

Different Phenotypes *in Vivo* Are Associated With ATPase Motif Mutations in *Schizosaccharomyces pombe* Minichromosome Maintenance Proteins

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ABSTRACT

The six conserved MCM proteins are essential for normal DNA replication. They share a central core of homology that contains sequences related to DNA-dependent and AAA⁺ ATPases. It has been suggested that the MCMs form a replicative helicase because a hexameric subcomplex formed by MCM4, -6, and -7 proteins has *in vitro* DNA helicase activity. To test whether ATPase and helicase activities are required for MCM protein function *in vivo*, we mutated conserved residues in the Walker A and Walker B motifs of MCM4, -6, and -7 and determined that equivalent mutations in these three proteins have different *in vivo* effects in fission yeast. Some mutations reported to abolish the *in vitro* helicase activity of the mouse MCM4/6/7 subcomplex do not affect the *in vivo* function of fission yeast MCM complex. Mutations of consensus CDK sites in Mcm4p and Mcm7p also have no phenotypic consequences. Co-immunoprecipitation analyses and *in situ* chromatin-binding experiments were used to study the ability of the mutant Mcm4ps to associate with the other MCMs, localize to the nucleus, and bind to chromatin. We conclude that the role of ATP binding and hydrolysis is different for different MCM subunits.

THE minichromosome maintenance (MCM) proteins were originally identified in a genetic screen for budding yeast mutants that were defective for minichromosome maintenance (MAINE *et al.* 1984) or cell cycle progression (MOIR and BOTSTEIN 1982; KEARSEY *et al.* 1996). The MCM family has six members (Mcm2p through Mcm7p), which are highly conserved in all eukaryotes (reviewed in PASION and FORSBURG 2001). Each of the MCM proteins is essential for initiation and elongation of DNA replication (reviewed in LABIB *et al.* 2000; PASION and FORSBURG 2001). These proteins share a central homology domain of ~200 amino acids containing motifs characteristic of DNA-dependent and AAA⁺ ATPases, including both Walker A and B motifs (WALKER *et al.* 1982; KOONIN 1993; NEUWALD *et al.* 1999). Biochemical studies indicate that the MCM complex is formed by stoichiometric assembly of all six MCM proteins. However, the purification of subcomplexes by glycerol gradients and co-immunoprecipitation studies suggests that the complex contains a tightly associated core formed by Mcm4p, -6, and -7, which is loosely bound by Mcm2p and the strongly associated Mcm3p-Mcm5p dimer (reviewed in LABIB and DIFFLEY 2001; PASION and FORSBURG 2001).

Interestingly, the intact MCM complex does not have any detectable biochemical activity (ADACHI *et al.* 1997; ISHIMI *et al.* 1998; LEE and HURWITZ 2000; SATO *et al.* 2000). However, a dimer formed by the trimer subcom-

plex Mcm4/6/7p possesses *in vitro* single-stranded DNA and ATP-binding activities as well as ssDNA-dependent ATPase and 3' to 5' DNA helicase activities (ISHIMI *et al.* 1998, 2000; LEE and HURWITZ 2000, 2001). Mutational analysis within the Walker A and B motifs of mouse Mcm4p and Mcm6p has demonstrated the importance of these domains for the *in vitro* DNA helicase activity of the hexameric Mcm4/6/7p complex (YOU *et al.* 1999).

The Walker A motif has the consensus sequence (G)xxxxGK[T/S], including the invariant lysine residue found in all ATP-binding proteins. The Walker A motif sequence in the MCMs contains a slight variation from the consensus, where the glycines in the GK(S/T) signature are substituted by alanine or serine (KOONIN 1993). On the basis of studies of known ATPases, it is suggested that the lysine in the A motif directly interacts with the phosphatyl group of bound ATP (WALKER *et al.* 1982). The B motif typically has a hydrophobic structure D[D/E]. Apparently the common denominator for all variants of the B motif is at least one negatively charged residue preceded by a stretch of bulky hydrophobic residues predicted to form a β -strand (KOONIN 1993). The acidic residues within this motif play an essential role in coordinating the magnesium ion and, probably, the water molecule that attacks the β - γ bond of the ATP. Consistent with this hypothesis, it is postulated that motif B is involved in ATP hydrolysis rather than ATP binding (PAUSE and SONENBERG 1992; BROSH and MATSON 1995).

Electron microscopy studies showed that the hexameric Mcm4/6/7p complex can actually form toroidal structures with a central cavity, comparable in size and

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shape to known hexameric DNA helicases (SATO *et al.* 2000). The MCMs are thus unique among hexameric helicases, which generally are homomeric rather than heteromeric (reviewed in PATEL and PICHA 2000). Interestingly, Archea *Methanobacterium thermoautotrophicum* contains one MCM-related protein that forms a double hexamer and possesses *in vitro* 3' to 5' DNA helicase activity (KELMAN *et al.* 1999; CHONG *et al.* 2000; SHECHTER *et al.* 2000). These findings and the fact that the MCMs are necessary not only for the initiation of DNA replication but also for the elongation of replication forks suggest that the MCM complex or the core subcomplex might actually be the eukaryotic replicative fork helicase similar to bacterial DnaB protein or simian virus 40 (SV40) large T antigen (T Ag; reviewed in BAKER and BELL 1998; TYE and SAWYER 2000; LABIB and DIFFLEY 2001).

To better understand the *in vivo* role of ATP-binding and hydrolysis motifs and to establish the importance of cyclin-dependent kinase (CDK) phosphorylation in the function of the MCM complex, we constructed mutations in the conserved Walker A and B motifs and the putative CDK phosphorylation sites of core MCMs. We show that equivalent mutations in the A and B motifs of Mcm4, -6, and -7 proteins have different *in vivo* effects, suggesting that the role of ATP binding and hydrolysis in the function of each core MCM protein is different. Both motifs in Mcm4p are essential, while in Mcm6p they are dispensable. Mcm7p shows an intermediate phenotype: Only a conservative substitution of the crucial lysine in the A motif resulted in a functional protein. We found that not all equivalent mutations that abolished the *in vitro* helicase activity of the mouse hexameric Mcm4/6/7p subcomplex (YOU *et al.* 1999) affect the *in vivo* function of the fission yeast MCM complex. Analysis of the Mcm4 mutant proteins showed different defects in MCM complex assembly, nuclear localization, or chromatin binding. Finally, mutations of the two putative CDK phosphorylation sites in Mcm4p and the single consensus site in Mcm7p had no effect upon the normal growth of cells, suggesting that these sites are not crucial for the regulation of MCM function.

MATERIALS AND METHODS

Strains and manipulation: Strains used in this study are listed in Table 1. Fission yeast strains were grown in yeast extract plus supplements (YES) or in Edinburgh minimal medium (EMM) with appropriate supplements as described (MORENO *et al.* 1991). In this study "wild type" refers to strain FY254. Transformations were carried out by electroporation (KELLY *et al.* 1993). Unless indicated, yeast plasmids used in this work contain the *ura4⁺* marker. The full-strength *nmtI⁺* promoter was used (BASI *et al.* 1993). When studying complementation using constructs with the *nmt* promoter, transformants were selected on plates containing 15 μ M thiamine (fully repressed). Different levels of induction in liquid culture were obtained by washing the cells twice with 10 ml of EMM followed by the addition of the indicated thiamine concentrations (JAVERZAT *et al.* 1996). Overexpression experiments in liquid cul-

ture were carried out for a maximum of 23 hr following the removal of thiamine from the media. Overexpression on plates was done by streaking individual colonies on media lacking thiamine and incubating for 3–5 days at 32°. Vectors without inserts were used as negative controls.

Complementation analyses of temperature-sensitive strains were carried out by streaking at least four independent colonies of each transformation on EMM plates with appropriate supplements and incubating the cells at 36° for 3–5 days. When testing complementation using the *nmt* promoter, colonies were streaked on media with three different concentrations of thiamine: 15 μ M thiamine (+ thiamine, maximum repression), 0.05 μ M thiamine (low thiamine, medium induction), and no thiamine (– thiamine, maximum induction).

Complementation of null strains was carried out using diploid strains containing one allele of the *mcm4⁺*, *mcm6⁺*, or *mcm7⁺* genes disrupted with *his3⁺* or *ura4⁺*. These strains were transformed with *ura4⁺* or *leu1⁺* plasmids. Transformants were selected on EMM plates lacking uracil and histidine. At least two independent transformed diploids for each transformation were induced to undergo meiosis, which was followed by a random spore analysis. The spore preparations were plated to identify *Ura⁺*, *His⁺* colonies, which were analyzed by FACS to determine DNA content. Plasmids were rescued from haploid strains and analyzed by restriction mapping or sequencing to confirm the presence of the mutation.

