Characterization of *Schizosaccharomyces pombe mcm7*$^+$ and *cdc23*$^+$ (*MCM10*) and Interactions With Replication Checkpoints

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ABSTRACT

MCM proteins are required for the proper regulation of DNA replication. We cloned fission yeast *mcm7*$^+$ and showed it is essential for viability; spores lacking *mcm7*$^+$ begin S phase later than wild-type cells and arrest with an apparent 2C DNA content. We isolated a novel temperature-sensitive allele, *mcm7-98*, and also characterized two temperature-sensitive alleles of the fission yeast homolog of *MCM10*, *cdc23*$^+$. *mcm7-98* and both *cdc23ts* alleles arrest with damaged chromosomes and an S phase delay. We find that *mcm7-98* is synthetically lethal with the other *mcmts* mutants but does not interact genetically with either *cdc23ts* allele. However, *cdc23-M36* interacts with *mcm4ts*. Unlike other *mcm* mutants or *cdc23*, *mcm7-98* is synthetically lethal with checkpoint mutants Δ*cds1*, Δ*chk1*, or Δ*rad3*, suggesting chromosomal defects even at permissive temperature. Mcm7p is a nuclear protein throughout the cell cycle, and its localization is dependent on the other MCM proteins. Our data suggest that the Mcm3p-Mcm5p dimer interacts with the Mcm4p-Mcm6p-Mcm7p core complex through Mcm7p.

THE MCM proteins are a conserved family of eukaryotic replication factors implicated in both the initiation and elongation phases of DNA replication (reviewed in Kearsey and Labib 1998; Tye 1999; Pasion and Forsburg 2001). They were first identified in budding yeast through screens for mutants that either arrested with 1C DNA content or were defective in minichromosome maintenance (MCM; Moir *et al.* 1982; Maine *et al.* 1984). The family consists of six distinct subclasses called MCM2–7, which are defined by sequence homology. Each subclass is essential for viability in both budding and fission yeasts; therefore they are not redundant factors. Late in the cell cycle, replication factors, including the MCM proteins, are loaded onto origin sequences bound by the origin recognition complex (ORC) by the action of the initiation factor cell division cycle (CDC)6/Cdc18, forming the prereplicative complex (preRC); loading of additional replication factors and initiation of DNA replication are promoted by the activity of the DBF4-dependent kinase (Cdc7) and cyclin-dependent kinase (CDK; Cdc2) (reviewed in Dutta and Bell 1997; Kelly and Brown 2000).

Although the MCM proteins were first thought to be initiation factors for replication, it is clear from recent evidence that the MCM proteins are also required for elongation of the replication fork. The temperature-sensitive allele of *mcm2*$^+$, *mcm2ts (cdc19-P1)*, interacts genetically with mutations in both the large and small subunits of DNA polymerase delta (Forsburg *et al.* 1997). Reduced levels of a single MCM protein impair completion of S phase, while complete absence blocks initiation of DNA replication (Liang *et al.* 1999). In budding yeast, the pattern of MCM association with chromatin mirrors that of DNA polymerase epsilon (Aparicio *et al.* 1997). Recently, the use of engineered degron mutants in budding yeast has shown that rapid elimination of any single MCM protein during S phase halts replication during elongation (Labib *et al.* 2000).

A role for MCMs throughout S phase is expected if they function as a replicative helicase; this has been suggested by biochemical experiments showing helicase activity associated with the subcomplex formed by MCM4-6-7. The six eukaryotic MCM proteins form a multisubunit complex with 1:1:1:1:1:1 stoichiometry. Helicase activity *in vitro* is associated with the MCM4-6-7 subcomplex and may be regulated by the other subunits (Ishimi *et al.* 1998; Sato *et al.* 2000). Intriguingly, the Archaea *Methanobacterium thermoautotrophicum* genome contains a single MCM protein that forms a dodecamer with helicase activity (Kelman *et al.* 1999; Chong *et al.* 2000; Shechter *et al.* 2000). A number of viral and prokaryotic replication helicases also form six-membered homomultimers (reviewed in Hingorani and O'Donnell 2000; Patel and Picha 2000). That eukaryotic MCM complexes have six different members suggests that the subunits have acquired individual functions that contribute to regulation of complex activity.

We are examining the roles of individual fission yeast

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MCMs in DNA replication and their relationship with other replication factors. In this article, we describe the cloning and characterization of *mcm7*$^+$, the last *Schizosaccharomyces pombe* MCM remaining to be characterized. Like other fission yeast *mcm* genes, *mcm7*$^+$ is essential for viability and is required for normal S phase progression. Interestingly, the *mcm7-98* strain does not arrest with the uniform *c*ell *d*ivision *c*ycle phenotype observed in other *cdc* mutants. *mcm7-98* cells arrest with a DNA content intermediate between 1C and 2C. *mcm7-98* cells require the checkpoint kinases Rad3, Chk1, and Cds1 for viability even at the permissive temperature. We examined the genetic interactions between *mcm7-98* and other mutants in initiation and elongation, as well as the physical interactions between Mcm7p and other replication proteins, including Cdc23p, the fission yeast homolog of MCM10. In budding yeast, *MCM10* and *MCM7* have allele-specific genetic and physical interactions. In fission yeast, we show that *cdc23-M36* has genetic interactions with *mcm4ts* and *rad4ts* but not with our *mcm7ts* allele. Crude cellular fractionation indicates that Cdc23p is insoluble, unlike the bulk of MCM proteins. These results further define the network of interactions among replication factors.

