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Essential Role of MCM Proteins in Premeiotic DNA Replication

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A critical event in eukaryotic DNA replication involves association of minichromosome maintenance (MCM2–7) proteins with origins, to form prereplicative complexes (pre-RCs) that are competent for initiation. The ability of mutants defective in MCM2–7 function to complete meiosis had suggested that pre-RC components could be irrelevant to premeiotic S phase. We show here that MCM2–7 proteins bind to chromatin in fission yeast cells preparing for meiosis and during premeiotic S phase in a manner suggesting they in fact are required for DNA replication in the meiotic cycle. This is confirmed by analysis of a degron *mcm4* mutant, which cannot carry out premeiotic DNA replication. Later in meiosis, Mcm4 chromatin association is blocked between meiotic nuclear divisions, presumably accounting for the absence of a second round of DNA replication. Together, these results emphasize similarity between replication mechanisms in mitotic and meiotic cell cycles.

INTRODUCTION

In meiosis, the normal alternation of S phase and chromosome disjunction seen in the mitotic cell cycle is altered so that a single round of premeiotic DNA replication is followed by two consecutive nuclear divisions, thus achieving a reduction in ploidy. Strict control to ensure a single round of DNA replication in premeiotic S phase is important to produce haploid cells containing a single complete copy of the genome, but the mechanism of this control has not been subject to as much analysis as in the vegetative cell cycle.

Analysis of mutants affecting replication enzymes such as DNA polymerases suggests that the basic mechanisms of S phase in the meiotic and mitotic cell cycles are similar (Schild and Byers, 1978; Johnston *et al.*, 1982; Budd *et al.*, 1989; Forsburg and Hodson, 2000). Furthermore, analysis of replication origin usage in *Saccharomyces cerevisiae* is consistent with basic conservation in the initiation mechanism (Collins and Newlon, 1994). In general, the same origins are used in both premeiotic and vegetative S phases, perhaps reflecting the fact that similar events at the origin recognition complex (ORC), which is bound to initiation sites, are occurring in both types of cell cycle. In the mitotic cell cycle, this involves association of six MCM2–7 proteins at ORC during late mitosis/early G1 in a process dependent on Cdc6/Cdc18 and Cdt1 (reviewed by Kearsey and Labib, 1998; Kelly and Brown, 2000; Maiorano *et al.*, 2000; Nishitani *et al.*, 2000). This process of prereplicative complex (pre-RC) formation confers replicative competence on the origin, allowing Cdc7 and cyclin-dependent kinase (CDK)-activated initiation of DNA synthesis during the subsequent S phase. During DNA replication, MCM2–7 proteins are thought to provide helicase activity for the elongation step of DNA replication (reviewed in Labib and Diffley, 2001). These proteins dissociate from chromatin, probably during replication termination, and cannot rebind because this step is inhibited by CDK activity and other mechanisms involving pre-RC components (Dahmann *et al.*, 1995; Tanaka *et al.*, 1997; Labib *et al.*, 1999; Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001). Thus, reinitiation is dependent on CDK inactivation in mitosis, limiting DNA replication to a single round per cell cycle.

In spite of these general similarities, some observations suggest that premeiotic S phase is not identical to vegetative DNA replication, and these differences could be related to specialized meiotic processes. Premeiotic S phase is universally longer than the S phase in cycling cells of the same organism (Holm, 1977; Cha *et al.*, 2000), and this may reflect the activity of proteins needed for reductional chromosome segregation, such as Rec8, whose correct function is intimately associated with DNA replication (Cha *et al.*, 2000; Watanabe *et al.*, 2001). In *S. cerevisiae*, deletion of replication origins can delay double-strand break (DSB) appearance, perhaps because of coupling between replication and recombination (Borde *et al.*, 2000) and preventing S phase activation also blocks DSB formation (Smith *et al.*, 2001). There is also evidence that pre-RC formation and the mechanism of initiation and elongation in premeiotic S phase may be significantly different. Specifically, *mcm2*, *mcm4,* and *cdc18* mu-

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Table 1. *S. pombe* strains used in this study

P1	*mcm4ts(cdc21-M68) leu1-32 h*$^-$	Nasmyth and Nurse, 1981
P886	*mcm4*$^+$*(cdc21*$^+$*)-GFP5::kan*^{r} *nmt1(41X)-cdc13::LEU2 cig1*Δ*::ura4*$^+$ *cig2*Δ*::ura4*$^+$ *cdc13*Δ*::ura4*$^+$ *ura4-*Δ*18 leu1-32 ade6-M210 h*$^-$	Derived from Fisher and Nurse (1995)
P900	*mcm4*$^+$*(cdc21*$^+$*)-GFP5::kan*^{r} *cyr1*Δ*::LEU2 sxa2*Δ*::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	Derived from Stern and Nurse (1997)
P937	*pat1-114 h*$^-$	
P958	*mcm4*$^+$*(cdc21)-GFP5::kan*^{r} *pat1-114 h*$^-$	
P978	*mcm4*$^+$*(cdc21)-GFP5::kan*^{r}*//mcm4*$^+$*(cdc21)-GFP5::kan*^{r} *pat1-114//pat1-114 ade6-M210//ade6-M216 leu1-32//+ h*$^-$*//h*$^-$	
P1025	*mcm6*$^+$*-GFP5::kan*^{r} *pat1-114 h*$^-$	
P1023	*mcm4ts(cdc21-M68)-td::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	
P1027	*mcm2*$^+$*-GFP5::kan*^{r} *pat1-114 h*$^-$	
P1037	*mcm4ts(cdc21-M68)-td::ura4*$^+$ *pat1-114 h*$^-$	
P1039	*mcm4ts(cdc21-M68) pat1-114 h*$^+$	
P1096	*mcm2*$^+$*-GFP5::kan*^{r} *mcm4ts(cdc21-M68)-td::ura4*$^+$ *pat1-114*	

tants of *Schizosaccharomyces pombe* are not arrested in premeiotic S phase or the subsequent nuclear divisions under conditions that block vegetative DNA replication (Forsburg and Hodson, 2000). Also, in budding yeast, although CDK activity is needed for activation of premeiotic S phase (Iino *et al.*, 1995; Dirick *et al.*, 1998; Stuart and Wittenberg, 1998), Cdc7 may not be required (Schild and Byers, 1978; Hollingsworth and Sclafani, 1993), which could reflect different regulatory controls over DNA replication.

