the steps when GBD-Sir3 first binds DNA and initiates silencing before the other Sirs are recruited. Perhaps *mcm10* mutations are inhibiting the first step in this artificial establishment of silencing such that GBD-Sir3 has trouble recruiting the other Sirs to begin the assembly and spreading of Sir complexes from the silencer (Figure 7C). In other words, GBD-Sir3 alone may not completely fulfill its proper function in *mcm10* strains and that transition of establishment to maintenance requires the participation of the other Sirs if Mcm10 is compromised. Furthering this idea (Figure 7C), we believe that Mcm10 cooperates with Sir3 during the normal transition of nucleation to spreading of heterochromatin at the silent mating-type and telomeric loci (Figures 1 and 2) independent of GBD-Sir3. This explanation is consistent with the established model of a cooperative mechanism of silencing between the Sir proteins. This explanation is also consistent with the hypothesized role of Mcm10 at replication origins as a stabilizing scaffold in the transition of the pre-replication complex to the pre-initiation complex (IC) and subsequently from the pre-IC to the elongation complex (HOMESLEY *et al.* 2000; WOHLSCHEGEL *et al.* 2002; LEE *et al.* 2003; FIEN *et al.* 2004; SAWYER *et al.* 2004). By studying the roles of Mcm10 in DNA replication and in gene silencing we may begin to elucidate the connection between DNA replication and heterochromatin assembly.

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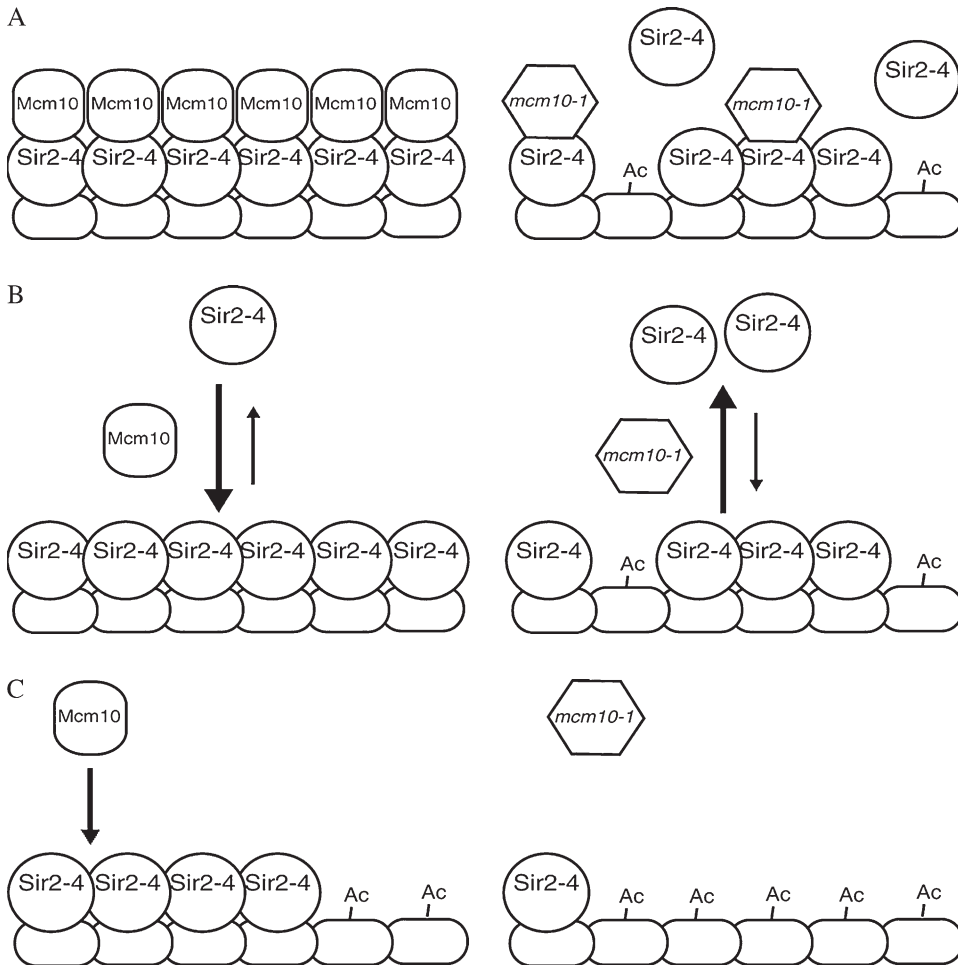


FIGURE 7.—Hypotheses for Mcm10 function in maintenance of silencing. (A) Mcm10 associates with the chromatin-bound Sir2-4 complex to stabilize silent chromatin. Mutant Mcm10 proteins are not able to maintain interaction with the Sirs. Dissociation of Sirs from chromatin results in derepression of silent loci. (B) Mcm10p acts to recruit Sir proteins to silent chromatin where needed. In the case of *mcm10* mutants, inability of Mcm10-1p to facilitate the binding of a sufficient number of Sir complexes to the silencer leads to the degradation of silent chromatin over time. (C) Mcm10 facilitates the transition from the nucleation of Sir2-4 complexes at the silencer to the spreading of Sir2-4 complexes along the silenced region. A defective Mcm10 does not allow the proper spreading of silent chromatin.

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