Mcm2 and Mcm3 Are Constitutive Nuclear Proteins That Exhibit Distinct Isoforms and Bind Chromatin during Specific Cell Cycle Stages of *Saccharomyces cerevisiae*

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The Mcm2–7 proteins are a family of conserved proteins whose functions are essential for the initiation of DNA synthesis in all eukaryotes. These proteins are constitutively present in high abundance in actively proliferating cells. In *Saccharomyces cerevisiae*, the intracellular concentrations of Mcms are between 100 and 500 times the number of replication origins. However, these proteins are limiting for the initiation of DNA synthesis at replication origins. Our studies indicate that only a small fraction of Mcm2 and Mcm3 tightly associates with chromatin, from late M phase to the beginning of the S phase. The rest of the Mcm2 and Mcm3 proteins are distributed to both the cytoplasm and nucleoplasm in relatively constant levels throughout the cell cycle. We also show that *S. cerevisiae* Mcm3 is a phosphoprotein that exists in multiple isoforms and that distinct isoforms of Mcm2 and Mcm3 can be detected at specific stages of the cell cycle. These results suggest that the localization and function of the Mcm proteins are regulated by posttranslational phosphorylation in a manner that is consistent with a role for the Mcm proteins in restricting DNA replication to once per cell cycle.

INTRODUCTION

In every cell cycle, the eukaryotic genome is replicated once, resulting in two daughter cells that each contain an exact complement of the parental genome. DNA replication occurs only during the S phase and rereplication within the same S phase is not normally observed. Although it is clear that the temporal order of cell cycle events is regulated by the rise and fall of the activities of the p34/cyclin protein kinases, the targets of these kinases are largely unknown. A number of models have been suggested to explain the strict coupling of DNA synthesis to the cell cycle. In mammalian cells, cells in G₁ phase, when fused to S phase cells, replicate their genomes once but cells in G₂ phase do not (Rao and Johnson, 1970). These experiments suggested that chromatin in cells in G₁ phase differs from chromatin in cells in G₂ phase in the

ability to support DNA synthesis in response to factor(s) present in S phase cytosol. Cell-free experiments using *Xenopus* egg extracts suggested that modification of G₁ chromatin is effected by *trans*-acting positive regulation (Coverley *et al.*, 1993). The accumulation of limiting nuclear factors during G₁ phase thus serves as a positive signal for entry into S phase. Rapid decay or inactivation of *trans*-acting factors prevents repetition of this signal until the next cell cycle, when nuclear envelope breakdown again allows access to the chromatin. This process of limiting access of specific replication initiation factors to chromatin and of modifying chromatin in a cell cycle-specific manner to allow DNA replication initiation to occur has been termed replication licensing (Blow and Laskey, 1988).

The Mcm2–7 proteins are members of a family of conserved proteins essential for the maintenance of minichromosomes (Yan *et al.*, 1993) and for S phase progression in yeast (Hennessy *et al.*, 1991). Six Mcm family members, Mcm2, Mcm3, Mcm4/Cdc54, Mcm5/Cdc46, Mcm6, and Mcm7/Cdc47, have been

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identified in *Saccharomyces cerevisiae*; each has a corresponding homologue in metazoans (Kawasaki and Tye, unpublished results; Gibson *et al.*, 1990; Hennessy *et al.*, 1991; Yan *et al.*, 1991; Dalton and Whitbread, 1995; Whitbread and Dalton, 1995). Each MCM gene is essential for the viability of cells, indicating that, although their products share significant sequence homology, each performs a unique function. Mutants defective in MCM gene products display diminished usage of replication origins, although the severity of the defect appears to be origin dependent (Maine *et al.*, 1984). Based in part on these observations, the Mcm proteins are believed to play a direct role in the regulation of initiation of DNA synthesis at replication origins.

The Mcm proteins are present at high levels (10^4 – 10^5 molecules per cell) relative to the few hundred replication origins present in the yeast genome (Lei *et al.*, 1996). Surprisingly, given the abundance of the Mcm proteins, they appear to be limiting for origin usage. Diploid yeast cells containing half the normal gene dosage of MCM2 show diminished usage of a subset of replication origins. Because the Mcm proteins interact with one another, and possibly other proteins, to form larger complexes, one explanation for the dosage effect of Mcm2 is that the assembly of these complexes may be limiting for replication initiation (Lei *et al.*, 1996). Functional interactions between the Mcm proteins and the origin recognition complex, which binds specifically to the consensus sequence at autonomously replicating sequences or replication origins (Bell and Stillman, 1992) have also been suggested by the results of genetic experiments (Li and Herskowitz, 1993).

In vivo footprinting analysis of ARSs indicated that these regions exist in different states as cells proceed through the cell cycle (Diffley *et al.*, 1994). In S and M phases, ARSs show footprints characteristic of protection by the origin recognition complex. In G₁ phase, ARSs appear to be protected by additional proteins. This G₁ specific conformational state has been named the prereplication state (Diffley *et al.*, 1994). Because of the involvement of the Mcm proteins in the initiation of DNA replication, and their apparent accumulation in the nucleus between late M and G₁ phase (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995), the Mcm proteins are candidates for components of prereplication complexes. The assembly of prereplication complexes at yeast replication origins may be part of the licensing of G₁ chromatin for replication.

To better understand the licensing of G₁ chromatin, Kubota *et al.* (1995) characterized proteins that are associated with G₁ but not G₂ chromatin in self-assembled nuclei in *Xenopus* egg extracts. They identified an abundant protein that was associated with G₁ DNA before the formation of nuclei but was dissociated

from nuclear DNA during the progression of replication (Kubota *et al.*, 1995). This protein, XMcm3, is the *Xenopus* homologue of the yeast Mcm3 protein. Independently, Chong *et al.* (1995) purified from *Xenopus* egg extracts an essential component of an activity that restricts DNA synthesis to one round per cell cycle and showed that this activity is in fact XMcm3 (Chong *et al.*, 1995). Although XMcm3 is constitutively nuclear, the timing of the binding of XMcm3 to chromatin corresponds to the replication licensing of G₁ chromatin (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995). An additional factor, which appeared to be a Cdc2-like kinase (Blow, 1993), is required for the initiation of this replication licensing process at anaphase.