Here we have investigated the role of MCM2–7 proteins as fission yeast cells exit the mitotic cell cycle and enter meiosis. By a combination of chromatin association assays and genetic analysis using novel degron alleles, we provide clear evidence that MCM2–7 proteins have an essential role in meiotic as well as vegetative S phases.

MATERIALS AND METHODS

Fission Yeast Strains and Methods

Strains used are shown in Table 1. Media and growth conditions and standard genetic methods were as described by Moreno *et al.* (1991). Diploid *pat1* strains were made by protoplast fusion. P-factor arrest was carried out as described in Stern and Nurse (1997) using a P-factor concentration of 1.5 μg/ml in minimal medium supplemented with leucine. Thiamine at 5 μg/ml was used to repress the *nmt1* promoter. Nitrogen starvation was carried out using EMM medium lacking NH_4Cl.

Tagging Mcm2, Mcm4, and Mcm6 with GFP

Mcm2$^+$ and *mcm6*$^+$ genes, expressed from their own promoters, were tagged to express C-terminally-fused GFP5, as described earlier for *mcm4*$^+$ (Kearsey *et al.*, 2000). We constructed a GFP-containing integration vector, pSMRG2+, containing GFP5 and the *kanMX6* selectable marker (EMBL accession no. AJ306910). This involved replacing the *Ngo*M IV fragment containing the *ura4*$^+$ gene in pSMUG (Kearsey *et al.*, 2000) with a *kanMX6* fragment, which was amplified using the primers 5′-atttagccggctgtttagcttgcctcgtccc-3′ and 5′-aattgccggcgagctcgtttaaactggatgg-3′ from pFA6a-kanMX6 (Bahler *et al.*, 1998). Also the linker region upstream of GFP was enlarged by inserting the sequence 5′-ctcgagggtagatctggtgcccggggtggtgctggtgccggagccggtgctggtgctgaagctt-3′ between the unique *Xho*I and *Hin*dIII sites. The C-terminal encoding region of the *mcm2*$^+$ gene was amplified using primers 5′- acgactcgagacactacaattcctttaat-3′ and 5′- ccaccccgggcaataagatatttagcaaatgttc-3′ and cloned into the *Xho*I and *Sma*I sites of pSMRG2+. Homologous integration into the *mcm2*$^+$ gene was directed by linearization with *Nhe*I. For *mcm6*$^+$, a similar procedure was used, using the primers 5′-gaacggggcccgcaagagcaaactgtgtag-3′ and 5′- cttgccccgggcgttcggaacatcgccattgc-3′ and cloning into the *Apa*I and *Sma*I sites. The plasmid was linearized using *Xho*I to direct integration into the *mcm6*$^+$ locus. Constructs were verified by sequencing. The Mcm2-GFP and Mcm6-GFP strains have a normal growth rate and DNA content by flow cytometry, indicating that the tagged proteins are functional.

For tagging the *mcm4*$^+$ gene in the background of the cyclin B shut-off strain (*nmt1(41X)-cdc13*, *cdc13*Δ *cig1*Δ *cig2*Δ; Fisher and Nurse, 1995) we replaced the *ura4*$^+$ marker in pSMUG+mcm4-GFP (Kearsey *et al.*, 2000) with an *Ngo*M IV fragment containing the *kanMX6* (kan^r) marker (see above), to give pSMRG+mcm4-GFP. This was linearized with *Hpa*I to direct integration at the *mcm4*$^+$ locus, thus generating strain P886. Because this strain is thiamine sensitive and kanMX6 cannot be selected for on minimal (EMM) medium, we devised a modified medium (KsnoT, Kanamycin-selective, no thiamine: bacto-peptone 10 g/l, 3% glucose, 2% agar, 250 mg/l adenine, 250 mg/l uracil, 250 mg/l leucine, 75 μg/ml geneticin), which allows kanamycin selection in a thiamine-deficient medium. pSMRG+mcm4-GFP was also used to tag *mcm4*$^+$ in *pat1ts* strains.

Degron Construction

To construct the degron *mcm4* strain, a plasmid was constructed containing the *mcm4*$^+$*/cdc21*$^+$ promoter, expressing an N-terminal ubiquitin-degron-HA cassette fused to the *mcm4*$^+$*/cdc21* gene. The degron was amplified as an *Apa*I-*Xho*I fragment using primers 5′-atagggccctgcttatctttcttcttcc-3′ and 5′-atactcgaggcttgccctcctaaaaatgc-3′, with plasmid pPW66R (Dohman *et al.*, 1994) as template. The *mcm4*$^+$ promoter was amplified as a *Kpn*I-*Apa*I fragment using the primers: 5′-ataggtaccccgcatttgatggtttcgcc-3′ and 5′-atagggcccgtggtgggtgtagaaagac-3′. Both fragments were cloned into *Kpn*I and *Xho*I cleaved pSMUG2+ (*ura4*$^+$-containining integration vector, identical to pSMRG2+, except containing the *ura4*$^+$ gene instead of the *kanMX6* marker; EMBL accession no. AJ306911) to give pSMUG2+degron. The 5′ region of the *mcm4*$^+$ reading frame was amplified as a *Xho*I-*Bgl*II fragment using the primers 5′-atactcgaggtcctctagtcagcaaagtg-3′ and 5′-ataagatcttcaatttgtcaatgtcaccag-3′. This fragment was cloned into the *Xho*I-*Bgl*II region of pSMUG2+degron to give pSMUG2+degron+mcm4. The final construct was verified by sequencing. This plasmid was cleaved with *Spe*I to direct integration into the *mcm4*$^+$ locus and thus tag the endogenous gene with the degron. The same strategy was used to construct the *mcm4ts-td* allele as the *mcm4ts* mutation (*cdc21-M68*) causes a Leu to Pro substitution at position 238, i.e., is not in the N-terminal region of the protein (S. Montgomery and S.E. Kearsey, manuscript in preparation).