MATERIALS AND METHODS

Strains and genetic manipulations: All *S. pombe* strains (Table 1) were maintained on yeast extract plus supplement (YES) agar plates or under selection on Edinburgh minimal media (EMM) with appropriate supplements using standard techniques (Moreno *et al.* 1991). Yeast cells were transformed by electroporation. Standard molecular biology techniques were used (Sambrook *et al.* 1989). Double mutants were constructed by standard tetrad analysis or random spore analysis. Spore germinations, chromosome gels, and viability assays were performed as described previously except that the strains were grown and plated on minimal plates for the viability assays (Liang *et al.* 1999). Cells were treated with hydroxyurea as indicated in the figure legends. For nitrogen starvation, cells were washed and grown overnight in EMM lacking ammonium chloride and supplemented with 7.5 μg/ml adenine. FY1067 (*cdc23HA*) was a generous gift of Jeff Hodson. FY1364 was constructed by crossing FY1067 with FY1092 (*orp1HA*) and screening for strains containing both hemagglutinin-tagged proteins.

Cloning of *mcm7*$^+$: Using primers based on the previously published partial sequence of the *mcm7* (Adachi *et al.* 1997), MCM7_1 5′-GCTTTACTTCTGTTGCTTGTCGGAGGTG-3′ and MCM7_2 5′-CGTCACTGGATCACGCATTACAGCAGC-3′, we amplified a *mcm7*$^+$-specific probe by PCR and screened a *S. pombe* genomic library (Barbet *et al.* 1992) and a *S. pombe* cDNA library (Fikes *et al.* 1990) by hybridization. The entire *mcm7*$^+$ gene (pDTL14) was sequenced on both strands (The Salk Institute DNA Sequencing Facility). The GenBank accession number is AF070481.

Construction and characterization of *mcm7*$^+$ disruption: A gene disruption of *mcm7*$^+$ was constructed as follows. We subcloned the last 261 bp of *mcm7*$^+$ and 537 bp of downstream sequence into pBluescript II KS+ (Stratagene, La Jolla, CA); we then amplified 703 bp of upstream sequence and the first 244 bp of *mcm7*$^+$ by PCR and subcloned the product into the previous plasmid. We inserted the *his3*$^+$ cassette from pAF1 (Ohi *et al.* 1996) to create pDTL43, disrupting amino acids 82–673 of Mcm7p. We transformed the disruption construct into a wild-type diploid strain with the genotype *h*$^-$/*h*$^+$ *his3-D1/his3-D1 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216*. His$^+$ diploids were selected and analyzed, and the disruption was confirmed by Southern blot.

We confirmed that the genomic clone could rescue the disruption phenotype as follows. We transformed the Δ*mcm7::his3*$^+$/*mcm7*$^+$ diploid with the *mcm7*$^+$ genomic clone on a *ura4*$^+$ plasmid (pDTL14), sporulated the strain, and then germinated the spores on plates lacking histidine. When we examined the phenotypes of the resulting colonies, we recovered His$^+$ haploids only if they were also Ura$^+$, indicating the plasmid is required for viability.

Plasmid and mutant construction: Plasmid pDTL87 contains *mcm7*$^+$ with a triple HA tag at the C terminus. It was constructed by using PCR to introduce a *Xho*I site upstream of the initiation ATG and a *Not*I site before the stop codon; the PCR product was cloned into pBluescript II KS+, creating pDTL60. The *Not*I-*Xho*I fragment of pDTL60 was subcloned into the triple-HA-tagging *nmt* expression vector pSLF172 (Forsburg and Sherman 1997) to create pDTL70. Because the PCR amplification was inefficient, we replaced the internal portion of *mcm7*$^+$ coding region with genomic sequence from the genomic clone (pDTL14) and sequenced the 5′ and 3′ ends to verify structure. First we subcloned the *Sma*I-*Xho*I fragment from pDTL70 into the same sites in pBluescript II KS+ to create pDTL77. Then the *Hin*cII-*Mlu*I fragment from pDTL77 was replaced with the identical fragment from pDTL14 to create pDTL82. The 5′ and 3′ ends were sequenced to ensure there were no PCR errors. Last, the *Not*I-*Xho*I fragment from pDTL82 was subcloned into the same sites in pSLF172 to create pDTL87.

Plasmid pDTL29 was constructed by subcloning the *Pvu*II-*Sal*I fragment of pDTL87, containing the 3′ end of the tagged gene, into pJK148 (Keeney and Boeke 1994). Following linearization with *Mlu*I, pDTL29 was integrated into the *mcm7*$^+$ locus, creating a partial tandem duplication of the gene and expressing the tagged form under the endogenous promoter (FY1021).

The *mcm7-47* mutant, S229Y, was constructed using a Stratagene QuickChange site-directed mutagenesis kit according to manufacturer's instructions and two complementary mutagenic oligonucleotides.

Plasmid shuffle and integration of *mcm7-98*: We transformed a Δ*mcm7::his3*$^+$ strain carrying wild-type *mcm7*$^+$ on a *ura4*$^+$ plasmid with a mutagenized library of *mcm7*$^+$ on a *leu1*$^+$ or *LEU2* plasmid. The plasmid was mutagenized with 1 M hydroxylamine as described previously (Snaith *et al.* 2000). The plasmid carrying wild-type *mcm7*$^+$ was lost from cells that were replica plated onto plates containing 0.1% 5-fluoroorotic acid (FOA) and 50 μg/ml uracil. The resulting Leu$^+$ colonies were screened for temperature sensitivity. We isolated 3 temperature-sensitive plasmids out of 10,000 plasmids screened (pDTL97/98/99). We isolated the plasmids from the yeast strains and confirmed that they would confer temperature sensitivity when retransformed into the Δ*mcm7* strain. The *mcm7*$^+$ open reading frame (ORF) was sequenced (The Salk Institute DNA Sequencing Facility) to identify the following mutations. pDTL97 contained a mutation in codon 374 from GCT to CCT, resulting in a change from alanine to proline. pDTL98 and pDTL99 contained the same mutation in codon 644 from CGT to CAT, resulting in a change from arginine to histidine. The pDTL98 mutation (*mcm7-98*) was integrated into the genome as follows. The *Eco*RV-*Xho*I fragment from pDTL98, containing the last 1.4 kb of *mcm7-98* sequence, was subcloned into pJK210 (cut with *Kpn*I, end filled, cut with *Xho*I; Keeney and Boeke 1994) to construct pDTL140. The *Xho*I fragment (*mcm7*$^+$ downstream sequence) from pDTL12 (genomic *mcm7*$^+$ clone) was subcloned into pBluescript II