Recent studies suggested that posttranslational modifications, such as phosphorylation, may be important for the chromatin association of the mammalian (Kimura *et al.*, 1994; Todorov *et al.*, 1995) and amphibian (Coue *et al.*, 1996) Mcm proteins. Analysis of Mcm proteins in yeast extracts also suggested that the phosphorylation of Mcm3 may play a role in the assembly of Mcm complexes (Lei *et al.*, 1996). Multiple potential protein kinase phosphorylation sites are present throughout Mcm2 (891 amino acids) and Mcm3 (971 amino acids) (Yan *et al.*, 1991; Kimura *et al.*, 1994). In this study, we investigated the cell cycle-regulated posttranslational modification of two Mcm proteins, Mcm2 and Mcm3, by examining the changes in their phosphorylation states at defined cell cycle stages in yeast. We show that Mcm3 is a phosphoprotein, and we show the presence of cell cycle-specific isoforms of Mcm2 and Mcm3. We also investigated the cell cycle timing of binding of Mcm2 and Mcm3 to chromatin. Contrary to earlier interpretations, based on immunofluorescence (IF) data, that yeast Mcm proteins are localized to the nucleus in a cell cycle-specific manner, we now show that Mcm2 and Mcm3 are present in nuclei throughout the cell cycle but bind to chromatin only from late M phase to early S phase.

MATERIALS AND METHODS

Strains

Yeast strains used in this work were BJ2168 (MATa *leu2 trp1 ura3-52 prb1-1122 prc1-407 pep4-3 gal2*), MY103 (MATa *leu2 trp1 ura3-52 prb1-1122 prc1-407 pep4-3 gal2 mcm3::MCM3-HIS6*), MY107 (MATa *cdc28-1 ura3-52 leu2-3, 112 trp1 ade5 pep4::URA3*), YJL924 (MATa *cdc9-1 leu2-3, 112 trp1-289 bar1::LEU2 pep4::TRP1 ura3-52*), and MY105 (MATa *ura3-52 leu2-3, 112 trp1 ade5 cdc28-HA dbf2 pep4::URA3*).

MY103 was constructed by integration of an MCM3 gene, carrying six histidine codons inserted immediately 5' of the coding region stop codon, into the MCM3 locus of BJ2168 cells. The integration of the MCM3-HIS6 gene at the MCM3 locus provides a partial, nonfunctional, copy of MCM3 as well as the expressed MCM3-HIS6 gene. MY105 was constructed by disruption of the PEP4 gene by a *pep4::URA3* cassette in L181-6B (*dbf2-1*) cells (Toyn and Johnston, 1994). MY107 was similarly constructed by disruption of the PEP4 gene in K285-1 (*cdc28-1*) cells.

Antibodies

Immunoblotting, immunoprecipitation, and IF were performed using standard techniques (Ausubel *et al.*, 1992). The ampholyte mixture for first-dimension isoelectric focusing in the two-dimensional (2D) protein gel assays was 3 parts pH 5–7 ampholytes (Bio-Rad, Richmond, CA) to 1 part pH 3.5–10 ampholytes (Pharmacia, Piscataway, NJ). Second-dimension gels were 7.5% SDS gels. For immunoblotting 7.5% homogeneous SDS gels and 4–20% gradient gels (Bio-Rad) were used. The primary antibodies used for immunoblotting were affinity-purified monospecific anti-Mcm2 or anti-Mcm3 antibodies (Yan *et al.*, 1993), crude anti-phosphoglycerate mutase (PGM) antiserum (Chen and Tye, 1995), and histone H2B (a gift from M. Grunstein, UCLA), with secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies (Bio-Rad) and DuPont Renaissance reagents used for chemiluminescent detection. For indirect IF, the primary antibodies used were affinity-purified monospecific anti-Mcm2 or anti-Mcm3 antibodies and anti-tubulin monoclonal antibody YOL134 (Accurate Chemical, Westbury, NY). Secondary antibodies used were 1:200 dilutions of fluorescein isothiocyanate-conjugated anti-rat (Cappel, Durham, NC) and 1:500 dilutions of Cy3-conjugated anti-rabbit (Jackson ImmunoResearch, West Grove, PA).

³²P Labeling and Immunoprecipitation

Ten milliliters of early logarithmic phase BJ2168 cells ($A_{600} \approx 1$; 10^7 cells/ml) grown in phosphate (P_i)-free YEPD (Rubin, 1973) were arrested with α -factor and then released from the pheromone block into 10 ml of P_i -free YEPD, and 1 mCi of [³²P]orthophosphate (10 mCi/ml) was added. Incubation at 30°C was continued for 2 h, and then cells were harvested, washed twice with YEPD to remove free radioisotope, and resuspended in 500 μ l of breaking buffer (20 mM HEPES, pH 7, 50 mM Na_2HPO_4/NaH_2PO_4 , pH 7, 100 mM NaCl, 10% glycerol) plus protease inhibitors (75 μ g/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone, 40 μ g/ml 7-amino-1-chloro-3-tosylamido-2-heptanone, 0.7 μ g/ml pepstatin A, 1 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 0.25 mM Pefabloc) and phosphatase inhibitors (5 mM sodium pyrophosphate, 0.1 mM sodium metavanadate, 50 mM sodium fluoride) before disruption by vortexing with glass beads (five 1-min periods). The crude extract was precleared by incubation with Immunoprecipitin (Life Technologies, Gaithersburg, MD), and then Mcm3 protein was immunoprecipitated with monospecific anti-Mcm3 antibodies followed by collection of the immunoprecipitate with protein A-Sepharose. Immunoprecipitates were washed twice with low salt buffer, one time with high salt buffer, and one time with detergent-free buffer before release of Mcm3 from the protein A-Sepharose beads by boiling in Laemmli buffer. Labeled Mcm3 was detected by autoradiography following separation on 7.5% SDS gels. Unlabeled Mcm3 was immunoprecipitated in a like manner and detected on immunoblots with the anti-Mcm3 antibodies.

For purification of Mcm3-HIS6, proteins were labeled and crude proteins were isolated as described above. Mcm3-HIS6 was purified on Ni-NTA columns by the procedure provided by the supplier (Qiagen, Chatsworth, CA) and detected by autoradiography following separation on 7.5% SDS gels.