Chromatin Binding Assay

Chromatin binding of GFP-tagged proteins was analyzed using a modified version of the protocol described in Kearsey *et al.* (2000). Instead of ZM buffer, cells were resuspended in ZM2 buffer (15 mM potassium hydrogen phthalate, 15 mM Na_2HPO_4, pH 7.0, 90 mM NH_4Cl, 1.2 M sorbitol, 10 mM dithiothreitol) and zymolyase 20-T was added to 2 mg/ml. Cells were washed twice in ZM buffer, once in EB2 (20 mM PIPES-KOH, pH 6.8, 0.4 M sorbitol, 1 mM EDTA, 0.5 mM spermidine-HCl, 1.5 mM spermine-HCl, 150 mM KAc, 1/1000 volume protease inhibitor cocktail; P-8215, Sigma, St. Louis, MO), and cells were extracted in EB2 containing 1% (wt/vol) Triton X-100 for 5 min at 20°C. Cells were fixed with methanol/acetone and analyzed by fluorescence microscopy as previously described (Kearsey *et al.*, 2000). At least 100 cells were counted for each data point, and error bars show the range of two experiments. For flow cytometry, methanol/acetone fixed cells were rehydrated in 10 mM EDTA, pH 8.0, 0.1 mg/ml RNase A, 2 μg/ml propidium iodide or 1 μM sytox green, and incubated at 37°C for 2 h. Cells were analyzed using a Coulter Epics XL-MCL (Fullerton, CA).

Protein Analysis

Protein extracts were made by TCA extraction and analyzed by Western blotting as described previously (Grallert *et al.*, 2000). Mcm4 was detected using a mouse mAb KL2.2, which was generated against full-length, bacterially expressed Mcm4 (Maiorano *et al.*, 1996). The recognized epitope is in the N-terminal 302 amino acids (unpublished observations). α-Tubulin was detected using Sigma T5168 at a dilution of 1/10,000.

RESULTS

Mcm4 Is Chromatin Bound in Cells Arrested in G1 Phase by Mating Pheromone

To determine the relevance of MCM2–7 proteins for premeiotic DNA replication, we examined the chromatin association of these proteins as cells exit vegetative growth and prepare for mating and meiosis. In fission yeast, meiosis is induced by nutrient deprivation, which causes haploid cells to express mating pheromones, arrest in G1, and conjugate to form a diploid zygote. Usually, meiosis then commences immediately, followed by sporulation to form a four-spored ascus. To follow pre-RC formation during this process we used an *in situ* detergent-washing procedure to examine the chromatin binding of GFP-tagged MCM2–7 proteins in single fission yeast cells. MCM proteins that are bound to chromatin are resistant to detergent extraction and remain nuclear, whereas unbound nucleoplasmic protein is washed away. Using this method, we have previously shown that chromatin association of Mcm4 (Cdc21) is restricted to the interval from mid-anaphase to S phase and shows an expected dependence on ORC and Cdc18 (Kearsey *et al.*, 2000). To study the effect of the P-factor mating pheromone on Mcm4 chromatin binding without the need for simultaneous nutrient deprivation, we used a genetic background that allows pheromone-induced G1 arrest of nonstarved cells. Deleting the *cyr1*$^+$ gene lowers intracellular cAMP levels, thus activating genes needed for the mating pheromone reponse (Maeda *et al.*, 1990), and deleting the *sxa2*$^+$ gene reduces P-factor proteolysis (Imai and Yamamoto, 1992; Ladds *et al.*, 1996). Thus, by introducing Mcm4-GFP into a *cyr1*Δ *sxa2*Δ genetic background, Mcm4 chromatin binding could be monitored during P-factor arrest of exponentially growing cells (Figure 1A). Before addition of P-factor, Mcm4

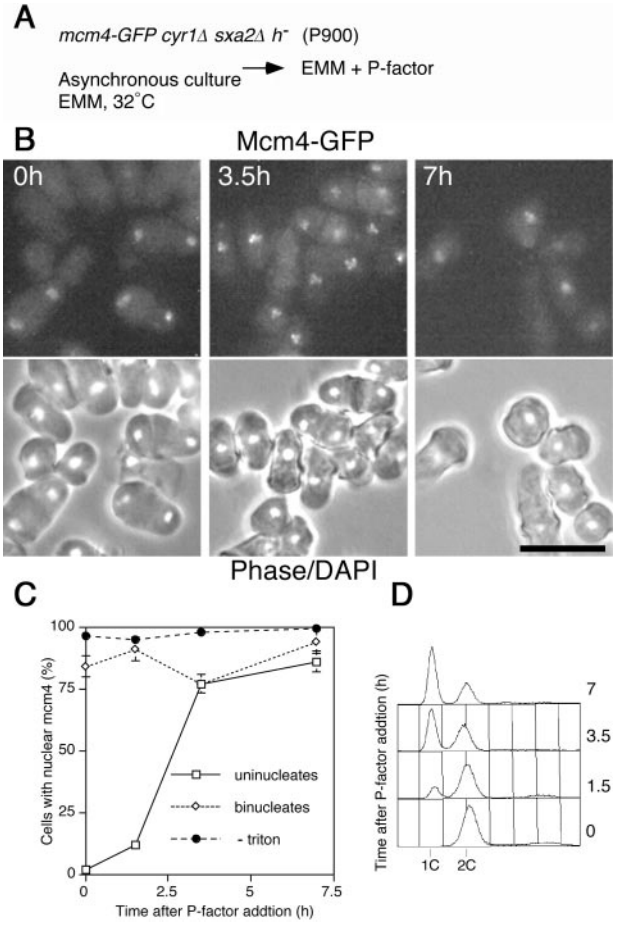


Figure 1. P-factor arrests cells with chromatin-associated Mcm4. (A) Experimental procedure. (B) Chromatin binding assay showing Mcm4-GFP chromatin association (top panels) and phase/DAPI (bottom panels) at various times after P-factor addition. Bar: 10 μm. (C) Quantitation of chromatin binding assay, showing percentage of uninucleate and binucleate cells positive for Mcm4-chromatin binding. "- triton" shows percentage of cells with nuclear localization of Mcm4 when the Triton-extraction step is omitted. Error bars show the range of data from two or more experiments. (D) Flow cytometric analysis of cells for data points shown in B and C).