TABLE 1

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

Strain	Genotype	Source
FY80	*h*$^+$ *ura4-D18 leu1-32 ade6-M210*	Our stock
FY243	*h*$^-$ *cdc19-P1 ura4-D18 leu1-32 ade6-M210*	Our stock
FY254	*h*$^-$ *ura4-D18 leu1-32 ade6-M210 can1-1*	Our stock
FY421	*h*$^-$ Δ*chk1::ura4*$^+$ *ura4-D18 leu1-32 ade6-704*	T. Carr
FY458	*h*$^+$ *nuc2-663 his2 leu1-32*	R. West
FY527	*h*$^-$ *his3-D1 ura4-D18 leu1-32 ade6-M216*	Our stock
FY528	*h*$^+$ *his3-D1 ura4-D18 leu1-32 ade6-M210*	Our stock
FY562	*h*$^-$ *cdc10-V50 ura4-D18 leu1-32 ade6-M216*	Our stock
FY583	*h*$^+$ *cdc22-M45 ura4-D18 leu1-32 ade6-M216*	Our stock
FY584	*h*$^+$ *cdc25-22 ura4-D18 leu1-32 ade6-M216 can1-1*	Our stock
FY786	*h*$^-$ *cdc21-M68 ura4-D18 leu1-32 ade6-M216*	Our stock
FY865	*h*$^-$ Δ*cds1::ura4*$^+$ *ura4-D18 leu1-32*	D. Griffiths
FY918	*h*$^+$ Δ*mcm7::his3*$^+$ *his3-D1 ura4-D18 leu1-32 ade6-M210* *h*$^-$ *mcm7*$^+$ *his3-D1 ura4-D18 leu1-32 ade6-M216*	This work
FY931	*h*$^+$ Δ*mcm7::his3*$^+$ *his3-D1 ura4-D18 leu1-32 ade6-M210 p[mcm7*$^+$*-ura4*$^+$*]*	This work
FY962	*h*$^-$ *mis5-268 ura4-D18 leu1-32 ade6-M216 can1-1*	Our stock
FY1021	*h*$^-$ *mcm7::[mcm7HA-leu1*$^+$*] his3-D1 ura4-D18 leu1-32 ade6-M210*	This work
FY1067	*h*$^-$ *cdc23::[cdc23HA-leu1*$^+$*] ura4-D18 leu1-32 ade6-M210 can1-1*	This work
FY1086	*h*$^+$ *cdc23-M36 ura4-D18 leu1-32 ade6-M216*	Our stock
FY1092	*h*$^+$ *orp1HA ura4-D18 leu1-32 ade6-M216*	Our stock
FY1105	*h*$^-$ Δ*rad3::ura4*$^+$ *ura4-D18 leu1-32 ade6-704*	P. Russell
FY1199	*h*$^-$ *mcm7-98 ura4-D18 leu1-32 ade6-M216*	This work
FY1364	*h*$^+$ *cdc23::[cdc23HA-leu1*$^+$*] orp1HA ura4-D18 leu1-32 ade6-M216*	This work
FY1365	*h*$^-$ *mcm7-98 rad4-116 ura4-D1 leu1-32 ade6-M216*	This work
FY1366	*h*$^-$ *mcm7-98 orp1-4 ura4-D18 leu1-32 ade6-M210*	This work
FY1367	*h*$^-$ *mcm7-98 cdc23-M36 ura4-D18 leu1-32 ade6-M216*	This work
FY1368	*h*$^-$ *cdc23-M36 orp1-4 ura4-D18 leu1-32 ade6-M216*	This work
FY1369	*h*$^+$ *cdc23-M36 rad4-116 ura4-D18 leu1-32 ade6-M210*	This work
FY1370	*h*$^-$ *cdc21-M68 rad4-116 ura4-D18 leu1-32 ade6-M216*	This work
FY1401	*h*$^+$ *cdc23-M30 ura4-D18 leu1-32 ade6-M216*	Our stock
FY1444	*h*$^+$ *cdc23-M36* Δ*chk1::ura4*$^+$ *ura4-D1 leu1-32 ade6-704*	This work
FY1445	*h*$^+$ *cdc23-M30* Δ*chk1::ura4*$^+$ *ura4-D1 leu1-32 ade6-704*	This work
FY1446	*h*$^-$ *cdc23-M36* Δ*cds1::ura4*$^+$ *ura4-D18 leu1-32*	This work
FY1447	*h*$^+$ *cdc23-M30* Δ*cds1::ura4*$^+$ *ura4-D18 leu1-32*	This work
FY1448	*h*$^+$ *cdc23-M36* Δ*rad3::ura4*$^+$ *ura4-D18 leu1-32 ade6-M216*	This work
FY1449	*h*$^+$ *cdc23-M30* Δ*rad3::ura4*$^+$ *ura4-D18 leu1-32 ade6-M216*	This work
FY1502	*h*$^-$ *mcm7-98 hsk1-1312 ura4-D18 leu1-32 ade6-M210*	This work
FY1503	*h*$^+$ *mcm7-98* Δ*rqh1::ura4*$^+$ *ura4-D1 leu1-32*	This work
FY1504	*h*$^+$ *mcm7-98 cdc23-M30 ura4-D18 leu1-32 ade6-M216*	This work
FY1617	*h*$^-$ *cdc21-M68* Δ*cds1::ura4*$^+$ *ura4-D18 leu1-32 ade6-M210*	This work

KS+ (with *Bam*HI site destroyed) cut with *Sal*I and *Xho*I to construct pDTL130 and then the *Xba*I-*Xho*I fragment was moved from pDTL130 into the same sites of pDTL140 to construct pDTL143. pDTL143 was linearized with *Msc*I, which cuts upstream of the *mcm7-98* mutation, and transformed into a wild-type *ura4-D18* strain to obtain a partial-tandem duplication. Stable Ura$^+$ isolates were tested for temperature sensitivity and temperature-sensitive isolates were streaked onto FOA plates to induce loss of the *ura4*$^+$ marker to loop out the duplicated sequences. We confirmed the FOA-resistant strains were temperature sensitive and showed the genomic structure was normal using Southern blot analysis (data not shown).