For construction of strains FY1602, FY1603, FY1604, and FY1605 (Table 1), plasmids pEBG56 (pJK148 *nmt-mcm4HA leu1⁺*), pEBG57 (pJK148 *nmt-mcm4D* → AHA *leu1⁺*), pEBG58 (pJK148 *nmt-mcm4K* → AHA *leu1⁺*), and pEBG59 (pJK148 *nmt-mcm4K* → RHA *leu1⁺*) were linearized with *NruI* within the *leu1⁺* sequence and integrated into the haploid strain FY254. *Leu⁺* transformants were selected and the integration event was confirmed by streaking and replica plating the cells on YES media. Expression of wild-type and mutant Mcm4-HA proteins was verified by immunoblot.

Protein extracts, immunoblotting, and immunoprecipitations: Cell lysates were prepared by glass bead lysis according to MORENO *et al.* (1991) in lysis buffer (50 mM HEPES, pH 7.0, 50 mM KCl, 5 mM MgAc, 100 mM sorbitol, 0.1% Triton X-100) with the addition of 1 mM dithiothreitol (DTT), 1 mM ATP, and protease inhibitors. Lysates were cleared by spinning at 16,000 \times g for 20 min at 4°. Total protein concentrations were determined by BCA protein assay (Pierce Chemical, Rockford, IL). For immunoblot analyses, samples were fractionated by 7% SDS-PAGE and transferred to Immobilon-P (Millipore, Bedford, MA). Antibodies to Mcm6p (serum 5899), Mcm5p (serum 5897), and Mcm4p (serum 5898) were previously described in SHERMAN *et al.* (1998); to Mcm2p (serum 5616) in FORSBURG *et al.* (1997); to Mcm3p (serum 6178) in SHERMAN and FORSBURG (1998); and to Mcm7p (serum 6184) in LIANG and FORSBURG (2001). Monoclonal anti-hemagglutinin (HA) 12CA5 antibody was a kind gift of Tony Hunter, and monoclonal anti- α -tubulin antibody was purchased from Sigma (St. Louis; T5168). Detection was carried out using anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Sigma or Jackson ImmunoResearch Laboratories, West Grove, PA) and enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Immunoprecipitations were performed with 600 μ g of total protein at a concentration of 1 μ g/ μ l and 1 μ l of 12CA5 antibody, for 4 hr at 4°. Fifty microliters of Protein A-Sepharose CL-4B (Sigma; 1:1 in lysis buffer) were added and incubated for 1–2 hr at 4°. Pellets were washed four times with 1 ml of cold lysis buffer and boiled after the addition of 80 μ l of 2 \times sample buffer. Ten microliters per lane of each immunoprecipitation were loaded on a SDS-PAGE.

***In situ* chromatin-binding assay and immunofluorescence analysis:** An *in situ* chromatin-binding assay was adapted from the protocol described in KEARSEY *et al.* (2000) as follows.

TABLE 1
Strains

Strain	Genotype	Source
FY254	<i>h⁻ can1-1 ura4-D18 leu1-32 ade6-M210</i>	Our stock
FY784	<i>h⁺ cdc21-M68 ura4-D18 leu1-32 ade6-M216</i>	Our stock
FY786	<i>h⁻ cdc21-M68 ura4-D18 leu1-32 ade6-M216</i>	Our stock
FY793	<i>h⁻ orp1HA ura4-D18 leu1-32 ade6-M210</i>	Our stock
FY798	<i>h⁻ Δcdc19::[cdc19HA::leu1⁺] ura4-D18 leu1-32 ade6-M210</i>	Our stock
FY856	<i>h⁺/h⁻ Δmis5::his3⁺/+ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6/M210/ade6-M216 his3-D1/his3-D1</i>	LIANG <i>et al.</i> (1999)
FY857	<i>h⁺/h⁻ Δcdc21::his3⁺/+ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 his3-D1/his3-D1</i>	LIANG <i>et al.</i> (1999)
FY918	<i>h⁺/h⁻ Δmcm7::his3⁺/+ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210 his3-D1/his3-D1</i>	LIANG and FORSBURG (2001)
FY961	<i>h⁺ mis5-268 ura4-D18 leu1-32 ade6-M210 can1-1</i>	Our stock
FY1199	<i>h⁻ mcm7-98 ura4-D18 leu1-32 ade6-M216</i>	LIANG and FORSBURG (2001)
FY1602	<i>h⁻ ura4-D18 leu1-32::[pJK148nmt-mcm4HA leu1⁺] ade6-M210 can1-1</i>	This work
FY1603	<i>h⁻ ura4-D18 leu1-32::[pJK148nmt-mcm4DAHA leu1⁺] ade6-M210 can1-1</i>	This work
FY1604	<i>h⁻ ura4-D18 leu1-32::[pJK148nmt-mcm4KAHA leu1⁺] ade6-M210 can1-1</i>	This work
FY1605	<i>h⁻ ura4-D18 leu1-32::[pJK148nmt-mcm4KRHA leu1⁺] ade6-M210 can1-1</i>	This work
FY1718	<i>h⁻ Δmcm4::ura4⁺ ura4-D18 leu1-32::[pJK148P_{mcm4}- mcm4HA leu1⁺] ade6-M210 can1-1</i>	This work

After the Zymolyase treatment, cells were washed with cold STOP buffer as indicated KEARSEY *et al.* (2000) and once with 10 ml of PEMS buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM MgSO₄, 1.2 M sorbitol). Cells were resuspended in 4 ml of PEMS + protease inhibitors and split in two. One-tenth volume of PEMS + 10% Triton X-100 was added to one of the tubes and 1/10 volume of PEMS + 0.25% Triton X-100 was added to the other. After an incubation for 10 min at room temperature, the cells were spun down and resuspended in 20 ml of FIX solution (0.1 M K-phosphate, pH 6.5, 10% methanol, 3.7% formaldehyde) for 20 min at room temperature. After pelleting cells and washing once in 10 ml of PEMS, the cells were blocked in 1 ml PEMBAL buffer (1× PEM, 1% BSA, 0.1% NaN₃, 100 mM L-lysine monohydrochloride) for 30 min at room temperature. Cells were incubated with monoclonal anti-HA 16B12 antibody (BabCO) overnight at room temperature at a 1:5000 dilution. After three 1-ml washes with PEMBAL the cells were incubated with Cy3-conjugated donkey anti-mouse Ig-G antibody (no. 715-165-150; Jackson Immuno-Research Laboratories) for 1 hr at room temperature in the dark at 1:500 dilution. Following washes with PEMBAL, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and heat fixed onto microscope slides. Microscopy was performed with a Leitz Laborlux S microscope or a Leica DMR microscope. Images were captured with a SPOT2 charge-coupled device digital camera or a Hamamatsu digital camera directly into Adobe Photoshop or Improvisation Openlab, respectively.

Flow cytometry: Cells were fixed in ice-cold 70% ethanol and stained for flow cytometry as described previously (SAZER and NURSE 1994) except that the cells were stained in a final concentration of 1 μM Sytox Green (Molecular Probes, Eugene, OR). Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA), and data analysis was carried out using Cell Quest software for Macintosh.

Plasmid and mutant construction: Plasmid features are described in Table 2. Nucleotide changes were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The constructs were sequenced to confirm the mutations and that PCR mutagenesis introduced no extra changes. Details of plasmid construction are available upon request.

Kinase assays: To purify substrate protein, 500-ml cultures of BL21(DE3)pLysS *Escherichia coli* (Invitrogen, San Diego) transformed with pMGC28 or pMGC29 were induced to express protein, and then his-tagged N terminus Mcm4 was purified under native conditions using Ni-NTA agarose (Qiagen, Valencia, CA) according to manufacturer's instructions.