was only chromatin associated in binucleate (late M/G1/S phase) cells (Figure 1, B and C) as in a wild-type strain. In contrast, 3.5 h after addition of P-factor, 1C cells were prominent by flow cytometry and a high proportion of uninucleate cells were positive for Mcm4 chromatin binding, implying that pre-RC assembly had occurred in G1-arrested cells (Figure 1, B–D). Chromatin association of Mcm4 appeared to be stable at least up to 7 h, but it was not possible to investigate this for longer periods because pheromone-arrested cells start to enter S phase after about 8 h of arrest (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). We also observed that wild-type cells arrested in G1 phase by a brief period (4–7 h) of nitrogen starvation alone showed chromatin-associated Mcm4, although this association was not stable on longer periods of arrest (>12 h, see below). These results show that cells arrested in a state competent

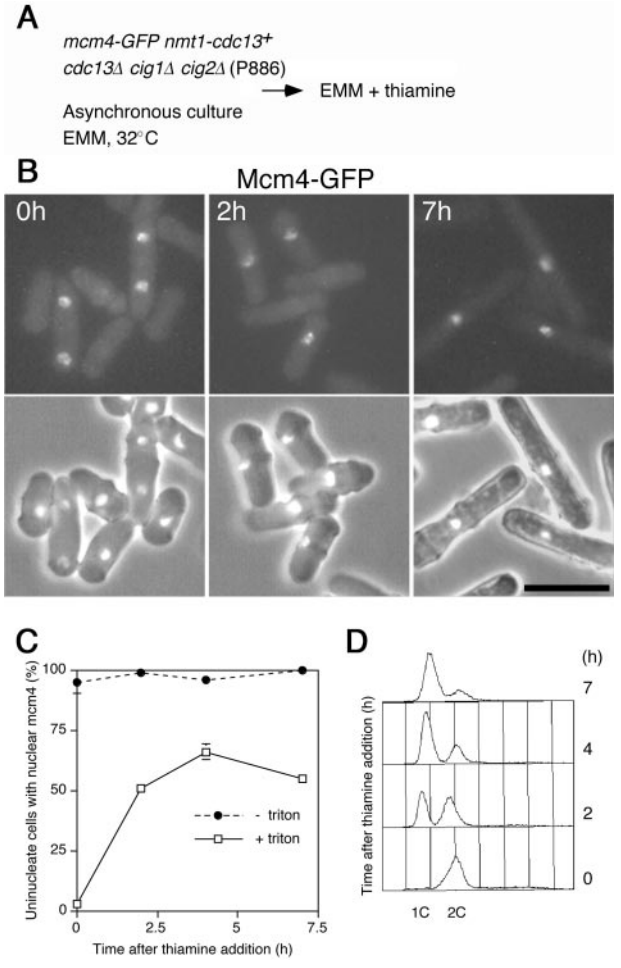


Figure 2. Cells arrested in G1 by cyclin B shut off have chromatin-associated Mcm4p. (A) Experimental procedure. (B) Chromatin binding assay showing Mcm4-GFP chromatin association (top panels) and phase/DAPI (bottom panels) at various times after addition of thiamine to the medium. Bar: 10 μm. (C) Quantitation of chromatin binding assay, showing percentage of uninucleate cells positive for Mcm4-chromatin binding with or without Triton extraction. (D) Flow cytometric analysis of cells for data points shown in B and C.

for mating and meiosis have chromatin-bound Mcm4, implying this could be relevant to execution of DNA replication in the meiotic cycle.

Because previous studies have shown that inhibition of Cdc2 activity is responsible for cell cycle arrest in G1 by mating pheromone (Stern and Nurse, 1997, 1998), we examined the effect of directly inhibiting Cdc2 activity on Mcm4 chromatin association. This was examined in a strain containing a thiamine-repressible *cdc13*$^+$ gene in the background of cyclin B gene deletions (*cig1*Δ, *cig2*Δ, and *cdc13*Δ), which arrests mainly in G1 after addition of thiamine to the medium (Figure 2, A and D; Fisher and Nurse, 1995). Mcm4 was shown to be chromatin associated in G1-arrested cells (Figure 2, B and C), implying that inhibition of Cdc2 activity alone is sufficient to explain Mcm4 chromatin association seen in cells arrested by mating pheromone.

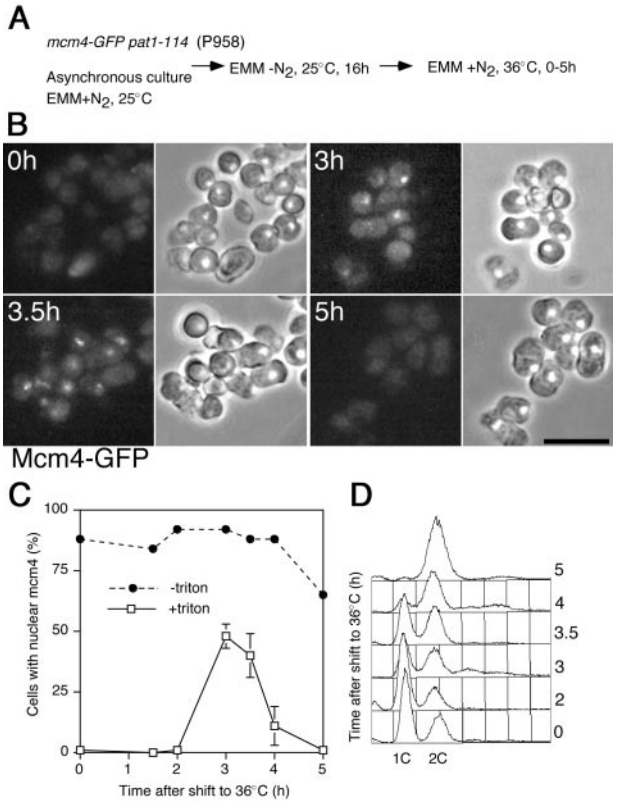


Figure 3. Mcm4p associates with chromatin during premeiotic S phase. (A) Experimental procedure. See text for details. (B) Chromatin binding assay on cells after induction of meiosis, showing Mcm4-GFP chromatin association (left panels) and phase/DAPI (right panels). Bar: 10 μm. (C) Quantitation of chromatin binding assay, showing percentage of cells positive for Mcm4-chromatin binding with or without Triton extraction. (D) Flow cytometric analysis of cells for data points shown in B and C.