Sequencing of *cdc23ts* alleles: The conditional alleles were cloned from the genome by PCR. Duplicate PCR reactions were set up using genomic DNA prepared from the mutant strains to characterize independent isolates and screen out PCR errors. The duplicate clones for each allele were sequenced. Because the same lesions were found in duplicate independent clones from independent PCR reactions, we conclude that they are unlikely to be PCR artifacts and represent the actual mutations. *cdc23-M36* contained a mutation in codon 232 from GAC to GCC, changing aspartic acid to glycine. *cdc23-M30* contained a mutation in codon 187 from CTA to CCA, changing leucine to proline.

Antibodies: We raised polyclonal rabbit antibodies to amino acids 18–290 of Mcm7p, purified as a 6xHis-tagged fusion protein, as described previously (Sherman *et al.* 1998). The expression plasmid, pDTL31, contains the *Hinc*II-*Eco*RV fragment from pDTL25 subcloned into the pRSETB *Pvu*II site (Invitrogen, San Diego, CA). pDTL25 was constructed by subcloning the *Cla*I-*Eco*RV fragment from pDTL24 (isolated from Dam$^-$ cells) into the same sites in pBluescript II KS+. pDTL24 contains the 1.5-kb *Eco*RV fragment from pDTL14 subcloned into the *Eco*RV site of pBluescript II KS+.

Antibodies were affinity purified from Western blots using purified protein, as described previously (Sherman *et al.* 1998). Antibodies to Mcm2p (Cdc19p), Mcm3p, Mcm4p (Cdc21p), Mcm5p (Nda4p), and Mcm6p (Mis5p) have been

described previously (Forsburg *et al.* 1997; Sherman and Forsburg 1998; Sherman *et al.* 1998). Monoclonal anti-HA 12CA5 antibody was a kind gift of Jill Meissenholder and Tony Hunter. Monoclonal anti-α-tubulin antibody was purchased from Sigma (St. Louis; T5168). Donkey-anti-rabbit::Cy3 conjugated secondary antibody used for indirect immunofluorescence was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Protein extracts, immunoblotting, and immunoprecipitation: Cell lysates were prepared by glass bead lysis (Moreno *et al.* 1991) in lysis buffer (20 mm HEPES, pH 7.0, 50 mm potassium acetate, 5 mm magnesium acetate, 100 mm sorbitol, 0.1% Triton X-100) with the addition of 1 mm ATP; 1 mm dithiothreitol (DTT); 2 μg/ml of leupeptin, pepstatin, and aprotinin; and 1 mm phenylmethylsulfonyl fluoride. Lysates were cleared by centrifuging at 20,000 × *g* for 20 min at 4°. When noted, total protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). For Western blotting, samples were fractionated by SDS-PAGE (National Diagnostic Protogel) and transferred to Immobilon-P (Millipore, Bedford, MA). Detection was carried out using anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Sigma or Jackson Labs) and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Immunoprecipitations were performed as described previously (Sherman and Forsburg 1998; Sherman *et al.* 1998) and washed with either lysis buffer or modified RIPA buffer (50 mm Tris, pH 7.5, 150 mm sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate).

Soluble/insoluble treatment: To compare soluble and insoluble fractions of yeast lysates, we modified the standard procedure (Moreno *et al.* 1991). For each sample, lysate was prepared from 1.5×10^8 cells in 250 μl buffer. Before the lysates were cleared, we removed unlysed cells and cell debris by centrifuging at 1000 × *g* for 1 min at 4°. After the lysates were cleared by centrifuging at 20,000 × *g* for 20 min at 4°, the soluble fraction was removed (1× volume) and the pellet was washed once in 1× volume lysis buffer. The pellet was then either resuspended in 1× volume 8 m urea or treated with DNase I, micrococcal nuclease, or NaCl as described below. DNase I (277 units; Sigma) was added to the pellet and treated for 10 min at 25° in 20 mm HEPES, pH 7.9, 1.5 mm magnesium acetate, 50 mm potassium acetate, 10% glycerol, 0.5 mm DTT, 150 mm NaCl, and protease inhibitors. Micrococcal nuclease (250 units; Amersham) was added to the pellet and treated for 3 min at 30° in 10% glycerol, 1 mm calcium chloride, 100 mm potassium chloride, 50 mm HEPES, pH 7.9, 2.5 mm magnesium chloride, 0.25% Triton X-100, and protease inhibitors. For treatment with 1 m sodium chloride, the pellet was resuspended in 10% glycerol, 1 m sodium chloride, 50 mm HEPES, pH 7.9, 2.5 mm magnesium chloride, 0.25% Triton X-100, and protease inhibitors and incubated on ice for 20 min. After treatment, the extractable material was separated from the pellet with another 20-min 20,000 × *g* clearing spin. The pellet was washed once in 1× volume buffer and resuspended in 1× volume 8 m urea. Ten microliters of each sample was fractionated on an 8% SDS-PAGE gel.