For kinase assays, wild-type yeast (FY254) transformed with pSLF172-*cdc13*-HA (BROWN *et al.* 1997) was grown at 32° in EMM + supplements + thiamine and then washed and grown in EMM + low thiamine (0.05 μM thiamine) for 18 hr at 32°. Cell lysates were prepared by glass bead lysis in HB buffer (25 mM MOPS, pH 7.2, 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and protease inhibitors) as described above. To precipitate Cdc2/Cdc13-HA kinase, 0.5 μl 12CA5 antibody was added to 300 μg extract and rotated at 4° overnight. Fifty microliters Protein A-Sepharose CL-4B (Sigma; 1:1 in lysis buffer) was then added and rotated at 4° for 1 hr. Pellets were washed four times in HB buffer, and then all HB was removed and 20 μl kinase reaction buffer (5 μCi [γ-³²P]ATP and 25 μM cold ATP in HB buffer) was added. After addition of 2 μg substrate protein [histone H1 (Boehringer Mannheim, Indianapolis), N terminus Mcm4 wild type, or N terminus Mcm4 T15A T112A], reactions were incubated at 32° for 30 min and boiled after addition of 4 μl 5× sample buffer. Proteins were fractionated by 10% SDS-PAGE. The Coomassie-stained gel was scanned into Canvas 5 for Macintosh and ³²P incorporation was determined using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Rationale: Mutations of the crucial lysine in the Walker A motif of several ATPases disrupt ATP hydrolysis, abolishing biological activity (MA *et al.* 1994; SINGH and MAURIZI 1994; YAHRAUS *et al.* 1996). Interestingly, in some proteins, a conservative substitution of arginine for this lysine may not significantly affect nucleotide binding or nucleotide-dependent protein association,

TABLE 2
Plasmids

Plasmid name	Features	Source
pSLF172	<i>nmt</i> promoter, 3' HA cloning vector	Our stock
pTZ18mcm4	<i>pTZ18R-mcm4</i> (bacterial plasmid)	Our stock
pMGC32	<i>nmt-mcm4⁺-HA</i>	This work
pEBG4	<i>nmt-mcm4</i> D → A-HA	This work
pEBG5	<i>nmt-mcm4</i> K → A-HA	This work
pEBG6	<i>nmt-mcm4</i> K → R-HA	This work
pEBG7	<i>P_{mcm4}-mcm4</i> D → A-HA	This work
pEBG8	<i>P_{mcm4}-mcm4</i> K → A-HA	This work
pEBG9	<i>P_{mcm4}-mcm4</i> K → R-HA	This work
pEBG10	<i>P_{mcm4}-mcm4⁺-HA</i>	This work
pSLF191	<i>nmt-mcm4⁺</i>	S. L. FORSBURG (unpublished data)
pEBG69	<i>nmt-mcm4</i> D → A	This work
pEBG70	<i>nmt-mcm4</i> K → A	This work
pEBG71	<i>nmt-mcm4</i> K → R	This work
pMGC39	<i>P_{mcm4}-mcm4</i> 2T → 2A	This work
pMGC40	<i>P_{mcm4}-mcm4</i> 2T → 2S	This work
pMGC41	<i>P_{mcm4}-mcm4</i> 2T → 2E	This work
pMGC28	<i>pRSETA-mcm4</i> N terminus	This work
pMGC29	<i>pRSETA-mcm4</i> 2T → 2A N terminus	This work
pEBG36	<i>nmt-HA-mcm6⁺</i>	This work
pEBG37	<i>nmt-HA-mcm6</i> DE → AA	This work
pEBG38	<i>nmt-HA-mcm6</i> K → A	This work
pEBG39	<i>nmt-HA-mcm6</i> KS → AA	This work
pEBG60	<i>nmt-mcm6⁺-HA</i>	This work
pEBG64	<i>nmt-mcm6⁺</i>	This work
pEBG65	<i>nmt-mcm6</i> DE → AA	This work
pEBG66	<i>nmt-mcm6</i> K → A	This work
pEBG67	<i>nmt-mcm6</i> KS → AA	This work
pDTL87	<i>nmt-mcm7⁺-HA</i>	LIANG and FORSBURG (2001)
pEBG30	<i>P_{mcm7}-mcm7⁺</i>	This work
pEBG31	<i>P_{mcm7}-mcm7⁺-HA</i>	This work
pEBG40	<i>P_{mcm7}-mcm7</i> D → A	This work
pEBG41	<i>P_{mcm7}-mcm7</i> K → A	This work
pEBG42	<i>P_{mcm7}-mcm7</i> K → R	This work
pEBG43	<i>P_{mcm7}-mcm7</i> T → A	This work
pEBG44	<i>P_{mcm7}-mcm7</i> D → A-HA	This work
pEBG45	<i>P_{mcm7}-mcm7</i> K → A-HA	This work
pEBG46	<i>P_{mcm7}-mcm7</i> K → R-HA	This work
pEBG48	<i>nmt-HA-mcm7⁺</i>	This work
pEBG49	<i>nmt-HA-mcm7</i> D → A	This work
pEBG50	<i>nmt-HA-mcm7</i> K → A	This work
pEBG51	<i>nmt-HA-mcm7</i> K → R	This work

although it abrogates ATPase activity (SUNG *et al.* 1988; REHRAUER and KOWALCZYKOWSKI 1993; SUNG and STRATTON 1996). Previous data from our laboratory showed that when the invariant lysine present in the A motif of Mcm2p is substituted with arginine, this protein is able to complement both *mcm2-ts* and $\Delta mcm2$ strains, but a K to A change gives a nonfunctional protein, suggesting that it is unlikely that this Mcm2p domain functions as a *bona fide* ATPase (FORSBURG *et al.* 1997).

To determine the importance of the nucleotide-binding and hydrolysis motifs for the *in vivo* function of the MCM complex, we introduced mutations into the conserved Walker A and B domains of the core MCM

proteins. These sequences are shown in Figure 1. In particular, we made conservative K → R and nonconservative K → A mutations in the Walker A motif and D → A mutations in the Walker B motif. The mutant genes were expressed on plasmids and tested for complementation of the cognate *mcm-ts* and Δmcm mutations. Where possible, these results were correlated with complex assembly, nuclear localization, and chromatin binding by expressing epitope-tagged forms of the proteins in wild-type cells. However, in several cases the epitope tag caused additional phenotypes, preventing this analysis.

For clarity, we use the MCM designation for each of

		Walker A	
		**	
Mcm4p	546	GDP	STSKS
Mcm7p	404	GDP	GVAKS
Mcm6p	477	GDP	STSKS
Mcm2p	535	GDP	GTAKS
Mcm5p	372	GDP	GTAKS
Mcm3p	357	GDP	STAKS
consensus		Gxx	GxxKS

		Walker B	
		**	
Mcm4p	596	GALVLS	DGGIICCIDE
Mcm7p	454	GALVLA	DNGIICCIDE
Mcm6p	527	GALMSA	DNGIICAIDE
Mcm2p	585	GALVLA	DKGVCLIDE
Mcm5p	422	GAMVLA	DGGIVCIDE
Mcm3p	407	GAMVLA	DRGVVCLIDE
consensus		Gxxxxxxx	GxxxxDE

FIGURE 1.—Protein sequence alignment of the *S. pombe* MCM Walker A and B motifs. The MCM homology domains of Mcm2p through Mcm7p were aligned with the ClustalW 1.8 program and formatted with MacBoxShade v2.01 and Microsoft Word 98. The Walker A and B motifs alignment is shown. White text in black boxes indicates identical amino acids present in the six MCMs. Black text in gray boxes indicates identical amino acids present in four or more MCMs. Asterisks indicate mutated amino acids. Walker A and B motifs consensus sequences are shown. Numbers to the left of each sequence indicate position of first amino acid in the Walker motifs.

these genes. However, several are also known by other gene names in the literature: *mcm2*⁺ corresponds to *cdc19*⁺ and *nda1*⁺ (MIYAKE *et al.* 1993; FORSBURG and NURSE 1994); *mcm4*⁺ is *cdc21*⁺ (COXON *et al.* 1992); *mcm5*⁺ is *nda4*⁺ (MIYAKE *et al.* 1993); and *mcm6*⁺ is *mis5*⁺ (TAKAHASHI *et al.* 1994).

Mcm4p nucleotide-binding and hydrolysis motifs are essential for its function *in vivo*: The wild-type *mcm4*⁺ and mutant derivatives were cloned under the control of the *nmt* promoter with or without a 3' triple-HA epitope tag and under their own promoter with a 3' triple-HA epitope tag (Table 2 and MATERIALS AND METHODS). We verified that the wild-type Mcm4p clones were functional by ensuring that they could rescue both the temperature-sensitive and null alleles of *mcm4*⁺ (Table 1, Figure 2A, and data not shown). Next we tested if the mutant proteins Mcm4D → A, K → A, and K → R, with and without a C-terminal HA tag, were functional. None of the Mcm4 mutant proteins could rescue the *mcm4-M68* temperature-sensitive or null phenotype (Figure 2, A and B). When expressing the untagged Mcm4K → R protein, microcolonies with very elongated and sick cells were sometimes observed but could not be propagated in liquid media (data not shown). Taken together, these results indicate that conservative and nonconservative mutations in the Walker A motif, and the nonconservative change in the B motifs, give rise to nonfunctional Mcm4p proteins.

Single point mutations in the ATP-binding and hydrolysis motifs of Mcm6p do not affect the function of the protein: Equivalent mutations were constructed for the *mcm6*⁺ gene. In our lab it was previously shown that the protein expressed by pSLF225 (*nmt-HA-mcm6*⁺; FORSBURG *et al.* 1997) could rescue both the *mcm6* temperature-sensitive (*mcm6-268*; TAKAHASHI *et al.* 1994) and null alleles (SHERMAN *et al.* 1998). HA-*mcm6* mutant plasmids, derived from pSLF225, were constructed (Table 2).