Cell Cycle Arrest and Protein Extraction

BJ2168 cells growing at 30°C in YEPD medium were arrested by addition of α -factor to final concentrations of 10 μ g/ml, hydroxyurea (HU) to 0.2 M (final concentration), or nocodazole (NOC) to 0.1 M (final concentration). For double arrests, the α -factor-arrested cells were washed three times with YEPD and resuspended in YEPD, and the second arresting agent was added. For primary arrests, cells were incubated with the arresting agent for 3 h; for double arrests, the cells were incubated for an additional 2 to 3 h after the second arresting agent was added. Pelleted cells were resuspended in 500 μ l of breaking buffer plus protease inhibitors

and, where indicated, phosphatase inhibitors and disrupted by vortexing for six 1-min cycles, at 4°C, in the presence of glass beads. Typically, ~50% of the cells ruptured in this procedure.

During growth to stationary phase, we monitored the cell density in cultures by measuring the A_{600} of aliquots removed at intervals. Previous work showed that increases in A_{600} correlated well with increases in the number of cells/ml, as counted in a hemacytometer.

Subcellular Fractionation

Where appropriate, cells were arrested prior to fractionation, as described above. Growing or arrested cells were harvested and resuspended in spheroplasting buffer (1 M sorbitol, 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol). The cell walls were removed by incubation at 30°C with 25–50 U/ml recombinant lyticase enzyme (ICN Biochemicals, Costa Mesa, CA). We noted that the proteases present in most commercial zymolyase preparations overwhelmed our protease inhibitors in most strains that were not protease deficient. Recombinant lyticase is substantially free of proteases and enabled us to more easily obtain intact proteins. The spheroplasts were washed twice with spheroplasting buffer and then resuspended in Ficoll buffer [18% Ficoll, 20 mM piperazine- N,N' -bis(2-ethanesulfonic acid), pH 6.3, 0.5 mM $CaCl_2$, 1 mM dithiothreitol, 1 mM EDTA] plus protease and, where indicated, phosphatase inhibitors. The suspension was treated with 30 strokes of a tight pestle in a Dounce homogenizer and the unbroken cells were removed by two low-speed spins (4500 rpm in a Microfuge for 5 min). The nuclei were pelleted by a high-speed spin (14,500 rpm in Microfuge for 20 min) and the cytosolic fraction (C) removed. The nuclei were resuspended in 100 μ l of extraction buffer [10 mM HEPES, pH 7.5, 1 mM EDTA, NaCl as required (0.5 M or 2.0 M)] plus protease and phosphatase inhibitors and incubated on ice for 20 min. After another high-speed spin, the nucleosolic fraction (NS) was removed and the nuclear pellet was resuspended in 100 μ l of extraction buffer containing 0.5 M NaCl. Four hundred units of DNase I (BRL) were added and the tube was incubated at 37°C for 2 h. The debris was pelleted and the nuclear pellet fraction (NP) was removed. Typical volumes recovered were 1–1.5 ml of cytosolic fraction and 100 μ l of nucleosolic fraction and 100 μ l of nuclear pellet fraction.

RESULTS

Mcm3 Is Present in High Abundance Only in Proliferating Cells

Saccharomyces cerevisiae cells continuously monitor their environment and adjust their metabolism and growth rate to reflect the available nutrients. In the presence of high levels of nutrients, such as growth on rich medium, cells achieve a high rate of growth. This logarithmic increase in cell density persists until the readily metabolized nutrients are depleted. Yeast cells then undergo a shift in metabolic activity from a rapidly dividing fermentative growth state to a slower respiratory growth state, at a point known as the diauxic shift (Figure 1A). Once the remaining nutrients are depleted during respiratory growth, the cells enter a nongrowing stationary phase. Multiple genes are involved in each growth phase and the relative abundance of certain proteins changes during the different phases. Because the growth rate varies during distinct phases, we were interested in whether the levels of replication proteins reflected the rate of growth.

Figure 1. Mcm3 is present in high abundance only in proliferating cells. S288C cells in YEPD were grown in continuous culture to stationary phase or induced to enter a G₀-like state by shifting a growing culture to medium lacking nitrogen. The cell density was monitored at intervals during growth to stationary phase and aliquots were removed for protein isolation. For G₀ induction, mid-logarithmic phase cultures (A_{600} , 1.5) were pelleted and resuspended in YN medium (Diffley *et al.*, 1994). After 6 h of incubation in YN, cells were harvested and proteins were isolated by bead-beating lysis. (A) Equal concentrations of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Levels of Mcm3 and tubulin, as a loading control, were analyzed by immunodetection of each protein fraction. Passage through the diauxic shift and entry into stationary phase were monitored by plotting the cell density at each time point. (B) Points at which proteins were isolated are shown on the graph. Essentially no further increase in cell density occurred after 100 h of continuous culture.

S288C cells were grown in a continuous culture for 5 d and the culture density was monitored at various intervals during growth to stationary phase by optical absorbance (A_{600}). At several points during growth to stationary phase, aliquots of cells were harvested and proteins were isolated by bead-beating lysis. Equal amounts of protein were separated by SDS-PAGE and the levels of Mcm3 and tubulin were analyzed by immunoblotting. Although tubulin levels were relatively constant at each cell density, Mcm3 levels decreased significantly when cells passed the diauxic shift and the growth rate of the cells slowed, after approximately 30 h of growth (Figure 1B, lane 5). By

the time cells reached stationary phase, characterized by no further increase in the density of cells in the culture, Mcm3 was no longer detectable (Figure 1B, lane 8).

In addition, we induced a nongrowing G₀-like state in early logarithmic phase cells by switching them to medium lacking nitrogen. In these nonproliferating G₀ cells, Mcm3 levels were significantly lower than in the logarithmic phase culture (Figure 1B, lane 9). Under similar conditions, Diffley *et al.* (1994) have shown that the prereplicative complex is absent from cells. At this point we do not know whether the abundance of Mcm3 in nonproliferating cells is controlled by rapid turnover of the protein or by a decrease in MCM3 transcription. However, we conclude that higher levels of Mcm3 are present in actively growing cells than in slow growing or quiescent cells.