Flow cytometry, microscopy, and indirect immunofluorescence: Cells were fixed in 70% ethanol and stained for flow cytometry as described (Sazer and Sherwood 1990), except that the cells were stained in a final concentration of 1 μm Sytox Green (Molecular Probes, Eugene, OR). We used a Becton Dickinson FACScan and CellQuest software to analyze the data (The Salk Institute Center for Cytometry and Molecular Imaging). Fixed cells were rehydrated in 50 mm sodium citrate and then resuspended in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PEMBAL (100 mm Pipes, pH 6.9, 1 mm EDTA, 1 mm magnesium sulfate, 1% BSA, 0.1% sodium azide, 100 mm lysine hydrochloride) for microscopy. Indirect immunofluorescence was performed as described (Pasion and Forsburg 1999).

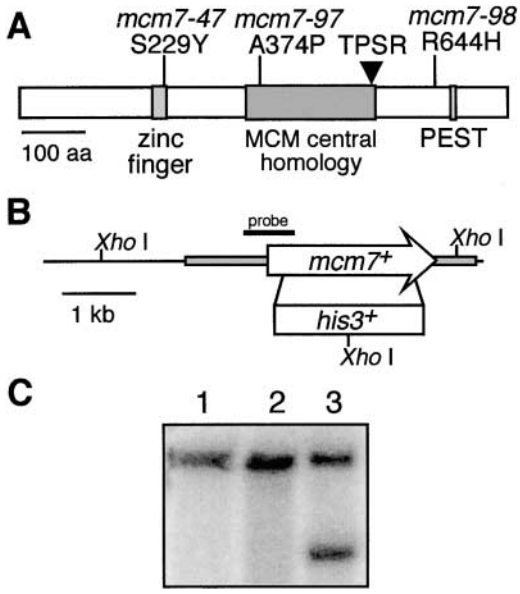

Figure 1.—Structure and disruption of *mcm7*$^+$. (A) Schematic of Mcm7p. Locations of zinc finger ($CX_2CX_{18}CX_4C$), MCM central homology domain, and putative PEST sequence are shown. The triangle denotes a consensus Cdc2 phosphorylation site (546-TPSR-549). The locations of the *mcm7-47*, *mcm7-97*, and *mcm7-98* lesions are shown. (B) Schematic of *mcm7*$^+$ locus. Location of *his3*$^+$ in the Δ*mcm7::his3*$^+$ disruption is shown. Shaded box and *mcm7*$^+$ arrow indicate fragment used to disrupt *mcm7*. The *Sca*I fragment used to probe the Southern blot in C is indicated above the map. (C) Southern blot analysis of chromosomal *mcm7* deletion. Genomic DNAs from a wild-type haploid (lane 1), wild-type diploid (lane 2), and Δ*mcm7::his3*$^+$/*mcm7*$^+$ diploid (lane 3) were digested with *Xho*I and then resolved on a 0.7% agarose gel. DNAs were blotted to membrane and hybridized with the probe fragment indicated in B. Bands were visualized by phosphorimager.

RESULTS

Cloning of fission yeast *mcm7*$^+$: We cloned *mcm7*$^+$, the last uncharacterized MCM in *S. pombe*, using colony hybridization of a fission yeast genomic library (Barbet *et al.* 1992). Our probe was a previously published sequence fragment that we amplified using PCR (Adachi *et al.* 1997). We isolated several overlapping clones, one of which contained the entire *mcm7*$^+$ gene with flanking sequences. The fission yeast *mcm7*$^+$ ORF is 2283 bp, without any introns. Conceptual translation indicates that Mcm7p is 760 amino acids with a predicted molecular weight of 83.6 kD. As is the case with the other MCM subclasses, fission yeast Mcm7p is more similar to its orthologs in other species than to the other fission yeast MCM proteins. Mcm7p contains several domains depicted in Figure 1A, including the MCM central homology domain, which includes an ATPase domain found in each of the MCM proteins, a zinc finger, and one

consensus CDK phosphorylation site (S/T)PX(K/R). The Mcm7p sequence does not contain any obvious nuclear localization signals. Between amino acids 667 and 679 there is a weak PEST sequence (PESTfind score +1.76, Rechsteiner and Rogers 1996).

Disruption of fission yeast *mcm7*$^+$: We determined if *mcm7*$^+$ is essential for viability by replacing the coding sequence for amino acids 82–673 of Mcm7p with the *his3*$^+$ gene (Figure 1B). We confirmed that we had disrupted the *mcm7*$^+$ locus by Southern analysis (Figure 1C). A 0.7-kb *Sca*I fragment containing the coding start site was used as a probe (Figure 1B). The *mcm7*$^+$ gene is present on an ~4.8-kb *Xho*I fragment. Two bands were detected in the diploid heterozygous for Δ*mcm7::his3*$^+$ (Figure 1C). The upper band corresponds to the 4.8-kb *Xho*I fragment of the wild-type gene locus, whereas the lower band corresponds to the 3.5-kb *Xho*I fragment of the null allele, due to the introduction of a *Xho*I site in *his3*$^+$. Of nine tetrads dissected, all showed 2:2 segregation of viable:inviable spores and all of the viable spores were His$^-$. Microscopically, the dead spores typically consisted of a single cell with a partially extended germination tube; thus *mcm7*$^+$ is an essential gene and the null mutant arrests during the first cell cycle. Expression of genomic *mcm7*$^+$ from a plasmid was able to complement the lethality of this disruption (data not shown).