Surprisingly, all of these changes gave rise to functional Mcm6 proteins; the three mutants could complement the *mcm6-ts* mutation and the *mcm6* null strain (FY857; Table 2).

Previous published results using baculovirus recombinant mouse MCM proteins showed that the double mutant Mcm6 K401A S402A or Mcm6 D459A E460A had significantly reduced or completely abolished helicase activity *in vitro* (YOU *et al.* 1999). Thus, we were surprised to find that single substitutions in the Walker motifs of Mcm6p gave functional *in vivo* proteins in *Schizosaccharomyces pombe*. To compare our *in vivo* results with the *in vitro* mutational analysis previously reported (YOU *et al.* 1999), we constructed the double mutants Mcm6p KS477/478AA and Mcm6p DE527/528AA (see Table 2) and tested their ability to complement *mcm6-ts* and null *mcm6* strain. Interestingly, the Walker B motif Mcm6DE → AA mutant was functional while the Walker A domain double mutant Mcm6KS → AA was not.

During the construction of the *mcm6* mutants we discovered that the plasmids used to make our mutant vectors (pTZ*mcm6* and pSLF225), which were constructed using the originally published sequence (TAKAHASHI *et al.* 1994), were missing 73 nucleotides on the basis of the sequence in the *S. pombe* genome database (accession no. AL139314). As a result, all our constructs also had a premature stop codon, resulting in a shorter but functional Mcm6p. The correct 3' end was introduced by PCR (Table 2). These revised constructs behaved the same as their counterparts lacking the last 73 nucleotides, suggesting that the last 29 amino acids are not required for Mcm6p activity.

Similar results were observed using plasmids without a tag under the *nmt* promoter (Figure 2, A and B). Complementation at 36° of the temperature-sensitive strain occurred with all three thiamine concentrations, indicating that overproduction of Mcm6p and the mutant derivatives is not toxic for the *mcm6-ts*. There was no deleterious phenotype associated with overproduction in wild-type cells (data not shown).

Unexpectedly, when the expression of the N-terminally tagged HA-Mcm6p was analyzed by Western blot we observed that an extremely high percentage of the Mcm6p-tagged protein lost its tag (data not shown). We constructed a C-terminally HA-tagged Mcm6p but this protein was unable to complement the null *mcm6* strain and could rescue the *mcm6-ts* defect only when overpro-

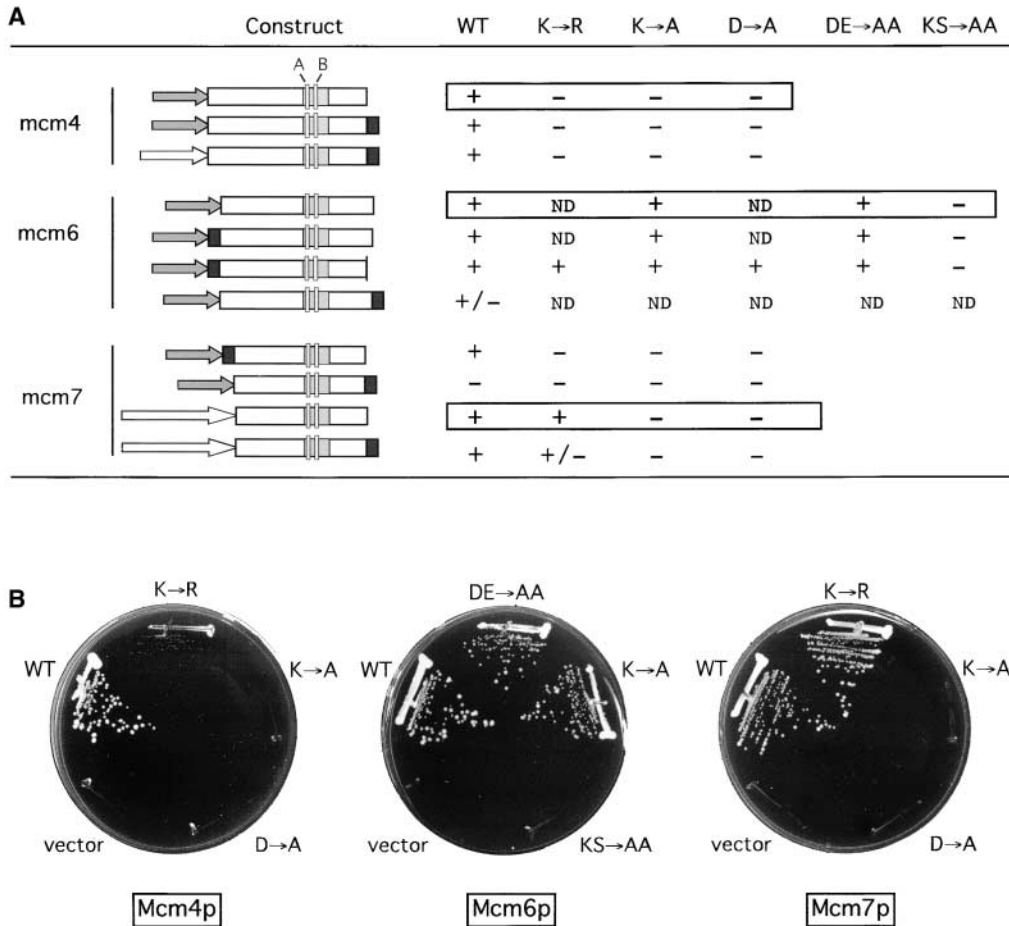


FIGURE 2.—Mcm4p, Mcm6p, and Mcm7p Walker A and B motifs mutational analysis. *mcm4⁺*, *mcm6⁺*, and *mcm7⁺* and the corresponding mutant derivatives were cloned in different expression vectors. (A) The complementation results. A schematic depiction of each construct is shown. Shaded arrows represent the *nmt* promoter. Open arrows symbolize endogenous promoters. Open rectangles stand for *mcm4* (Mcm4p), *mcm6* (Mcm6p), and *mcm7* (Mcm7p) genes as indicated. The vertical line at the end of the *mcm6* construct symbolizes the 3' truncated *mcm6* gene. Shaded boxes represent the MCM central homology domains, where the relative positions of the Walker A and B motifs are shown. The triple-HA epitope tags are represented by solid boxes. + and - signs indicate complementation and no complementation of the temperature-sensitive and null alleles, respectively. ND, not determined. +/- indicates complementation of the temperature-

sensitive strains and no complementation or partial complementation of the null alleles. DE → AA and KS → AA double mutants were tested for Mcm6p only. (B) Complementation results boxed in A. Plasmids with the untagged *mcm4⁺*, *mcm6⁺*, and respective mutant genes, under the control of the *nmt* promoter, were transformed into the *mcm4-ts* strain FY786 or *mcm6-ts* strain FY961, respectively. Isolated transformants were streaked on EMM + adenine + leucine + thiamine. Plasmids with the untagged *mcm7⁺* and mutant genes under the *mcm7* promoter were transformed into *mcm7-ts* strain FY1199, and the isolated transformants were streaked on EMM + adenine + leucine. Cells were grown at 36° for 2–3 days.

duced (Figure 2A, Table 2). Without a tag to distinguish wild-type and mutant proteins further analysis of the Mcm6p mutants was not possible.

Nonconservative mutations in the ATP-binding and hydrolysis motifs of Mcm7p abolish its activity *in vivo*: Similar mutations in the Walker A and B motif sequences were introduced in the *mcm7⁺* gene. Wild-type and mutant genes *mcm7D* → A, *mcm7K* → A, and *mcm7K* → R were cloned into the pUR19-*Hind*III vector (Table 2). These constructs included the complete *mcm7⁺* open reading frame plus 2549 nucleotides upstream and 540 nucleotides downstream, corresponding to the *mcm7⁺* promoter and its endogenous stop sequence, respectively. As shown in Figure 2, A and B, the wild-type Mcm7p as well as the Mcm7K → R mutant could rescue the *mcm7-98* temperature defect, while D → A and K → A could not. Equivalent results were obtained when testing the ability of these proteins to complement the null allele (Figure 2A). These results show that nonconservative changes in crucial residues in the A and B motifs abolish the function of Mcm7p.

To study in more detail which aspects of the Mcm7p function were affected by the mutations, we needed tagged versions of these proteins and an inducible system to control their expression. *mcm7⁺* under the control of the *nmt* promoter with a 3' triple-HA epitope tag could not complement the *mcm7-98* defect or the null strain (Figure 2A and LIANG and FORSBURG 2001). Curiously, as shown in LIANG and FORSBURG (2001), *mcm7HA* in the chromosome is functional, suggesting that the cells are very sensitive to precise levels of the Mcm7p protein or *mcm7* mRNA.