Mcm4 Associates with Chromatin during Premeiotic S Phase but not during the Interval Between Meiosis I and II

To examine more directly whether Mcm4 associated with chromatin in G1-arrested cells is relevant to meiosis, we determined whether this and other MCM2–7 proteins bind to chromatin during premeiotic S phase. It is difficult to examine this process in a wild-type diploid, because entry to meiosis on nutrient starvation is rather asynchronous. We therefore made use of a temperature-sensitive *pat1* allele (*pat1ts*), which encodes a defective negative regulator of meiosis, and even haploid strains containing this allele can be induced to enter meiosis by shifting to the restrictive temperature (Iino and Yamamoto, 1985; Nurse, 1985; McLeod and Beach, 1986). Pat1 inactivation leads to a meiosis that is very similar to that induced physiologically by nutrient deprivation and has been generally used for analyzing meiotic mechanisms. A *pat1ts* strain was arrested in G1 by nitrogen starvation for 16 h, after which meiosis was induced by shifting to the restrictive temperature and refeeding (Figure 3A). Initially Mcm4 was not chromatin bound, although binding increased after 2 h, and peaked

slightly in advance of the time of premeiotic S phase (Figure 3, B–D). As premeiotic S phase finished, Mcm4 chromatin association was lost. Similar results were obtained using a diploid *pat1* strain shifted to 34°C (Figure 4, A and D), these conditions being compatible with a viable meiosis (Bähler *et al.*, 1991), although the timing of premeiotic S phase was a little advanced compared with the haploid strain (Figure 4C). Thus, the timing of Mcm4 association with chromatin suggests that MCM proteins function in premeiotic DNA replication as in a normal S phase.

Following later stages of the diploid meiosis showed that Mcm4 remained nuclear during meiosis I and II, but Mcm4 chromatin association was not seen at any stage between these nuclear divisions (Figure 4, A, B, and D, 5–6 h). Thus, a block to MCM chromatin association and pre-RC formation could account for absence of DNA replication between meiosis I and II. The resistance of spores to zymolyase digestion made it difficult to examine Mcm4 chromatin binding after meiosis II, but a high proportion of cells showed absence of Mcm4 chromatin binding (Figure 4A, 6 h; 4D, 5–6 h) before obvious spore formation. Thus, MCM chromatin association may have to be re-established after spore germination.

Arresting Premeiotic DNA Replication with Hydroxyurea Prevents Displacement of Mcm2, 4, and 6 Proteins from Chromatin

To test if the correlation of Mcm4 chromatin association with premeiotic DNA replication reflects a direct involvement with DNA synthesis, we examined how blocking S phase affected MCM2–7 chromatin binding. In the vegetative cell cycle, arresting the elongation step of DNA synthesis with hydroxyurea (HU) prevents the displacement of Mcm4 (Kearsey *et al.*, 2000). To extend the meiotic analysis to other MCM2–7 proteins, we also tagged Mcm2 and Mcm6 with GFP. In vegetative cells, these proteins are similar to Mcm4 in terms of cell cycle changes in chromatin binding. *Pat1ts* strains were arrested in G1 and induced to undergo premeiotic S phase after refeeding and shifting to 37°C as before, except that HU was added to half the culture (Figure 5A). In the presence of HU, most cells showed chromatin binding of Mcm2, Mcm4, and Mcm6 after 5.5 h, whereas control cultures without HU had completed premeiotic S phase and chromatin binding of these proteins was not detected (Figure 5, B and D). Similar results were obtained using a diploid *pat1ts mcm4-GFP* strain. When the same experiment was carried out with an Mcm2-GFP strain containing a temperature-sensitive *mcm4* mutation (*mcm4ts-td*, see below), Mcm2 chromatin association was blocked (Figure 5, C and D). Thus, there is mutual dependency of MCM2–7 proteins for chromatin association, as has been shown in the vegetative cell cycle (Pasion and Forsburg, 1999; Labib *et al.* 2001). Overall, these results suggest that premeiotic S phase involves chromatin association of MCM2–7 proteins and that displacement of these proteins from chromatin requires completion of DNA replication as in the mitotic cell cycle.

A Degron Mutant Reveals a Requirement for Mcm4 in Premeiotic S Phase

To investigate whether chromatin association of MCM2–7 proteins in premeiotic S phase reflects a functional require-