To determine the arrest point of Δ*mcm7* spores, we performed a spore germination experiment using the Δ*mcm7::his3*$^+$*/mcm7*$^+$ diploid and a wild-type *his3*$^+$*/his3-D1* diploid control. We prepared spores from the two diploid strains and inoculated the spores into media lacking histidine. This allows only the wild-type *his3*$^+$ or Δ*mcm7::his3*$^+$ spores to germinate. The ability of germinating spores to synthesize DNA and divide can be analyzed by flow cytometry and microscopy. As shown in Figure 2, His$^+$ spores from the wild-type diploid completed S phase between 6 and 8 hr. The Δ*mcm7* spores entered S phase with delayed timing relative to the wild-type spores, arresting with an ~2C DNA content. Although the Δ*mcm7* cells initiate DNA synthesis and appear to have a replicated DNA content, the cells arrest with a single nucleus and do not go on to divide. No evidence for premature mitotic entry or a <1C phenotype was observed, in contrast to initiation mutants such as *cdc18* (Kelly *et al.* 1993). This suggests that in the Δ*mcm7* spores DNA replication is not complete and a checkpoint has been activated in response to incomplete DNA replication or DNA damage; this is consistent with observations of the other Δ*mcm* spores (Sherman and Forsburg 1998; Liang *et al.* 1999).

Isolation of a temperature-sensitive allele of *mcm7*: To facilitate genetic analysis of *mcm7*$^+$, we isolated a temperature-sensitive allele. In budding yeast, the *mcm7* mutant *cdc47-1* is the result of a single-amino-acid substitution of serine 286 to tyrosine (Moir *et al.* 1982; Dalton and Hopwood 1997). We constructed *in vitro* the

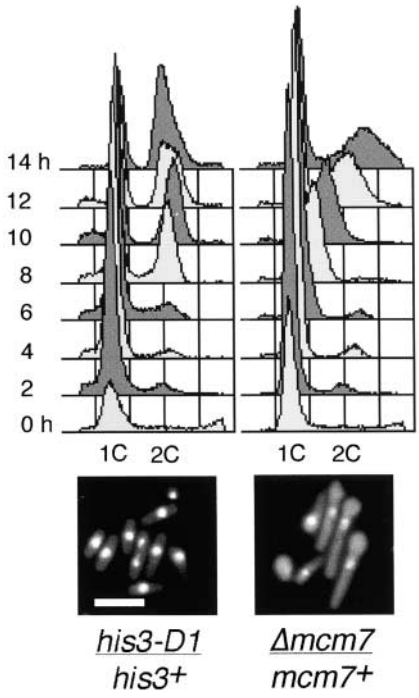

Figure 2.—Spore germination of Δ*mcm7* spores. Δ*mcm7* spores undergo a delayed S phase. Spores prepared from wild-type (*his3-D1/his3*$^+$) and Δ*mcm7* (Δ*mcm7/mcm7*$^+$) diploids were inoculated into medium lacking histidine at 32°. Samples were taken every 2 hr for 14 hr and analyzed by flow cytometry and microscopy. The persistent 1C peak in each of the panels is from the His$^-$ spores that cannot germinate. Photomicrographs are of DAPI-stained spores after 12 hr at 32°. Bar, 10 μm.

equivalent mutation in fission yeast Mcm7p (S229Y) and tested the ability of the mutant (*mcm7-47*) to complement the null allele Δ*mcm7::his3*$^+$. However, *mcm7-47* did not complement the null at 25°, suggesting that in fission yeast, this mutation results in a nonfunctional protein. We therefore generated a conditional allele of *mcm7*, using a plasmid shuffle strategy (see materials and methods). Out of 10,000 transformants screened, we identified three temperature-sensitive plasmids. We sequenced the entire *mcm7* open reading frame to determine the mutations in the temperature-sensitive alleles and found that two of the three mutants were identical. *mcm7-97* consisted of a G to C transition resulting in a change of codon 374 from alanine to proline. *mcm7-98* consisted of a G to A transition resulting in a change of codon 644 from arginine to histidine. We replaced the wild-type chromosomal copy of *mcm7*$^+$ with *mcm7-98* as described in materials and methods and confirmed the structure by Southern blot (data not shown). *mcm7-97* could not be recovered in the chromosome, suggesting that this mutant is nonfunctional in single copy. *mcm7-98* clones formed colonies at a similar rate to wild type on minimal media at the permissive temperature of 25° (Figure 3A). *mcm7-98* is unable to form colonies on minimal media at 29° or higher temperatures, indi-