Mcm2 and Mcm3 Are Present Throughout the Cell Cycle in Proliferating Cells

We have shown that Mcm3 is abundant in actively proliferating cells. Because the Mcm proteins are known to perform cell cycle-specific functions, we wanted to know whether the Mcm activities were regulated, at least in part, by fluctuations in the levels of Mcm2 and Mcm3 during the cell cycle. Logarithmic phase BJ2168 cells were arrested in G₁ by the addition of α -factor to the medium for 3 h and then released from the α -factor block into YEPD. Aliquots were removed from the culture every 10 min, and crude protein extracts were prepared. The morphology of the cells was noted at each time point of harvest, as an approximate indicator of the cell cycle stage (Figure 2A, top). Proteins from equal volumes of extract at each time point were separated electrophoretically in SDS protein gels. After transfer to membrane for Western blot detection, one membrane was probed with anti-Mcm3 antiserum, and the other membrane was probed with anti-Mcm2 antiserum. Both membranes were also probed with anti-tubulin. Both Mcm2 and Mcm3 were detected throughout the cell cycle in proliferating cells, as shown in Figure 2A. The time in minutes after release from α -factor block is shown beneath the tubulin blot, and the approximate stage of the cell cycle, displayed as a cartoon of the predominant morphology seen at those time points, is shown at the top. In Figure 2B, a quantitation of the intensity of each Mcm2 and Mcm3 band, normalized to tubulin levels, is depicted. These experiments have been repeated, harvesting cells at other time intervals, with similar results (our unpublished results). Variations in protein levels at different time points were likely due to differences in recovery of protein from each aliquot. Overall, these data show that both Mcm2 and Mcm3 are present at relatively constant levels

Figure 2. Mcm2 and Mcm3 are present throughout the cell cycle in proliferating cells. BJ2168 cells in early logarithmic phase were arrested by the addition of 10 $\mu\text{g}/\text{ml}$ synthetic α -factor. After 3 h of incubation in the presence of α -factor, the cells were washed three times with YEPD, then resuspended in YEPD, and returned to growth conditions. Aliquots of cells were removed every 10 min and total cellular proteins were prepared. Equal-volume aliquots of crude protein extracts from cells harvested at each time point were electrophoresed on parallel 7.5% SDS protein gels and the separated proteins were transferred to Immobilon-P. (A) One membrane was probed with anti-Mcm3 and anti-tubulin and the other membrane was probed with anti-Mcm2 and anti-tubulin. (B) Plot of Mcm2 and Mcm3 protein concentrations during the cell cycle. Normalized levels of Mcm2 and Mcm3 (Y-axis) versus minutes after release from α -factor arrest (X-axis) are shown. After exposure of the chemiluminescent membranes to film, the intensity of each band was determined densitometrically, and the Mcm2 and Mcm3 bands were normalized to the tubulin levels at the corresponding time points. Cells were examined under a microscope at various points after release from α -factor, and the predominant cell morphology (>80% of cells at each time point) was noted, as shown in cartoon form at the top of A. Single unbudded cells are in G_1 phase, small budded cells are in early S phase, and large budded cells are in late S or M phase.

during all stages of the cell cycle in actively growing cells.

Mcm3 Is a Phosphoprotein

After showing that control of Mcm2 and Mcm3 function is probably not exerted at the level of protein abundance, we investigated other common regulatory mechanisms. Mcm3 is a serine- and threonine-rich protein (>14% Ser or Thr residues) that contains numerous potential protein kinase phosphorylation sites, including 11 potential Cdc28 protein kinase phosphorylation sites. To investigate whether Mcm3 indeed undergoes phosphorylation in vivo, we examined the

Figure 3. Mcm3 is a phosphoprotein. (A) In lanes 1 and 2, proteins in BJ2168 cells were labeled in vivo by the addition of 1 ml of 10 mCi/ml [^{32}P]orthophosphate to 10-ml cultures released from an α -factor arrest into P_i -free YEPD. Mcm3 was immunoprecipitated from total cell lysates, and the presence of Mcm3 was detected by autoradiography after electrophoresis in a 7.5% SDS gel. Immunoprecipitation reaction in lane 1 used 4 μl of anti-Mcm3 antibody and lane 2 used 20 μl of antibody. In lane 3, proteins in MY103 cells were labeled in vivo by the addition of 1 ml of 10 mCi/ml [^{32}P]orthophosphate to 10-ml cultures grown in inorganic phosphate-free YEPD. Mcm3-HIS6 was isolated from crude cell lysates by Ni-NTA column chromatography and detected by autoradiography after electrophoresis in a 7.5% SDS gel. In lane 4, Mcm3 was immunoprecipitated from extracts of unlabeled BJ2168 cells, and the presence of Mcm3 was detected by immunoblotting after separation on a 7.5% SDS gel. (B) Mcm3 was detected in immunoblots of 2D protein gels. Aliquots of a crude cell lysate of logarithmic-phase BJ2168 cells were incubated at 30°C for 3 h without (–PPase) or with (+PPase) 1000 U of lambda phosphatase (New England Biolabs) prior to 2D separation.

Mcm3 protein immunoprecipitated from BJ2168 cells that were grown in medium depleted of P_i , which was supplemented with [^{32}P]orthophosphate for 3 h before protein extraction. From preliminary experiments, it appeared that Mcm3 is modified as cells progress through S phase (see Figure 4). We took advantage of these data to attempt to label Mcm3 to higher specific activity by releasing G_1 -synchronized cultures into medium containing [^{32}P]orthophosphate. BJ2168 cells growing in P_i -free YEPD were arrested with α -factor and then released from α -factor arrest into P_i -free YEPD. After incubation in the presence of the radioactive isotope, cells were harvested and crude protein extracts were prepared. Figure 3A (lanes 1 and 2) indicates the position of an ~120-kDa ^{32}P -labeled protein band immunoprecipitated from these labeled extracts by anti-Mcm3 antibodies, which have been shown previously to be specific for Mcm3 (Yan *et al.*, 1993). We also purified ^{32}P -labeled Mcm3, tagged with six histidine residues, by Ni-NTA chromatography (Figure 3A, lane 3). To verify the specificity of the immunoprecipitation reaction, unlabeled protein extracts were also precipitated by the anti-Mcm3 antibodies and detected by anti-Mcm3 antibodies in an

immunoblot (Figure 3A, lane 4). The immunoprecipitated *in vivo* ^{32}P -labeled Mcm3 migrated to the same relative positions as the Mcm3 detected by immunoblotting and the Mcm3-HIS6 protein purified by nickel column chromatography (Figure 3A), and all three bands migrated to the same position as Mcm3 in immunoblots of crude (nonimmunoprecipitated) yeast protein preparations.