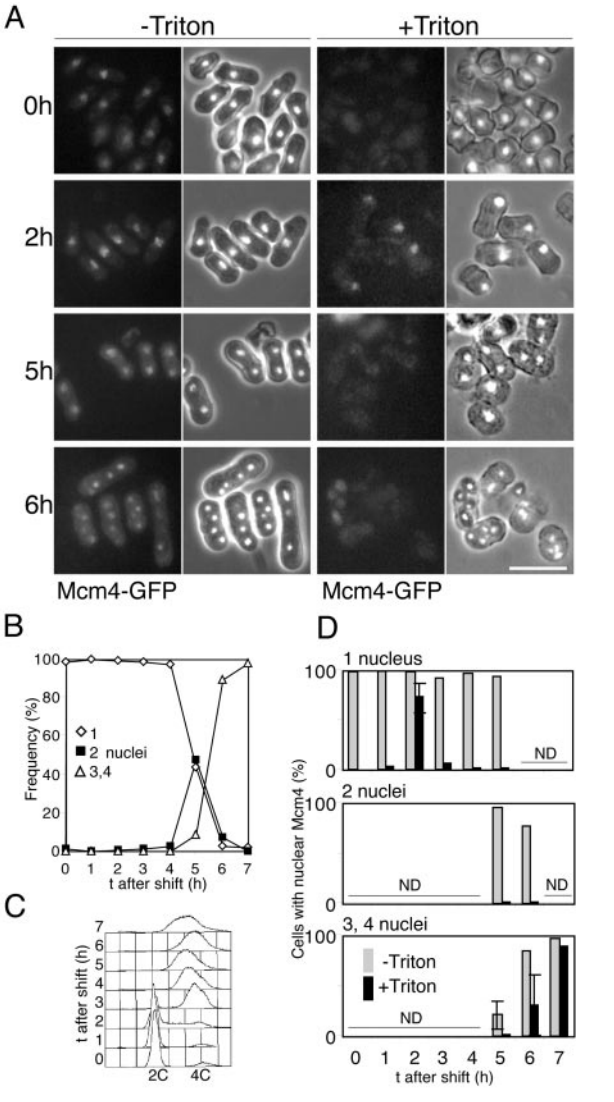


Figure 4. Mcm4 remains nuclear and associates with chromatin during premeiotic S phase in diploid cells, but chromatin binding is blocked between meiosis I and II. Strain P978 was arrested in G1 and induced to enter meiosis as in Figure 3A, except that a temperature shift to 34°C was used. (A) Nuclear localization (-Triton) and chromatin binding (+Triton) of Mcm4-GFP was determined at times shown after the shift to 34°C. Right-hand images show phase/DAPI-staining. Cells were either fixed in methanol and acetone without extraction (-Triton), or detergent extracted using the chromatin binding assay (+Triton). Bar: 10 μm. Progress of meiosis was monitored by following nuclear divisions (B) and flow cytometry (C). (D) Quantitative analysis of data for experiment shown in A–C, indicating percentage of cells showing Mcm4 nuclear localization (-Triton) and chromatin association (+Triton) at different stages of meiosis. Note chromatin binding of Mcm4 during premeiotic S phase (top panel, 2 h) and absence of chromatin binding but maintenance of nuclear localization of Mcm4 between meiosis I and II (middle panel, 5–6 h). Increase in nuclear Mcm4 in extracted cells with 4 nuclei (bottom panel, 6–7 h) reflects formation of spores that are resistant to zymolyase digestion and thus detergent extraction. ND, not determined.

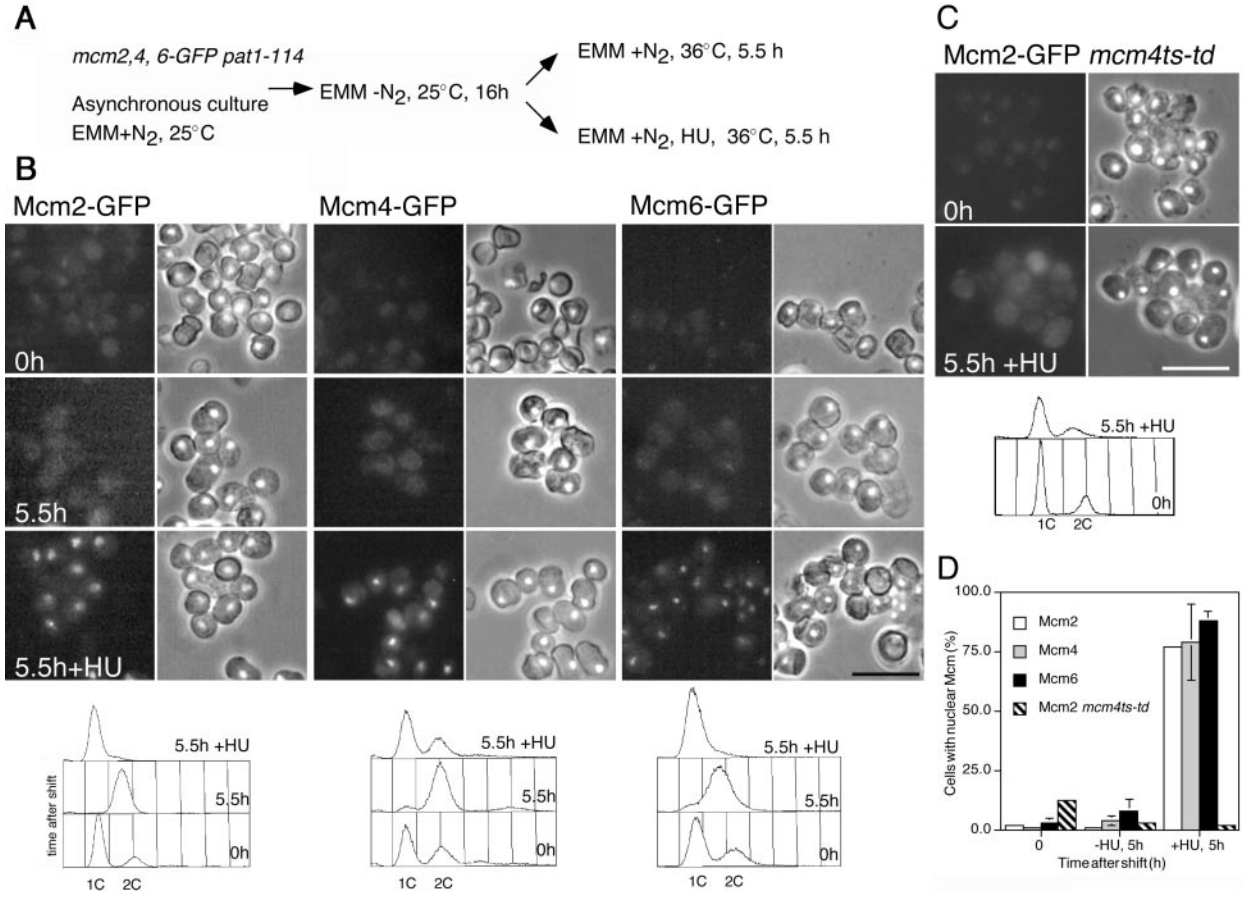
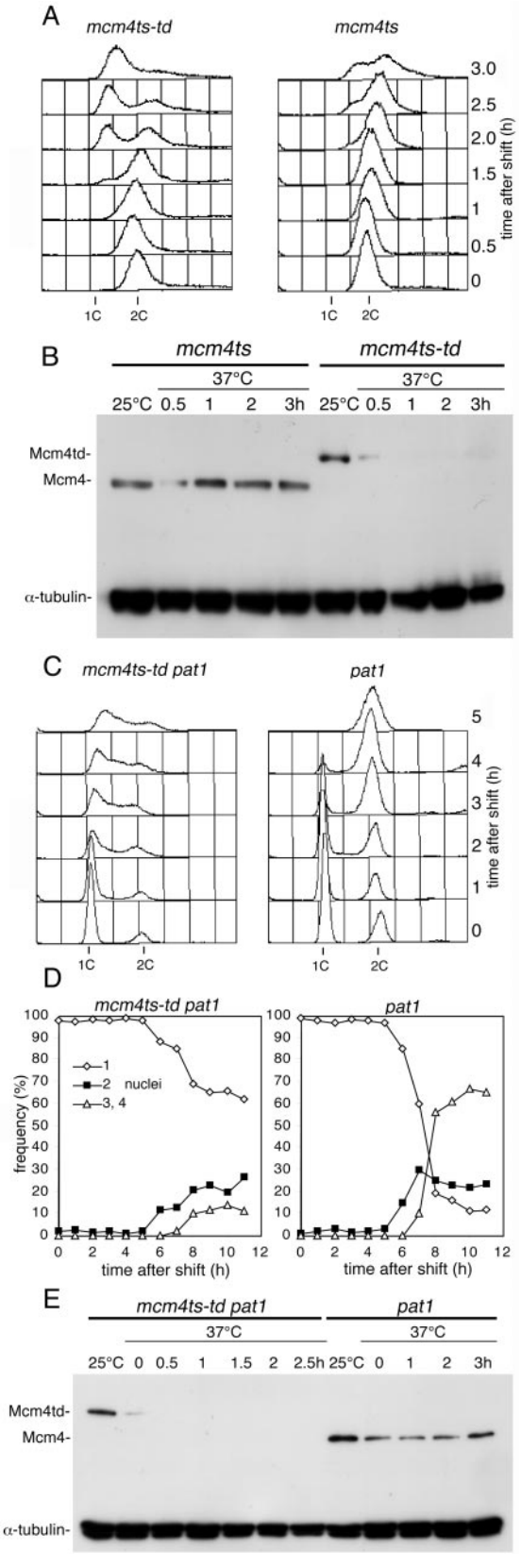