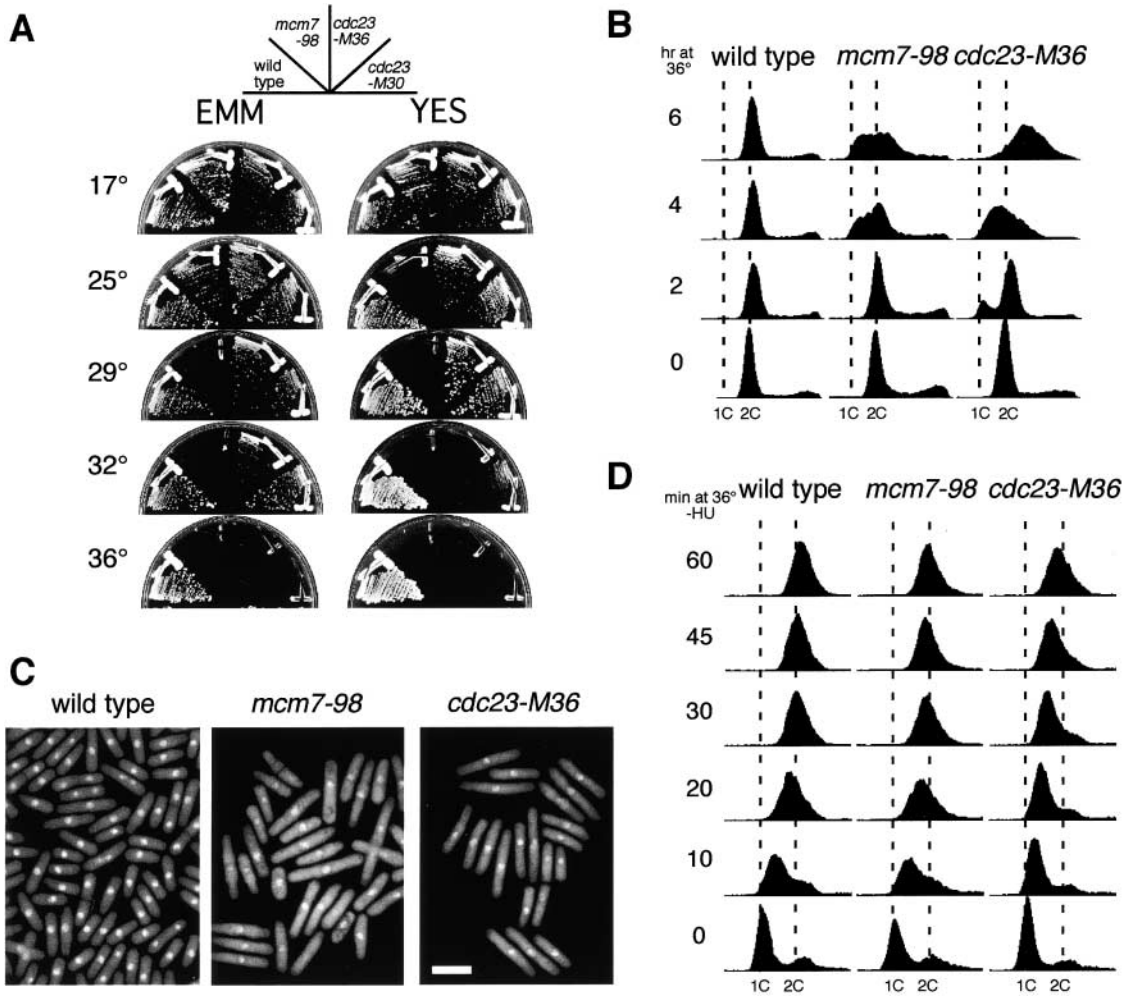

FIGURE 3.—*mcm7-98* and *cdc23ts* arrest phenotype. (A) *mcm7-98*, *cdc23-M36*, and *cdc23-M30* cells are temperature and media sensitive. Wild-type, *mcm7-98*, *cdc23-M36*, and *cdc23-M30* cells were streaked onto minimal (EMM) plates at 17° for 17 days; rich (YES) plates at 17° for 10 days; EMM plates at 25° for 5 days; and YES plates at 25° or both EMM and YES plates at 29°, 32°, or 36° for 3 days. (B) Flow cytometry of *mcm7-98* and *cdc23-M36* cells. Cells were grown at 25° in minimal media before being shifted to 36° for 6 hr. Aliquots were removed at 0, 2, 4, and 6 hr and analyzed by flow cytometry. The positions of 1C and 2C DNA contents are indicated. (C) *mcm7-98* and *cdc23-M36* cells arrest with a heterogeneous phenotype. Photomicrographs are of DAPI-stained cells after 6 hr at 36°. Bar, 10 μm. (D) *cdc23-M36* cells complete S phase slowly after hydroxyurea block and release. Wild-type, *mcm7-98*, and *cdc23-M36* cells were grown at 25° in minimal media, then treated with 15 mM hydroxyurea for 4 hr, shifted to 36° for 30 min, and then washed out of hydroxyurea and returned to 36°. Aliquots were removed 0, 10, 20, 30, 45, and 60 min after release from hydroxyurea and prepared for flow cytometry. The positions of 1C and 2C DNA contents are indicated.

cating that 29° is the restrictive temperature. Although the *mcm7-98* strain formed colonies at a similar rate to wild type on rich media at 17°, it is unable to form colonies on rich media at 25° or higher temperatures (data not shown). Perhaps the cells grow more slowly on minimal media and are thus able to repair any problems in replication. However, this phenotype is observed only on solid media, since the growth rate of *mcm7-98* is the same in both rich and minimal liquid media at 25° (data not shown).

Genetic interactions of *mcm7-98*: In fission yeast and budding yeast, there is a network of genetic interactions among conditional alleles of MCMs and other initiation genes that predict physical associations. We examined the effects of *mcm7-98* in combination with other mutants (Table 2). We were unable to construct double mutants of either *mcm2ts (cdc19-P1)*, *mcm4ts (cdc21-M68)*, or *mcm6ts (mis5-268)* with *mcm7-98*, suggesting *mcm7-98* is synthetically lethal when combined with these other *mcm* mutants. Previous work showed that *mcm2ts (cdc19-P1)* and *mcm4ts (cdc21-M68)* are synthetically lethal with each other (FORSBURG and NURSE 1994), as are *mcm4ts (cdc21-M68)* and *mcm6ts (mis5-268)* (TAKAHASHI *et al.* 1994).

We next investigated whether there were genetic interactions between *mcm7-98* and mutants in other proteins implicated in origin function and DNA metabolism (Table 2). We combined *mcm7-98* with *rad4-116* (check-

TABLE 2

Summary of genetic interactions of *mcm7-98* and *cdc23-M36* mutants

Mutant	Function	*mcm7-98*	*cdc23-M36*	*mcm4-M68*
Δ*rad3*	Checkpoint kinase	SL	Red. RT	NT
Δ*chk1*	Checkpoint kinase	SL	None	NT
Δ*cds1*	Checkpoint kinase	SL	None	None
cdc19-P1	MCM2	SL	None[a]	SL[b]
mcm4-M68	MCM4	SL	SL	
mis5-268	MCM6	SL	NT	SL[c]
mcm7-98	MCM7		None	SL
hsk1-1312	Cdc7 kinase	Slow at 25°	Red. RT[d]	None[d]
rad4-116	Initiation/checkpoint protein	None	Red. RT	Red. RT
orp1-4	ORC1	None	None	Red. RT[e]
Δ*rqh1*	DNA helicase	Slow at 25°	NT	Red. RT[f]

Genetic interactions were determined by comparing growth of the double mutant strains to the parental strains at 25°, 29°, 32°, 34°, and 36°. None, no interaction; SL, synthetic lethal; Red. RT, reduced restrictive temperature; NT, not tested.