To further investigate whether Mcm3 is phosphorylated, we analyzed the isoforms of Mcm3 by Western blotting of 2D gels after *in vitro* treatment of unlabeled crude protein extracts with phosphatase. Protein extracts were prepared from BJ2168 cells by lysis of unlabeled logarithmic phase cultures grown in YEPD. Mcm3 was analyzed by 2D protein gel electrophoresis and immunoblotting before (Figure 3B, top) and after (Figure 3B, bottom) treatment of crude protein extracts with λ phosphatase. Multiple isoforms of Mcm3 were detectable in immunoblots of untreated extracts. After limited phosphatase treatment, the number of Mcm3 isoforms detected was reduced through removal of some of the charged phosphate groups (Figure 3B, bottom). These results indicate that Mcm3 is phosphorylated *in vivo* and the phosphorylation can be analyzed *in vitro*.

Mcm2 and Mcm3 Are Modified in a Cell Cycle-specific Manner

If the cell cycle-specific functions of Mcm2 and Mcm3 are regulated by posttranslational phosphorylation, changes in the phosphorylation states of these proteins would be expected when these specific functions are executed. On the basis of this model, the different isoforms of Mcm3 observed in logarithmic phase cultures represent a mixture of isoforms from various cell cycle stages. To determine whether phosphorylation varies with the cell cycle stage, we synchronized parallel cultures of BJ2168 cells in G₁ phase with α -factor, in S phase with HU, and in M phase with NOC. After 3 h of incubation in the presence of inhibitors, cells were harvested and crude cellular extracts were prepared.

High-resolution analysis of Mcm3 isoforms was achieved by Western blots of 2D protein gels of extracts from cells arrested at different cell cycle stages. To enhance the separation of the different isoforms, we used an isoelectric focusing gradient biased toward resolution of acidic isoforms. Protein species that migrate toward the more acidic end are presumed more highly phosphorylated than those that migrate toward the more basic end (see Figure 3B). Protein extracts prepared from asynchronous cultures displayed a number of Mcm3 isoforms that migrated in the central portion of the isoelectric focusing gradient (Figure 4A, part 1). In contrast, extracts from α -factor-arrested cells displayed predominantly basic forms of Mcm3; a small population of more acidic forms was also ob-

served (Figure 4A, part 2). A similar distribution of isoforms was also observed in Western blots of extracts prepared from *cdc28* cells arrested in G₁ at START, indicating that the appearance of these isoforms is not an artifact of the pheromone arrest (our unpublished results). In S phase (HU-arrested) protein extracts, the acidic forms were more abundant than the basic forms (Figure 4A, part 3). M phase (NOC-arrested) cell extracts had predominantly acidic forms (Figure 4A, part 4). The absence of the basic Mcm3 isoforms in the asynchronous population was surprising (Figure 4A, part 1). Overexposure of the Western blot revealed additional Mcm3 isoforms at the basic end of the gradient, similar to those observed in α -factor-arrested cells (Figure 4A, part 5). These results suggest that this basic form of Mcm3 is a minor component of an actively growing population and that it occurs at a very narrow window at or near Start in G₁ phase ("G₁") of the cell cycle. We concluded that the cell cycle-specific isoform patterns were likely to be due to changes in the phosphorylation state of the Mcm3 protein.

A similar analysis of Mcm2 was conducted. Mcm2 displays less dramatic differences in isoforms at various stages of the cell cycle than was observed for Mcm3. Basic forms were predominant in G₁ phase (α -factor-arrested) cell extracts (Figure 4B, part 2) and acidic forms predominated in S phase (HU-arrested) cell extracts (Figure 4B, part 3). M phase (NOC-arrested) cell extracts also displayed unique Mcm2 isoforms (Figure 4B, part 4). On the basis of 2D gel analysis, Mcm2 also undergoes cell cycle-specific modifications but these modifications appeared to confer only modest changes in the pI of Mcm2 isoforms. It is important to note, however, that isoforms of proteins that comigrate in 2D gels do not necessarily contain identical modifications, because the isoelectric focusing separates isoforms on the basis of overall charge not on the basis of specific modified sites or even types of modification. Therefore, each isoform pattern may represent several unique species of protein.

Our analysis of Mcm2 and Mcm3 showed that both of these proteins undergo dynamic posttranslational modification. The data in Figure 3 showed that Mcm3 is modified by phosphorylation and the data in Figure 4 showed that both Mcm2 and Mcm3 undergo cell cycle stage-specific posttranslational modification.

Mcm2 and Mcm3 Are Present in Three Distinct Subcellular Fractions

Our previous work showed that a portion of Mcm2 and Mcm3 is bound to chromatin, even at salt concentrations high enough to release histones from the chromatin (Yan *et al.*, 1993), and is released only by treatment with DNase. We followed up on these observations by further analysis of Mcm2 and Mcm3 present in distinct subcellular fractions. Asynchronous

Figure 4. Mcm2 and Mcm3 exhibit cell cycle stage-specific isoforms. Total proteins were isolated from logarithmic phase BJ2168 cells grown asynchronously (lanes in part 1), arrested in G_1 by the addition of $10 \mu\text{g/ml}$ α -factor (lanes in part 2), arrested in S phase by the addition of HU to 0.2 M (lanes in part 3), or arrested in M phase by the addition of NOC to 0.1 M (lanes in part 4). The isoform distributions of Mcm2 or Mcm3 were detected by immunoblots of 2D protein gels (B and D) and the mobility of Mcm2 and Mcm3 were detected by immunoblots of one-dimensional protein gels (A and C) with monospecific antibodies. Cells arrested at START (" G_1 ") by α -factor are arrested at a discrete point in G_1 phase, and cells arrested by HU (S) or by NOC (M) arrest throughout S or M phases, respectively, depending on the cell cycle position at the time of addition of the drug. All 2D gels were aligned by using chemiluminescence signals from protein aggregates that did not enter the first-dimension gel.

logarithmic phase BJ2168 cells were sequentially fractionated into four separate fractions, as shown (Figure 5A). The final volumes of the NS and NP fractions were equal, and the volume of the cytoplasmic frac-

tion was 10- to 15-fold larger. Equal volume aliquots of each of these fractions were analyzed on Western blots and proteins were detected using anti-Mcm2 or anti-Mcm3 antiserum (Figure 5B). The intensity of

Figure 5. Mcm2 and Mcm3 exist in specific subcellular fractions. (A) Yeast cell proteins were sequentially fractionated into several subcellular fractions following the scheme shown in the fractionation flow chart and as described in MATERIALS AND METHODS. (B) Immunoblots of resulting fractions isolated from an asynchronous population of BJ2168 cells. Equal volumes of each fraction were loaded on 4–20% protein gels and processed for immunodetection by anti-Mcm2, anti-Mcm3, anti-PGM, or anti-H2B antibodies, as shown. With the methods described herein, we obtained cytoplasmic fraction volumes of 1.5 ml, and 100 μ l of protein extract were obtained from each of the other fractions.