Figure 5. Inhibition of premeiotic S phase with HU prevents displacement of MCM2–7 proteins. (A) Experimental procedure. Strains used were P958 (Mcm4p), P1027 (Mcm2p), and P1025 (Mcm6p). (B) Chromatin binding assay on cells before induction of meiosis and after induction of premeiotic S phase, showing Mcm2, 4, and 6-GFP chromatin association (left panels) and phase/DAPI (right panels). Bar: 10 μm. Flow cytometric analysis of cells is shown underneath. (C) Mcm4 function is essential for Mcm2 chromatin binding. The experiment shown in Figure 2B was repeated using an Mcm2-GFP strain (P1096) containing a temperature-sensitive *mcm4* mutation (*mcm4ts-td*, a degron allele). No chromatin association of Mcm2-GFP is seen after the temperature shift in the presence of hydroxyurea. (D) Quantitation of data shown in B and C.

ment for these proteins, we analyzed the effect on an *mcm4* mutation on meiosis. Because available temperature-sensitive alleles of *mcm2–7* genes are rather leaky and do not block vegetative S phase efficiently (e.g., Forsburg and Nurse, 1994; Takahashi *et al.* 1994), we explored the use of temperature-sensitive degron (td) alleles in *S. pombe*, because these have been useful for clarifying MCM2–7 function in *S. cerevisiae* (Labib *et al.*, 2000). We constructed a degron fusion of Mcm4 (*mcm4td*) where the N-terminus of Mcm4 is fused to the DHFR degron (Dohman *et al.*, 1994), expressed from the native *mcm4*$^+$ promoter. In *S. cerevisiae* this degron is stable at 25°C, but at 37°C the degron is ubiquitylated by Ubr1, probably because of an increase in the accessibility of its N-terminal arginine, leading to its rapid proteolysis (Dohman *et al.*, 1994; Lévy *et al.*, 1999). In *S. pombe*, the *mcm4td* strain grew normally and levels of the degron-Mcm4 protein were similar to Mcm4 levels at 25°C, but at 37°C cells were elongated, implying that the degron confers temperature sensitivity in this organism. Flow cytometry did not show a tight block to DNA replication; however, and Mcm4 levels were not dramatically reduced, implying that Mcm4 degradation is too inefficient to block DNA replication at initiation. To obtain a mutant with a tighter phenotype, we modified a temperature-sensitive *mcm4* allele (*mcm4ts/cdc21-M68*; Nasmyth and Nurse, 1981) by fusion to the degron, to give a *mcm4ts-td* strain. This mutant arrested with predominantly 1C DNA after shifting exponentially growing cells to the restrictive temperature in contrast to the *mcm4ts* mutant, which showed a leakier phenotype (Figure 6A). Western blotting showed that levels of the Mcm4 protein were similar in the *mcm4ts-td* and *mcm4ts* strains at 25°C and in the *mcm4ts* strain at 37°C, but a rapid reduction in protein levels in the *mcm4ts-td* strain was seen at 37°C (Figure 6B). To determine if the *mcm4ts-td* allele affected premeiotic DNA replication, we constructed a double mutant of *mcm4ts-td* with *pat1ts*, and G1-arrested cells were refed and shifted to 37°C. Control *mcm4*$^+$ pat1ts cells carried out premeiotic S phase around 3 h, whereas *mcm4ts-td pat1ts* cells did not replicate their DNA, although a minor fraction of cells exhibited partial replication (Figure



6C). This effect on premeiotic S phase was more severe than that seen with a *mcm4ts* (*cdc21-M68*) *pat1ts* mutant, which showed more extensive replication as previously reported (Forsburg and Hodson, 2000; Murakami and Nurse, 2001). Meiotic nuclear divisions were also reduced in the degron *mcm4* strain, consistent with a block in DNA replication (Figure 6D; Murakami and Nurse, 1999). Western analysis showed that levels of Mcm4 were significantly reduced in the *mcm4ts-td* mutant compared with *mcm4*$^+$ strain after the nitrogen starvation step, and there was a further reduction in protein levels only 30 min after the shift to 37°C (Figure 6E). Protein levels were also lower than in a *mcm4ts* strain, where we could detect Mcm4 throughout premeiotic S phase. We have also shown that a degron *mcm6* mutation blocks premeiotic S phase. Thus, these results, taken together with analysis of MCM chromatin binding, indicate that execution of premeiotic S phase requires the participation of MCM2–7 proteins.