We constructed N-terminally tagged Mcm7p and found that the protein complemented the *mcm7-ts* strain and the null allele. However, in contrast to the untagged protein, HA-Mcm7K → R could not rescue the *mcm7-98* defect at 36° (Table 2), suggesting that the HA epitope negatively affects the activity of the Mcm7p K409R mutant. These results indicate that the HA tag is not neutral in Mcm7p.

In a final attempt to have tagged Mcm7p proteins that would behave as the untagged counterparts, we cloned

the *mcm7* derivatives with a 3' HA epitope tag under the control of the *mcm7⁺* promoter (Table 2). The wild-type Mcm7p-HA protein was able to rescue *mcm7-98* and the null allele, in contrast to the episomal *nmt-mcm7⁺*-HA. Since the only difference is the promoter, we suggest that the *mcm7⁺* gene may require some form of transcriptional regulation. However, the mutant Mcm7K → R-HA did not complement as well as the untagged version (Figure 2A). We conclude that Mcm7K409R is a functional protein that becomes defective when a triple-HA epitope is added at either end: a synthetic phenotype.

Interestingly, overexpression of the functional wild-type HA-Mcm7p from an episome or an integrated allele was toxic for normal cell growth and viability.

Analysis of MCM complex formation by Mcm4p mutants: To investigate the ability of mutant MCMs to form a complex or localize properly *in vivo*, the proteins must be expressed in a wild-type cell (to maintain viability), with an epitope tag (to distinguish it from the wild-type protein). Only in the case of Mcm4 was there no significant difference in the behavior of tagged and untagged proteins, so our protein analysis focused on this MCM subunit.

To analyze complex assembly, we used a co-immunoprecipitation competition strategy in which mutant proteins are expressed at low levels and immunoprecipitated; other members in the MCM complex are detected by Western blot (SHERMAN *et al.* 1998). Plasmids coding for wild-type and mutant derivatives were transformed into a wild-type strain. To compare relative complex affinity, the expression levels of the tagged proteins have to be similar to one another and to the endogenous wild-type Mcm4p levels. When cells were grown with the same concentration of thiamine, we noted that the expression levels of the different tagged proteins varied reproducibly (data not shown). We accommodated this by culturing the cells in different thiamine concentrations as indicated in Figure 3A.

The HA-tagged proteins were immunoprecipitated and Western blot analysis was used to detect coprecipitating MCM proteins (Figure 3B). The amount of immunoprecipitated HA-tagged proteins was determined by blotting with an anti-Mcm4 antibody (Figure 3B). The endogenous wild-type Mcm4p was not detected in the anti-HA precipitates, suggesting that there is only one molecule of Mcm4p protein per complex. Similar results were reported for Mcm2p (SHERMAN *et al.* 1998).

Compared to the amount of MCMs bound to Mcm4p-HA, normal levels of MCMs coprecipitated with Mcm4K → R-HA, while somewhat reduced levels were associated with Mcm4D → A-HA and Mcm4K → A-HA (Figure 3B). These results show that all the mutants can assemble into a complex, although the nonconservative mutants have reduced affinity. Interestingly, all the coprecipitated MCMs were affected to the same extent. These data suggest that the Walker domains of Mcm4p contribute to the formation of a stable MCM2-7 complex.

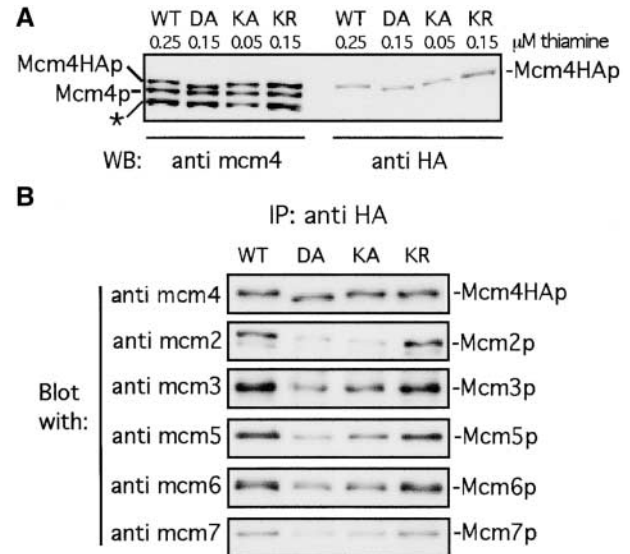


FIGURE 3.—Nonconservative mutations in Mcm4p Walker motifs reduce MCM complex formation. (A) Plasmids pMGC32, pEBG4, pEBG5, and pEBG6, encoding for Mcm4p-HA (WT), Mcm4D → A-HA (DA), Mcm4K → A-HA (KA), and Mcm4K → R-HA (KR), respectively, were transformed into the wild-type strain FY254. Cells were grown at 32° in minimal media plus the indicated concentrations of thiamine. Protein lysates were separated by SDS-PAGE and transferred to Immobilon. Endogenous Mcm4p and the HA-tagged proteins were detected with anti-Mcm4p antibodies (anti-mcm4), and HA antibodies (anti-HA) revealed the HA-tagged proteins. (B) The HA-tagged proteins were selectively immunoprecipitated with antibodies to HA. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon, and blotted with the indicated MCM antibodies. Note that Mcm4D → A-HA has a faster mobility.

In situ chromatin localization: Next, we investigated the ability of the mutant HA-tagged Mcm4p to localize to the nucleus and to bind to chromatin. In fission yeast, MCMs are nuclear throughout the cell cycle (MAIORANO *et al.* 1996; OKISHIO *et al.* 1996; SHERMAN and FORSBURG 1998; PASION and FORSBURG 1999; LIANG and FORSBURG 2001) but periodic association with chromatin has been reported (OGAWA *et al.* 1999; KEARSEY *et al.* 2000). Recently a new technique was published to analyze the chromatin association of fission yeast proteins on the basis of detection of Mcm4p-green fluorescent protein (GFP) fluorescence in permeabilized cells after extraction with a nonionic detergent (KEARSEY *et al.* 2000). In permeabilized cells that were not detergent washed, Mcm4p-GFP was nuclear throughout the cell cycle, but following detergent extraction, Mcm4p-GFP nuclear localization was visualized only in the nucleus of binucleate (M/G1/S phase) cells, where it is bound to chromatin.

We modified this technique to analyze fission yeast HA-tagged proteins during the cell cycle (see MATERIALS AND METHODS). Our revised method differs primarily in the use of 0.025% (low) Triton X-100 washes to enhance nuclear signal without delocalizing the HA-tagged pro-