Mcm2 and Mcm3 in cytoplasmic (lanes 1), NS–0.5 M (lanes 2), and NP fractions (lanes 4) was approximately equal, but Mcm2 and Mcm3 were undetectable in the NS–2.0 M fraction (lanes 3). As controls for the fractionation, we also assayed each fraction for the presence of PGM, which is predominately a cytoplasmic protein (Chen and Tye, 1995), and histone H2B, as a marker for chromatin-associated proteins. The high-speed spin that we used to separate the nuclei from the cytoplasmic fraction will also pellet other organelles. However, there is no evidence for Mcm proteins being present in any organelle other than the nucleus, and we consider it unlikely that a significant portion of Mcm2 or Mcm3 is found in other organelles. Therefore, we are confident that the NS frac-

tion represents nuclear Mcm proteins. In summary, we believe that at least two forms of Mcm2 and Mcm3 exist in the nucleus: a readily extractable form, as seen in lane 2, and a tightly chromatin-associated form that is released upon treatment of the fraction with DNase, as seen in lane 4.

Mcm2 and Mcm3 Are Constitutively Present in Both the Cytoplasm and Nucleus but Bind Chromatin in a Cell Cycle-specific Manner

After establishing that nuclear Mcm2 and Mcm3 are present in two distinct populations, we wanted to determine as precisely as possible when during the cell cycle these different forms are found. We ar-

rested yeast cells at defined stages of the cell cycle with specific blocking reagents or by *cdc* mutations, then fractionated the arrested cultures into cytosolic, nucleosolic, chromatin-bound fractions, and analyzed Mcm2 and Mcm3 by Western blotting. In all of these experiments, PGM, which is mostly cytoplasmic (Chen and Tye, 1995), and histone H2B, which is associated with chromatin and resistant to 0.5 M salt wash, were used as markers to monitor the purity and the yield of our cell fractions (Figure 6 and 7, C and D). Our concern during fractionation was in minimizing contamination of the soluble nuclear fraction with cytoplasmic protein. Hence, we checked our fractions with PGM antibody to verify that the Mcm proteins detected in the soluble nuclear fractions were not cytoplasmic in origin. We were less concerned with leakage from the nucleus to the cytoplasm, because it was clear that a large portion of Mcm proteins reside in the cytoplasm and loss of a portion of the nucleosolic protein to the cytosolic fraction would not affect our findings. Likewise, we used histone H2B as a marker for chromatin-bound protein, not as a general nuclear protein, to verify that we were indeed recovering chromatin-associated proteins after DNase treatment.

Using specific blocking reagents, cells were arrested in G₁ at START by incubation in medium containing α -factor, at the S phase boundary by release from α -factor arrest into HU-containing medium, and in early M phase by release from α -factor arrest into NOC-containing medium. The arrest morphology of these cells and the predominant subcellular locations of Mcm2 and Mcm3 in these cells were verified by indirect IF microscopy (Figure 6, E and F). In α -factor-arrested G₁ cells, all of the cells were single and unbudded with intense staining of the nuclei by Mcm2 (Figure 6E, top) or Mcm3 (Figure 6F, top) specific antibodies. In cells arrested by α -factor, released from the α -factor block, and then arrested by HU at the G₁-S phase boundary, about 80% of the cells were small budded and 20% unbudded, but the nuclei of all of these cells were intensely stained by Mcm2 (Figure 6E, middle) or Mcm3 (Figure 6F, middle) specific antibodies. These data extend the previous IF results by showing the presence of Mcm2 and Mcm3 in the nucleus at the earliest HU-sensitive point, which is generally considered the beginning of S phase. NOC-arrested M phase cells were large budded with dividing or divided nuclei located at the bud neck. Because NOC causes disassembly of microtubules, it was not possible to determine the exact point in M phase that these cells are arrested from spindle fiber morphology. However, the position of the nuclei at the bud neck suggests that the arrest occurred at metaphase or an early stage of mitosis. In these cells, homogeneous staining of cytoplasm and nuclei by Mcm2

(Figure 6E, bottom) and Mcm3 (Figure 6F, bottom) specific antibodies was observed.

Analysis of proteins after cell fractionation indicated that both Mcm2 and Mcm3 are present in the cytoplasm (C) and nucleoplasm (NS) at all stages of the cell cycle (Figure 6, A, lanes 1, 4, and 7; and B, lanes 2, 5, and 8). The abundance of these proteins in the two compartments did not seem to vary significantly in G₁, G₁-S, or early M phase. Mcm2 and Mcm3 were found tightly bound to chromatin in α -factor-arrested cells (Figure 6, A and B, lanes 3). However, as the cell cycle progresses from G₁ to S to M phase, the amounts of chromatin bound (NP) Mcm2 and Mcm3 decreased in abundance. In G₁ (α -factor arrested) cells, Mcm2 and Mcm3 were always readily detected in the chromatin bound (NP) fraction (Figure 6, A and B, lanes 3). In cells that were released from factor block into HU (G₁-S phase boundary), the amount of Mcm2 and Mcm3 in the NP fractions decreased (Figure 6, A and B, lanes 6), and in NOC-arrested cells (G₂-M phase), they were no longer detectable in the NP fraction (Figure 6, A and B, lanes 9).

To identify more precisely the points when Mcm2 and Mcm3 become associated with and dissociated from chromatin, yeast cells were arrested at G₁, S, and late M phase of the cell cycle by using well-characterized *cdc* mutations. For G₁ arrest, we used *cdc28-1*, which is defective in the p34 kinase whose function in G₁ phase defines START (Reed, 1992). For late S-G₂ phase arrest, we used *cdc9* or *cdc17*, which are defective in DNA ligase and DNA polymerase I, respectively. These enzymes are involved in the elongation phase of DNA replication. For late M phase arrest, we used *dbf2*, which encodes a protein kinase that has an execution point at the anaphase-telophase portion of M phase (Toyn and Johnston, 1994). IF microscopy confirmed that nuclear staining for Mcm2 and Mcm3 was more intense in G₁ and late M phase cells than in S phase and early M phase cells (Figures 6 and 7, E and F).