DISCUSSION

In this article we have investigated MCM2–7 chromatin binding and, by inference, pre-RC formation, during G1 arrest of the mitotic cycle and entry into meiosis. In summary, *S. pombe* cells arrested in G1 by mating pheromone have chromatin-bound Mcm4. This pre-RC formation is relevant to premeiotic S phase, because Mcm4 binds to chromatin around the time of premeiotic S phase and S phase completion is necessary to allow displacement of Mcm2, Mcm4, and Mcm6. Analysis of a degron mutant shows that Mcm4 is essential for premeiotic S phase and given that in the vegetative cell cycle MCM2–7 proteins interact (Adachi *et al.*, 1997; Pasion and Forsburg, 1999) and are required for licensing and the elongation steps of replication (Labib *et al.*, 2000; Prokhorova and Blow, 2000; Tye and Sawyer, 2000), it is likely that all MCM2–7 proteins are required for premeiotic DNA replication.

These results show pheromone arrest in *S. pombe* is similar to that in *S. cerevisiae*, where α-factor also arrests cells in G1 with pre-RCs assembled and MCM2–7 proteins bound to chromatin (Diffley *et al.*, 1994; Donovan *et al.*, 1997). The mechanisms of these cell cycle arrests are distinct in detail, although in both cases pheromone blocks CDK activation

Figure 6. Analysis of *mcm4* degron mutant. (A) Flow cytometric analysis of degron *mcm4* (*mcm4ts-td*, P1023) and *mcm4ts* (*cdc21-M68*, P1) strains during the vegetative cell cycle, after shifting asynchronous cultures to 37°C at t = 0, showing that the degron strain arrests with 1C DNA. (B) Mcm4 protein levels decline abruptly on shifting the *mcm4* degron strain (*mcm4ts-td*, P1023) to the restrictive temperature. In contrast, Mcm4 levels in the *mcm4ts* (*cdc21-M68*, P1) strain are not affected by the temperature shift. α-Tubulin is shown as a loading control. (C) *mcm4* degron mutation blocks premeiotic S phase. *pat1ts mcm4ts-td* (P1037) and *mcm4*$^+$ *pat1ts* (P937) strains were arrested in G1 and then shifted to 37°C and re-fed as in Figure 4A, except that cells were shifted to 37°C 30 min before refeeding. Progress of premeiotic DNA replication was analyzed by flow cytometry. (D) Analysis of meiotic progress for experiment shown in C and E, showing that inactivation of Mcm4 inhibits nuclear divisions. (E) Mcm4 levels are reduced in *pat1ts mcm4ts-td* (P1037) mutant on nitrogen starvation and induction of meiosis at 37°C compared with a *mcm4*$^+$ *pat1ts* (P937) strain (time after shift is shown). α-Tubulin is shown as a loading control.

needed for S phase entry (Peter *et al.*, 1993; Peter and Herskowitz, 1994; Stern and Nurse, 1998), whereas expression of Cdc18/Cdc6 needed for pre-RC formation is not prevented (Zwerschke *et al.*, 1994; Stern and Nurse, 1997). Budding and fission yeast cells have different fates after diploid formation, which is relevant to the function of chromatin-associated MCM2–7 proteins. Because *S. cerevisiae* haploid cells are constitutively competent for conjugation, MCM2–7 proteins in mating cells would generally function in a vegetative S phase. On the other hand, because *S. pombe* cells only fuse on nutrient limitation and zygotes progress directly to meiosis, MCM2–7 proteins assembled onto chromatin in mating competent cells are likely to function in premeiotic S phase.

It is likely that a previous report suggesting that MCM2–7 proteins are not needed for premeiotic S phase (Forsburg and Hodson, 2000) reflects the leaky nature of the original conditional alleles compared with more efficient inactivation of Mcm4 function in our degron allele While this article was in preparation, Murakami and Nurse (2001) reported that using a higher restrictive temperature than that used in the initial study does in fact prevent completion of premeiotic S phase, using *mcm2* and *mcm4* mutants. One factor that could be relevant to why premeiotic DNA replication is not blocked by *mcm* mutations under conditions that prevent completion of vegetative S phase is the nitrogen starvation step used for G1 synchronization before meiotic entry. Nitrogen-starved cells have increased levels of Rum1 compared with vegetative cells (Maekawa *et al.*, 1998) and thus depressed CDK activity, and CDK levels are further reduced by enhanced proteolysis of cyclin B (Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998; Kominami *et al.*, 1998). Both these changes could suppress mutations affecting Cdc18 or MCM2–7 function by promoting pre-RC formation, based on analysis of mutants with reduced CDK levels (Jallepalli and Kelly, 1996; Grallert *et al.*, 2000). For instance, enhanced Rum1 levels suppresses a *cdc18* mutation via inhibition of Cdc18 proteolysis (Jallepalli and Kelly, 1996) and deletion of the $cig2^+$ B-type cyclin suppresses *cdc18*, *mcm2*, and *mcm4* mutations (Grallert *et al.*, 2000). Another possibility is that the difference between premeiotic and vegetative DNA replication might be quantitative in that less MCM2–7 function is required for premeiotic S phase. It seems less likely that there is an alternative meiotic replication pathway capable of compensating for loss of MCM2–7 function, given the effective replication arrest seen in the degron *mcm4* mutant.

Mcm4 levels are maintained through meiosis (Forsburg and Hodson, 2000), but chromatin association of Mcm4 does not occur between meiosis I and II even though the protein remains nuclear (Figure 4), presumably accounting for the absence of a second round of DNA replication. Inactivation of components required for pre-RC formation would provide an obvious mechanism to block MCM chromatin association, and it is of interest that in *Xenopus* incomplete inactivation of Cdc2 after meiosis I is required for preventing DNA replication (Iwabuchi *et al.*, 2000; Nakajo *et al.*, 2000). If a similar situation applies to fission yeast, Cdc2-mediated destabilization of Cdc18 (Baum *et al.*, 1998) could constitute one mechanism to ensure that only a single round of DNA replication occurs in meiosis.

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