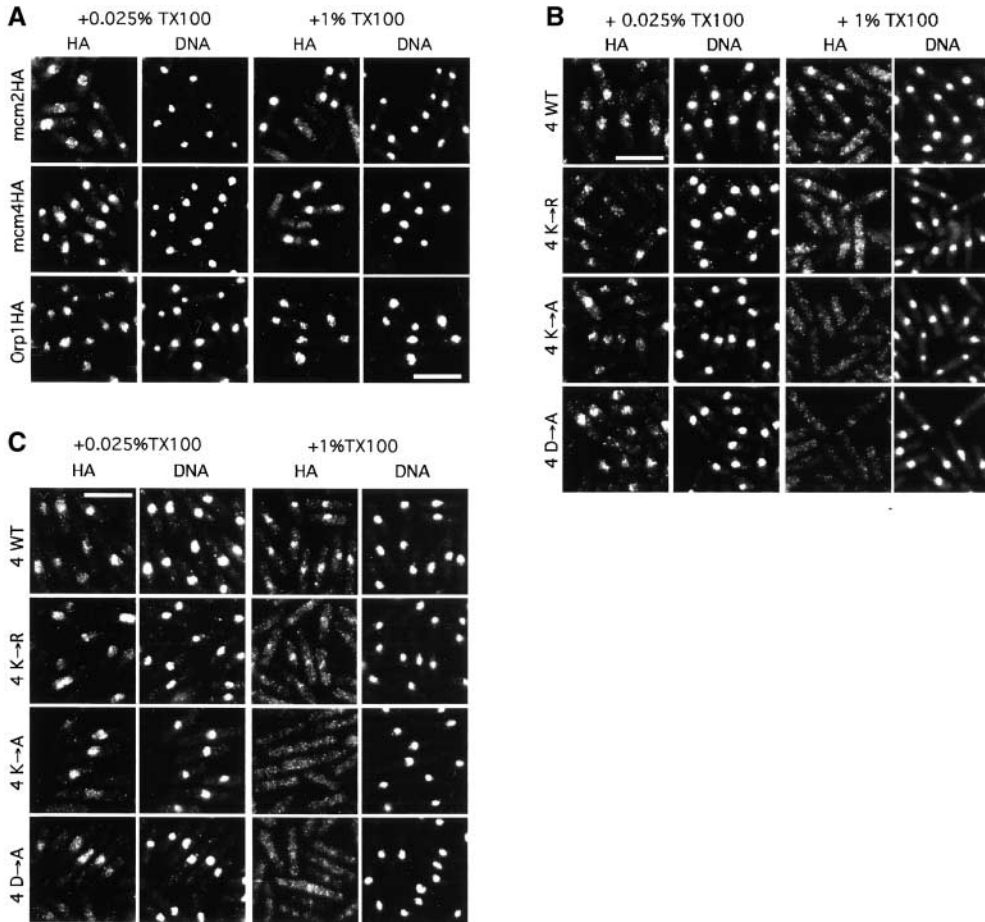


FIGURE 4.—The Mcm4p Walker A and B domain mutants can localize to the nucleus but only Mcm4p K to R can bind to chromatin. Cells were permeabilized and washed with 0.025% Triton X-100 or 1% Triton X-100 as indicated. DNA was visualized by DAPI staining (DNA). HA-tagged proteins were visualized using the HA antibody and a secondary antibody conjugated to Cy3 (HA). Bar, 10 μ m. (A) Chromatin-binding analysis of *S. pombe* HA-tagged Mcm2p, Mcm4p, and Orp1p. The Mcm2p-HA (FY798), Mcm4p-HA (FY1718), and Orp1p-HA (FY793) proteins were expressed from the native promoters as the only copies in the cells. (B) *mcm4⁺* and the mutant versions, HA tagged at the 3' end and under the control of the *nmt* promoter, were integrated at the *leu1* locus of strain FY254. Cells were grown at 32° in the presence of thiamine. Cells were photographed using the same exposure times. 4 WT, Mcm4p-HA; 4 K \rightarrow R, Mcm4K \rightarrow R-HA; 4 K \rightarrow A, Mcm4K \rightarrow A-HA; 4 D \rightarrow A, Mcm4D \rightarrow A-HA. (C) Mcm4p K to R mutants also bind to chromatin in hy-

droxyurea S-phase-arrested cells. Strains with the integrated HA-tagged *mcm4* genes under the control of the *nmt* promoter were incubated for 3.5 hr with 15 μ M hydroxyurea in the presence of thiamine. Cells were photographed using the same exposure times.

teins. To test this method we analyzed the localization and chromatin binding of Mcm2p-HA (FY798), Mcm4p-HA (FY1718), and Orp1p-HA (FY793) integrated at the endogenous locus (Figure 4A). The proteins were localized to the nucleus in all cells when treated with low Triton X-100. Orp1p-HA remained nuclear throughout the cell cycle when cells were washed with 1% (high) Triton X-100, while Mcm2p-HA and Mcm4p-HA were visible in binucleate cells only. These results agree with the previous Mcm4p-GFP published data indicating that Mcm2p and Mcm4p bind to chromatin only at the end of mitosis and during G1/S phase (binucleate cells; KEARSEY *et al.* 2000), while Orp1p associates with chromatin throughout the cell cycle (LYGEROU and NURSE 1999).

Mcm4-HA clones under the control of the *nmt* promoter were integrated into the *leu1⁺* locus of strain FY254 to eliminate cell-to-cell variation observed in expression from episomes (*e.g.*, PASION and FORSBURG 1999). As for the association assays, the HA-tagged proteins have to compete with the endogenous Mcm4p for association with the other MCMs, and the mutant MCM

complexes have to compete to enter the nucleus and to bind to chromatin. Cells were grown asynchronously in media containing thiamine, permeabilized, treated with low or high Triton X-100, and fixed. Western blot analysis confirmed the expression of similar levels of HA-tagged proteins (data not shown). In cells treated with low Triton X-100, Mcm4p-HA and the HA-tagged mutant proteins were nuclear throughout the cell cycle (Figure 4B), indicating that all the analyzed proteins can localize to the nucleus. After detergent extraction (+ 1% Triton X-100), nuclear localization was lost in mononucleate G2 cells. Binucleate cells retained nuclear localization only when Mcm4p-HA and Mcm4K \rightarrow R-HA proteins were expressed (Figure 4B). The intensity of the signal was weaker in the K to R mutant when compared with Mcm4p-HA. Thus chromatin binding appears particularly sensitive to mutations of the MCM4 Walker motifs.

To further analyze the ability of these mutants to bind to chromatin, we arrested the cells as mononucleates in early S phase with hydroxyurea (Figure 4C). After 3.5 hr in 15 mM hydroxyurea, a high proportion of the

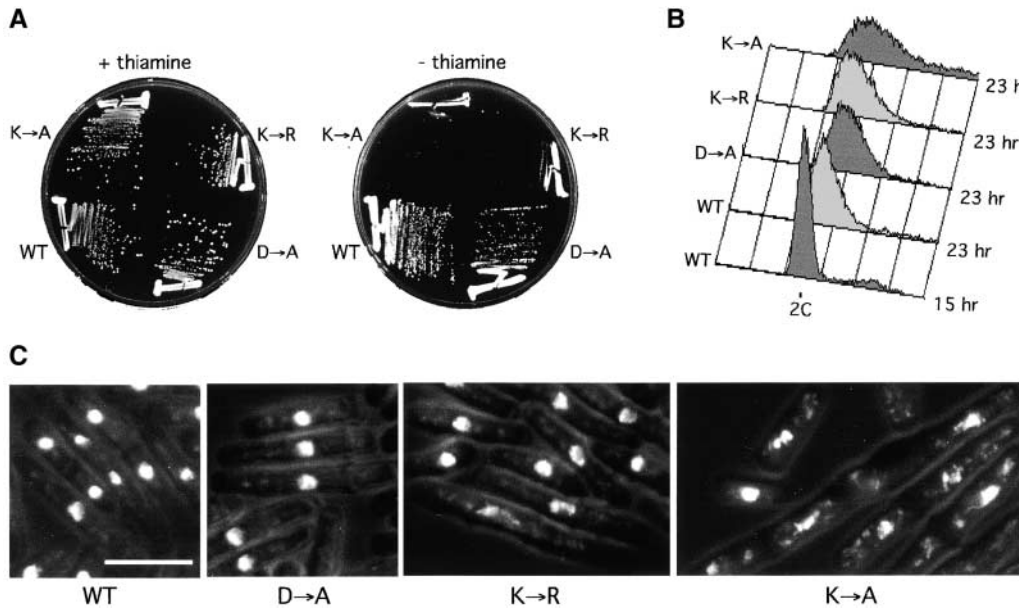


FIGURE 5.—Walker A motif mutant proteins have a dominant negative phenotype when overproduced. Strains carrying the *nmt-mcm4* HA-tagged wild-type and mutant genes integrated at the *leu1* locus of strain FY254 were analyzed. WT, D → A, K → A, and K → R correspond to cells expressing Mcm4p-HA, Mcm4D → A-HA, Mcm4K → A-HA, and Mcm4K → R-HA, respectively. (A) Cells were streaked on + thiamine and - thiamine plates as indicated and grown at 32° for 3 days. (B) Cells were grown at 32° in liquid medium lacking thiamine for a maximum of 23 hr. Cells were ethanol fixed and analyzed by flow cytometry; shown are histograms of DNA content. (C) DAPI staining of cells analyzed in B. Bar, 10 μ m.

cells had a 1C DNA content (data not shown). Cells washed with low Triton X-100 had the HA-tagged proteins in the nucleus (Figure 4C). When treated with high Triton X-100, only Mcm4p-HA and Mcm4K → R-HA proteins remained chromatin bound (Figure 4C).

From these results we conclude that the three non-functional Mcm4p mutant proteins can localize to the nucleus, but only the Mcm4K → R-HA mutant protein can bind to chromatin. However, the mutant Mcm4K → R-MCM complexes appear less efficient at binding to chromatin than wild-type MCM complexes.