At these defined cell cycle stages, no significant variations in the abundance of Mcm2 or Mcm3 were observed in the cytosolic (C) or nucleosolic (NS) fractions (Figure 7, A and B, compare lanes 1, 4, and 7 with lanes 2, 5, and 8). In contrast, chromatin-associated Mcm2 and Mcm3 were readily detectable only in cells arrested in G₁ (Figure 7, A and B, lanes 3) and late M phase (lanes 9) but not in late S-G₂ phase (lanes 6). The data in Figures 6 and 7 show that, although Mcm2 and Mcm3 were constitutively present in the cytoplasm and nucleus, their association with chromatin occurred in a cell cycle-specific manner, at anaphase-telophase. The association with chromatin persisted through G₁ phase and then terminated as cells progressed through S phase.

Figure 6. Mcm2 and Mcm3 are present in the cytoplasmic and nucleosolic fractions at specific points of the cell cycle. (A–D) Immunoblots of Mcm2, Mcm3, histone H2B, and PGM present in fractions (C, cytoplasmic; NS, nuclear soluble; NP, nuclear pellet) isolated from cells arrested with factor, from cells arrested with α -factor and released to medium containing 0.2 M HU, and from cells arrested with α -factor and released to medium containing 0.1 M NOC. (E and F) IF of Mcm2 (E) and Mcm3 (F) in cells arrested by the addition of α -factor, α -factor to HU, and α -factor to NOC. Four views of each IF field are shown: Nomarski optics, DAPI staining of DNA, tubulin staining, and Mcm2 or Mcm3 staining.

DISCUSSION

Mcm proteins have been shown to be highly abundant proteins in actively growing tissues of *Drosophila*, *Arabidopsis*, and *Zea mays* but to be present in low abun-

dance in differentiated tissues (Springer *et al.*, 1995; Treisman *et al.*, 1995; Sabelli *et al.*, 1996). Studies in mouse and HeLa cells showed that although the Mcm proteins are constitutively present in the nucleus, they

Figure 7. Mcm2 and Mcm3 are present in the cytoplasmic and nucleosolic fractions at defined cell cycle arrest points. (A–D) Immunoblots of Mcm2, Mcm3, histone H2B, and PGM present in fractions (C, cytoplasmic; NS, nuclear soluble; NP, nuclear pellet) isolated from cells arrested by shifting cultures to the nonpermissive temperature for *cdc28-1*, *cdc9-1*, and *dbf2-1*. (E and F) IF of Mcm2 (E) and Mcm3 (F) in cells arrested by shifting cultures to the nonpermissive temperature for *cdc28-1*, *cdc9-1*, and *dbf2-1*. Four views of each IF field are shown: Nomarski optics, DAPI staining of DNA, tubulin staining, and Mcm2 or Mcm3 staining.

alternate between subnuclear locations on chromatin and in the nucleoplasm in a cell cycle-specific manner

(Kubota *et al.*, 1995; Todorov *et al.*, 1995). In vivo labeling experiments showed that these proteins are

phosphoproteins that exhibit differential extractability from the nuclear pellet fraction at different cell cycle stages (Todorov *et al.*, 1995). These results suggest that the cell cycle-specific functions of the Mcm proteins in the initiation of DNA replication may be regulated by phospho-modifications.

The *S. cerevisiae* Mcm proteins are abundant proteins essential for the initiation of DNA synthesis at replication origins. These proteins were previously shown to have cell cycle-specific nuclear immunostaining, when assayed by IF microscopy. These observations led to the speculation that, unlike other eukaryotes, the functions of the *S. cerevisiae* Mcm proteins might be regulated by periodic nuclear import. In the work presented herein, we showed that the properties and behavior of the *S. cerevisiae* Mcm2 and Mcm3 proteins are very similar to those of higher eukaryotes. The Mcm3 protein is present in high abundance and at relatively constant levels throughout the cell cycle in actively proliferating cells. We showed that Mcm3, and most likely Mcm2 as well, are phosphoproteins and that modification of these proteins occurs in a cell cycle-specific manner. Furthermore, we showed that Mcm2 and Mcm3 are present in both the cytoplasm and the nucleus throughout the cell cycle, but they are bound to chromatin only during the period from late M phase to early S phase. Our findings emphasize the ubiquitous properties and functions of the Mcm proteins in all eukaryotes.

Indirect IF Microscopy versus Cell Fractionation Studies of Mcm2 and Mcm3

Previous studies in *S. cerevisiae* using IF microscopy, which detects the relative intensities of nuclear and cytoplasmic Mcm proteins by immunostaining, suggested that these proteins accumulate in the nucleus at late M phase and then abruptly disappear from the nucleus as cells enter S phase (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995). Improved methodology in cell fractionation enabled us to show that both Mcm2 and Mcm3 are present in the nucleus and in the cytoplasm throughout the cell cycle (Figures 6 and 7). The notion that the Mcm proteins are exclusively nuclear or cytoplasmic during different portions of the cell cycle (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995) needs to be reevaluated. Our data (Figures 6 and 7) do not contradict previous IF results. Indeed, we obtained similar patterns of fluctuations in cell cycle-specific nuclear immunostaining for Mcm2 and Mcm3, when we used indirect IF microscopy. However, direct measurements of the Mcm proteins by *in vitro* biochemical analysis enabled us to show the constitutive presence of Mcm2 and Mcm3 in both cytosolic and nucleosolic fractions. Through the course of these studies, we noted that cytosolic fractions are particularly suscep-

tible to proteolysis from endogeneous proteases and from the high level of proteases present in zymolyase enzyme preparations often used to remove the cell walls prior to Dounce homogenization. Thus, it is of critical importance to minimize proteolysis during fractionation to get a true picture of the presence or absence of proteins in each fraction and to use internal controls to verify the integrity of the fractions.