Walker A domain mutant proteins have a dominant negative phenotype when overproduced: As previously reported (MAIORANO *et al.* 1996; FORSBURG *et al.* 1997), when *mcm4⁺* is expressed by full-strength *nmt* promoter on an episome at 32° it is dominant negative in wild-type cells. When *nmt-mcm4HA* was present as a single copy in the cell (strain FY1602), overproduction of Mcm4p-HA protein was not toxic (Figure 5A). To test the effect of overproducing the Mcm4 mutant proteins, strains FY1603, FY1604, and FY1605 were streaked on plates without thiamine and incubated at 32°. After 3 days, overproduction of Mcm4D → A-HA had no phenotype: Colony formation was normal and cell and colony morphology was similar to cells overproducing Mcm4p-HA (Figure 5A). In contrast, overproduction of Mcm4K → A-HA and to a lesser extent Mcm4K → R-HA was toxic for the cells: Colonies were much smaller and cells were elongated (Figure 5A). To analyze this dominant negative overproduction phenotype, cells were grown in liquid media lacking thiamine for a maximum of 23

hr (Figure 5, B and C). The DNA fluorescence profiles of cells overproducing Mcm4D → A-HA and Mcm4K → R-HA were indistinguishable from one another and slightly wider than when Mcm4p-HA was overproduced (Figure 5B). In contrast, the peak of DNA content was much broader when Mcm4K → A-HA was overproduced (Figure 5B). Cells were DAPI stained and visualized under the microscope (Figure 5C). Cells overproducing Mcm4p-HA and Mcm4D → A-HA were slightly elongated and one DAPI-stained body was visualized per cell. When Mcm4K → R-HA was overproduced, cells were elongated and some nuclear fragmentation was detected; this was dramatically exacerbated when Mcm4K → A-HA was overproduced (Figure 5C). Interestingly, only the Walker A motif mutants had a dominant negative phenotype, suggesting that nucleotide-binding and hydrolysis domains might be important for different aspects of Mcm4p function. However, these phenotypes did not correlate with chromatin binding or complex assembly. Table 3 summarizes these results.

Mcm4p and Mcm7p with mutations in the consensus CDK phosphorylation sites are functional proteins: We investigated the role of CDK phosphorylation of Mcm4p and Mcm7p by mutating the two CDK consensus sites at positions 15 and 112 of Mcm4p to alanine (mimics an unphosphorylated protein), serine (alternate phosphate acceptor), or glutamate (mimics a constitutively phosphorylated protein). We found that Mcm4p with all of these mutations is fully functional. These mutant proteins complement the temperature sensitivity of *mcm4-M68* (Figure 6A) and are wild type for growth

TABLE 3
Summary of the Mcm4p mutant properties

Mcm4p mutant	Complementation	Interaction with other MCMs	Localization to the nucleus	Binding to chromatin	Dominant negative when OP
D → A	—	±	+	—	—
K → A	—	±	+	—	+
K → R	—	+	+	±	+

OP, overproduced; +, same activity as wild type; —, no activity; ±, reduced activity.

rate and cell cycle progression when integrated into the genome as the only copy of *mcm4* (data not shown). Further, these Mcm4p mutants did not affect the rate of rereplication in fission yeast cells overexpressing Cdc18p or Rum1p (data not shown). To determine if Mcm4p lacking the CDK consensus sites is no longer a substrate for CDK phosphorylation, we expressed the first 384 amino acids of wild-type or T15A T112A Mcm4p in bacteria. When purified protein was added to Cdc2p/Cdc13-HAp immunoprecipitated from fission yeast extracts, we found that both Mcm4p fragments were phosphorylated equivalently (Figure 6B). In addition, when we constructed a version of Mcm7p with a mutation of the single CDK consensus site at position 546 to alanine, we found that this protein was able to complement the growth defect of *mcm7-98* at the restrictive temperature (Figure 6A) as well as complement $\Delta mcm7$ (data not shown). Thus, the CDK consensus sequences are not required for protein activity.

DISCUSSION

The MCM proteins are members of the large AAA+ ATPase family and are required not only for replication

initiation but also for elongation. Mammalian (ISHIMI 1997; YOU *et al.* 1999) as well as fission yeast (LEE and HURWITZ 2000) “core” MCMs (MCM4, -6, and -7) have been shown to have helicase activity *in vitro*, leading to the model that the MCM complex is a replicative helicase at the moving fork. However, the complete MCM hexamer (MCM2–7), which is found *in vivo*, does not have helicase activity *in vitro* (ADACHI *et al.* 1997; ISHIMI *et al.* 1998; LEE and HURWITZ 2000; SATO *et al.* 2000).

We examined mutations that were shown to affect the *in vitro* DNA helicase activity of the core MCMs (YOU *et al.* 1999) to determine their effect upon *in vivo* function. We constructed point mutations in the Walker A and B motifs characteristic of ATPases. Surprisingly, not all corresponding mutations that abolished the *in vitro* DNA helicase activity of the mouse hexameric Mcm4/6/7p subcomplex (YOU *et al.* 1999) affected the *in vivo* function of fission yeast MCM complex (Table 4). That is, there is not a simple correlation between *in vitro* and *in vivo* phenotypes.

Substitutions in the mouse Mcm6p Walker B motif completely abolished the *in vitro* DNA helicase activity of the mutant subcomplex (YOU *et al.* 1999), although equivalent mutations in fission yeast Mcm6p resulted in

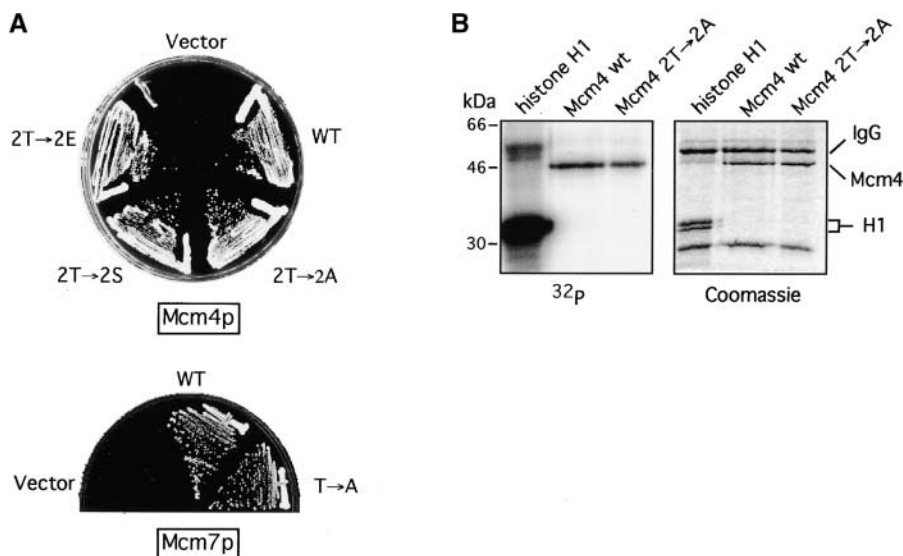


FIGURE 6.—Mcm4p and Mcm7p with mutations in the consensus CDK phosphorylation sites are functional proteins. (A) *mcm4-M68* (FY784) transformed with empty vector (pIRT2) or wild-type (pAC1), 2T → 2A (T15A T112A, pMGC-39), 2T → 2S (T15S T112S, pMGC40), or 2T → 2E (T15E T112E, pMGC41) *mcm4*-containing plasmid (top) and *mcm7-98* (FY1199) transformed with empty vector (pUR19-H), wild-type (pEBG30), or T → A (T546A, pEBG43) *mcm7*-containing plasmid (bottom) were grown at 36° for 3 days. (B) Cdc2/Cdc13 was immunoprecipitated from fission yeast extracts and incubated with [γ -³²P]ATP and histone H1 (positive control for kinase activity) or the first 384 amino acids of wild-type or 2T → 2A (T15A T112A) Mcm4p produced in bacterial cells. ³²P incorporation (left) and protein loading (right) are shown.

TABLE 4

Comparison of the *in vivo* results with the *in vitro* published activities of the MCM4/6/7 subcomplex

MCM mutant	<i>In vivo</i> complementation	Data from You <i>et al.</i> (1999) ^a	
		<i>In vitro</i> DNA helicase activity	<i>In vitro</i> ATPase activity
ATP-binding and hydrolysis motifs			
WT MCM	+	++++	++++
Mcm4 Walker A K → A	–	ND	ND
K → R	–	ND	ND
Walker B D → A	–	++ ^b	++++ ^b
Mcm6 Walker A K → A	+	ND	ND
K → R	+	ND	ND
KS → AA	–	+	+++
Walker B DE → AA	+	–	++++
Mcm7 Walker A K → R	+		
K → A	–		
D → A	–		
Mcm2 ^c Walker A K → R	+		
K → A	–		
Mcm2 ^c Walker B D → A	–		
CDK P sites			
Mcm4 CDK T15A T112A	+		
P site T15S T112S	+		
T15E T112E	+		
Mcm7 CDK P site T546A	+		

P, phosphorylation.