The cell cycle-specific changes in the relative intensity of staining of nuclear and cytoplasmic Mcm proteins, as seen by indirect IF, may be due to changes in the relative concentrations of nuclear and cytoplasmic Mcm proteins at these critical points. Because the nucleus represents less than 10% of the total cellular volume in yeast, slight influxes from the cytoplasm can bring about large increases in nuclear concentrations. These changes could be effected by either cell cycle-specific nuclear import or by cell cycle-specific nuclear retention (Schmidt-Zachmann *et al.*, 1993) of the Mcm proteins. An alternative explanation for the apparent discrepancies between the IF results and cell fractionation results is that conformational changes in the Mcm proteins may affect the accessibility of epitopes and hence the intensity of indirect IF staining at critical points of the cell cycle. Conformational changes or masking of protein domains induced by phospho-modification (Kimura *et al.*, 1996), DNA binding (Tan and Richmond, 1990), protein-protein interaction, and proteolytic cleavage (Henkel *et al.*, 1992) have been reported for a number of regulatory proteins.

Temporal Correlation between Landmark Cell Cycle Events in DNA Replication with the Phosphorylation States and Subcellular Locations of Mcm2 and Mcm3

Although Mcm2 and Mcm3 are constitutively present in the nucleus and in the cytoplasm at relatively constant levels, they exist in many different isoforms depending on the cell cycle stage. Analysis of Mcm3 in 2D protein gels indicated that Mcm3 undergoes phosphorylation or dephosphorylation at each cell cycle stage, giving rise to specific isoforms distinguishable by their isoelectric points. Most notable was the total conversion of Mcm3 from relatively acidic isoforms in M phase cells to more basic isoforms in G₁ cells and then back to more acidic isoforms as cells progress through S phase (Figure 4). More important, conversion of one set of isoforms to another is concurrent with landmark events of the cell cycle (Figure 8). Changes in the phosphorylation states of Mcm3 were observed between M phase and G₁ phase, during S phase when replication occurs and chromatin must be modified to prevent reinitiation, and during M phase when sister chromatids are separated and then mother and daughter cells separate.

Figure 8. Summary of various aspects of Mcm2 and Mcm3 behavior throughout the cell cycle. (A) Arrest points used in this work and the approximate point during each cell cycle stage where those arrests occur. (B) Cartoon of cell morphology at different cell cycle stages. Darker shading of nuclei indicates points when Mcm2 and Mcm3 nuclear immunostaining, as seen when using indirect IF microscopy, is pronounced and the light lines indicate points when Mcm2 and Mcm3 are bound to chromatin. (C) Graphic representation of the cycles of Mcm2 and Mcm3 chromatin binding (dashed line) and phosphorylation (solid line).

Interestingly, the association of the Mcm2 and Mcm3 proteins with chromatin also follows this timetable (Figure 8). At late M phase, both Mcm2 and Mcm3 become tightly associated with chromatin, which is defined by its association with histone and sensitivity to DNase I. Our studies indicate that the Mcm proteins bind chromatin at a specific point of the cell cycle—after the NOC block but before the *dbf2* block at the anaphase–telophase portion of the cell cycle. The Mcm proteins remain associated with chromatin throughout G₁ phase. Dissociation of the Mcm proteins from chromatin occurs as cells enter S phase. The correlation between the events that coordinate DNA replication initiation and the binding of Mcm proteins to chromatin are consistent with the involvement of the Mcm proteins in the assembly of the prereplication initiation complex that precedes DNA replication initiation in yeast.

Indeed, our previous work showed that Mcm3 interacts with itself and with other Mcm proteins to form multimeric complexes (Lei *et al.*, 1996). Mcm3 is less phosphorylated when in larger complexes than when present as monomers or in dimeric complexes. Herein, we show that Mcm3 is less phosphorylated in G₁ cells than in S or M phase cells. Thus, our results suggest a role for phospho-modification in regulating the assembly of Mcm complexes throughout the cell cycle. Our finding that only a minor fraction of the total Mcm proteins is chromatin bound suggests that only a fraction of these proteins is directly involved in DNA replication. This idea is supported by the low abundance of these proteins in slowly dividing cells. These observations may explain the limiting nature of these apparently abundant proteins (Lei *et al.*, 1996; Kawasaki and Tye, unpublished results).

From these results, we envision complicated cycles of phosphorylation and dephosphorylation of Mcm3, that may involve multiple protein kinases and phosphatases, to spatially and temporally regulate the function of Mcm3. A number of protein kinases have been impli-

cated in regulating the initiation of DNA synthesis in yeast. These include the Cdc7/Dbf4 kinase (Kitada *et al.*, 1992) and the cyclin-dependent Cdc28 kinase (Reed *et al.*, 1985). Indeed, we have recently shown that Mcm3 is apparently phosphorylated at several Cdc28 kinase recognition sites adjacent to its nuclear localization sequence and that Mcm proteins are in vitro as well as in vivo substrates of the Cdc7/Dbf4 protein kinase (Lei *et al.*, unpublished results). Furthermore, we have identified a suppressor of a temperature-sensitive allele of *MCM3*, *mcm3-10*, as *PTC2*, a protein phosphatase (Roberts and Tye, unpublished results). Our studies of the phosphorylation and dephosphorylation are ongoing, but the complexity of modifications, as revealed by the cell cycle-specific isoforms of Mcm3, will require extensive effort to fully understand.

The Complex Roles of Mcm Proteins

Although this study focuses mainly on Mcm3 and to a lesser extent on Mcm2, our findings pertaining to these proteins, if extrapolated to other members of the Mcm2–7 family, portray a complex picture for the regulation of DNA replication in eukaryotes. The Mcm proteins have ubiquitous properties in all eukaryotes studied so far. They are a family of six proteins that interact to form complexes with one another and with other proteins. They are abundant proteins essential for the initiation of DNA synthesis. Although constitutively present in the nucleus, they are tightly associated with G₁ but not G₂ chromatin (Kimura *et al.*, 1994; Todorov *et al.*, 1995). The existence of these proteins in multiple phospho-isoforms suggests a tightly orchestrated sequence of phosphorylation and dephosphorylation events that regulate their cell cycle-specific functions. Such functions involve cycles of complex assembly and disassembly and chromatin association and dissociation, consistent with the notion that these proteins may be components of the prereplication complex. Other cell cycle-regulated

functions for the Mcm proteins, such as chromatin decondensation and transcriptional regulation, have also been proposed. Our findings highlight the potential difficulties for biochemical analysis of these proteins if specific activities require the proper phosphorylation of each subunit of the Mcm complex. Until the active forms of the Mcms are known, in vitro analysis will remain a difficult task.

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