^a The activity of the subcomplex Mcm4/6/7 is shown by the number of plus signs. Wild-type Mcm4/6/7 is indicated as ++++ (You *et al.* 1999).^b Activity of mouse Mcm4p Walker B motif DE → AA double mutant.^c Data published in FORSBURG *et al.* (1997).

complementation. Thus, the Mcm6p Walker B motif appears to be essential for *in vitro* DNA helicase activity (YOU *et al.* 1999) but dispensable for *in vivo* MCM complex function. None of the single mutations in Mcm6p disrupted complementation, although the double Walker A motif mutant KS → AA was not able to complement. Data from other ATPases showed that single mutations of the crucial lysine in the Walker A motif abolish ATPase activity and *in vivo* function (LAURENT *et al.* 1993; SINGH and MAURIZI 1994; RIKKONEN 1996). It is possible that the double mutation introduced in fission yeast Mcm6p Walker A domain could alter the protein structure, rather than simply affect its ATP binding. Unfortunately, we could not examine the behavior of this protein *in vivo*, as it could not be epitope tagged.

Interestingly, YOU *et al.* (1999) showed that Mcm6p is responsible for ATP binding by MCM4/6/7 and Mcm6p mutant subcomplexes cannot bind ATP (YOU *et al.* 1999). However, our *in vivo* results suggest that Mcm6p is not directly involved in the MCM complex ATP-binding and hydrolysis activities. It is possible that *in vitro* Mcm6p Walker domains are essential for MCM4/6/7 subcomplex ATP binding and thus helicase activity, but *in vivo* this function can be carried out by a different

MCM protein in the intact hexamer. The *in vitro* studies were carried out using a subset of the MCM proteins, as helicase activity was not obtained with the complete MCM2–7 complex. In contrast, studies *in vivo* suggest that all MCMs are present and contribute similarly to complex function (APARICIO *et al.* 1997; SHERMAN *et al.* 1998; LABIB *et al.* 2000; LIANG and FORSBURG 2001), so perhaps Mcm2, -3, or -5 is responsible for the activity associated with Mcm6p in the *in vitro* experiments.

Previous results from our lab showed that a conservative substitution of the Mcm2p Walker A domain lysine was able to rescue *mcm2-ts* and $\Delta mcm2$ mutations, although a nonconservative substitution gave a nonfunctional protein (FORSBURG *et al.* 1997). These data resemble the results obtained with Mcm7p where the K409R was functional while K409A was not. In several proteins, mutations of the Walker A motif lysine to arginine abolish ATPase activity but not their ability to bind ATP (REHRAUER and KOWALCZYKOWSKI 1993; SUNG and STRATTON 1996; SUNG *et al.* 1988). These results suggest the crucial role of ATP in Mcm2p and Mcm7p may be in binding, rather than hydrolysis.

A recent published article (SCHWACHA and BELL 2001) shows some discrepancies with our *in vivo* muta-

tional analysis. Schwacha and Bell analyze the ability of *Saccharomyces cerevisiae* MCM Walker A and B mutant proteins to complement null MCM strains using tagged MCMp versions under the control of the MCM5 promoter. In our study we found that the HA epitope added to either end of fission yeast MCM proteins is not neutral, especially if combined with other mutations. We speculate that some of the observed differences could be attributed to the use of a tag. However, the intrinsic differences between fission and budding yeasts should also be considered.

An additional finding came out of this study; mutations of the consensus CDK phosphorylation sites (S/T P XX K/R) in Mcm4p and Mcm7p did not have any significant effect on the function of the proteins. In the case of Mcm4p, phosphorylation by CDKs has been demonstrated in higher eukaryotes and correlates with loss of chromatin association (COUE *et al.* 1996; HENDRICKSON *et al.* 1996; FUJITA *et al.* 1998). Mutation of the consensus sites in *S. pombe* Mcm4p did not have any effect on protein phosphorylation, suggesting that additional SP or TP residues may be phosphorylated; these are numerous throughout the protein. Similar CDK phosphorylation of sites that do not contain the final basic residue has been observed for Orp2p (VAS *et al.* 2001). As is seen for *S. cerevisiae*, it may be necessary to combine CDK mutations in multiple proteins to observe a phenotype (NGUYEN *et al.* 2001).

For all the MCMs analyzed, both K → A and D → A mutations always had the same phenotype. Some reports show that mutations in Walker B motifs abolish ATPase activity but do not affect ATP binding (PAUSE and SONENBERG 1992; BROSH and MATSON 1995). However, others reported that mutations in this domain abolished binding as well (KLEMM *et al.* 1997). Our findings suggest that neither mutant binds ATP, although *in vitro* biochemical studies will be required to confirm this.

Our most detailed analysis was carried out on Mcm4p, since a tagged version of this protein behaved identically to the untagged version, facilitating protein analysis in cells. We analyzed the importance of the ATP-binding and hydrolysis domains in the formation of the MCM complex. Interestingly, although none of these mutants are functional, their ability to assemble into the MCM complex is different (Figure 3B). Several reports show that formation and stability of some protein complexes depend on nucleotide binding (*e.g.*, SINGH and MAURIZI 1994; SAWAYA *et al.* 1999; SINGH *et al.* 1999; ZHANG *et al.* 2000; JERUZALMI *et al.* 2001). In most hexameric helicases, the formation of the ring hexamer structure requires nucleotide binding and Mg⁺⁺ (reviewed in PATEL and PICCHIA 2000). For some AAA+ family members, oligomerization is promoted both by nonhydrolyzable ATP analogs or by creating mutations that block ATP hydrolysis (reviewed in VALE 2000). The differences observed between conservative and nonconservative substitutions in the Walker A motif suggest that ATP

bound to Mcm4p is necessary for its association with the other MCMs. Interestingly, both K → A and D → A mutants were similarly affected in their ability to form a stable MCM complex, suggesting that *in vivo* they might have similar defects.

Although ATP binding is involved in oligomerization of T7 gp4 helicase subunits, other hydrophobic and electrostatic interactions also contribute to the association and stability of the hexameric structure (SAWAYA *et al.* 1999). The fact that neither Walker A nor B domain mutations completely abolished Mcm4p complex formation suggests that other regions of Mcm4p must be also involved in association. Because these mutant proteins were still able to form a MCM complex, we also conclude that none of these mutations seriously disrupts Mcm4p structure.

The ability of the Mcm4p mutants to localize to the nucleus and bind to chromatin was analyzed using an *in situ* chromatin-binding assay (KEARSEY *et al.* 2000). The three HA-tagged mutants could localize to the nucleus similarly to wild-type Mcm4p-HA (Figures 4 and 5). Previous reports from our lab showed that complex formation is necessary for proper MCM complex nuclear localization (PASION and FORSBURG 1999). All Mcm4p mutants can, to some extent, assemble into a complex and, therefore, be imported into the nucleus. Interestingly, there was no difference in cytoplasmic staining between Mcm4 wild-type and mutant proteins, suggesting that their nuclear localization is as effective as wild type.

In contrast, chromatin binding was defective in all three mutants. Mcm4-HA K → A and D → A mutants showed no chromatin binding, while for the conservative mutant K → R it was reduced. Since this mutant is likely to have residual ATP binding (SUNG *et al.* 1988; REHRAUER and KOWALCZYKOWSKI 1993; SUNG and STRATTON 1996), we suggest that ATP bound to Mcm4p is required for the association of the MCM complex with chromatin and/or association with other prereplicative complex proteins.

Either ATP or a nonhydrolyzable analog stabilizes chromatin binding of human MCMs (FUJITA *et al.* 1997). Similarly, DNA binding of SV40 and polyomavirus T-antigen is enhanced by ATP and also by AMP-PNP (DEB and TEGTMEYER 1987; LORIMER *et al.* 1991). In budding yeast, origin recognition complex (Orc)1p ATP binding, but not hydrolysis, is responsible for the ATP dependence of ORC DNA binding (KLEMM *et al.* 1997). These observations are consistent with our results with Mcm4p mutants defective in chromatin binding, but this could also indicate that ATP is required for complex assembly, which is in turn required for chromatin association.

The different phenotypes we observe in subunits with similar mutations suggest that each subunit has a different role in the activity of the MCM complex. Biochemical studies using the 4A' product of T7 g4 DNA helicase

showed that the six identical subunits are functionally distinct; similar to the F1-ATPase, 4A' T7 product also has noncatalytic and catalytic sites, and it was proposed that both proteins function in a similar way (HINGORANI *et al.* 1997). In the case of the bacterial clamp loader, the three γ -subunits have different tertiary structures (reviewed in ELLISON and STILLMAN 2001). As suggested by others (TYE and SAWYER 2000), perhaps in the MCM hexamer some subunits are catalytic and others are non-catalytic. This would be consistent with the different activities associated with subcomplexes *in vitro*. Our mutational analysis provides evidence supporting this hypothesis, showing that the nucleotide-binding and hydrolysis domains of MCM4, -6, and -7 proteins are not equivalent. Perhaps the most important conclusion from this study is that hypotheses derived from biochemical studies must be correlated with *in vivo* analysis.

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