[a] Forsburg *et al.* (1977).
[b] Forsburg and Nurse (1994).
[c] Takahashi *et al.* (1994).
[d] Snaith *et al.* (2000).
[e] Grallert and Nurse (1996).
[f] J. M. Bailis, personal communication.

point/replication factor), *orp1-4* (ORC component), *hsk1-1312* (Cdc7 kinase), and Δ*rqh1* (RecQ DNA helicase; Fenech *et al.* 1991; Grallert and Nurse 1996; Murray *et al.* 1997; Stewart *et al.* 1997; Snaith *et al.* 2000). We did not observe any synthetic phenotypes in the *mcm7-98 orp1-4* or *mcm7-98 rad4-116* double mutants but noted that both the *mcm7-98 hsk1-1312* and *mcm7-98* Δ*rqh1* double mutants grew more slowly at 25° than did either parent.

In budding yeast, MCM7 interacts physically and genetically with MCM10 (Merchant *et al.* 1997; Homesley *et al.* 2000). Despite its name, MCM10 is not a member of the MCM family and has no homology to MCM2–7 but is thought to associate with the MCM complex. The fission yeast homolog of *MCM10* is *cdc23*$^{+}$ (Aves *et al.* 1998), for which there are two mutant alleles, *M30* and *M36* (Nasmyth and Nurse 1981). We cloned these by PCR and sequenced them. To avoid PCR-generated mutations, each reaction was carried out in duplicate, and the product of each PCR reaction was independently cloned. Based on this analysis, the *cdc23-M36* allele changes a conserved aspartic acid to glycine (D232G). The *cdc23-M30* allele changes a conserved leucine to proline (L187P). Both mutations are in the MCM10 central domain (Izumi *et al.* 2000). As was the case with *mcm7-98*, the *cdc23ts* alleles had different maximum permissive temperatures on minimal *vs.* rich plates (Figure 3A). The maximum permissive temperature for either *cdc23ts* strain was 32° on minimal plates and 29° on rich plates. We combined *mcm7-98* with both temperature-sensitive alleles of *cdc23*; in neither case did we observe genetic interactions (Table 2; data not shown). We also crossed *cdc23-M36* with other initiation mutants (Table 2). There is no genetic interaction between *cdc23-M36* and *orp1-4*. However, *cdc23-M36* and *mcm4ts (cdc21-M68)* are synthetically lethal, and the *cdc23-M36 rad4-116* double mutant has a reduced restrictive temperature. Because of the interaction of *cdc23-M36* with both *rad4-116* and *mcm4ts (cdc21-M68)*, we constructed the *mcm4ts rad4-116* double mutant and observed that it too had a reduced restrictive temperature. The double mutants of *hsk1-1312 cdc23-M36* and *hsk1-1312 rad4-116* also exhibit reduced restrictive temperature (Snaith *et al.* 2000), suggesting these factors interact in a network to regulate initiation (see discussion).

Arrest phenotype of *mcm7-98* and *cdc23-M36*: We examined the arrest phenotypes of *mcm7-98* and *cdc23-M36* in liquid culture. Wild-type and mutant *mcm7-98* and *cdc23-M36* cells were shifted from the permissive temperature to the restrictive temperature and samples were taken for flow cytometry and microscopic analysis (Figure 3, B and C). Wild-type cells maintained a 2C DNA content throughout the 36° growth period. *mcm7-98* cells remained 2C through 2 hr at the restrictive temperature; by 4 hr, a distinct shoulder of cells with a DNA content between 1C and 2C appeared. By 6 hr, about one-half of the cells had an intermediate DNA content, suggesting an S phase delay. We also observed an S phase delay in *cdc23-M36* cells. There was a small peak of cells with 1C DNA content after 2 hr at the restrictive temperature, and by 4 hr there was a broad peak of cells with between 1C and 2C DNA content. However, in contrast to the *mcm7-98* strain, the *cdc23-M36* cells arrested at 6 hr with an elongated 2C DNA content peak. Although the *mcm7-98* and *cdc23-M36* strains arrested with different flow cytometry profiles,

the cells had similar morphologies. When we stained the cells with DAPI, we observed that most, but not all, of the cells were elongated. This mixed-arrest phenotype is similar to that observed in *mcm6ts (mis5-268)* (Takahashi *et al.* 1994) and distinct from the uniform elongated phenotype of *mcm2ts (cdc19-P1)* (Forsburg and Nurse 1994) and *mcm4ts (cdc21-M68)* (Coxon *et al.* 1992).

We treated wild-type and mutant *mcm7-98* and *cdc23-M36* cells with hydroxyurea to determine if they can complete DNA synthesis at the restrictive temperature after an early S phase block. Asynchronous cells were treated with hydroxyurea at the permissive temperature to block them in early S phase. Cells were shifted to the restrictive temperature for 30 min to inactivate the mutant proteins and then the hydroxyurea was washed out of the culture and the cells were released to the restrictive temperature (Figure 3D). Wild-type cells rapidly enter S phase when released from hydroxyurea and complete S phase within 30 min. *mcm7-98* cells enter and complete S phase with timing similar to that of wild-type cells. *cdc23-M36* cells were also able to complete S phase, albeit slower than both wild-type and *mcm7-98* cells.

We crossed *mcm7-98* to the checkpoint mutants Δ*rad3*, Δ*chk1*, and Δ*cds1* to see if the arrest was dependent on replication or damage checkpoints. Rad3 (ATM/ATR homolog) is required for both checkpoints and is thought to act upstream of Chk1 and Cds1; Cds1 responds to the replication checkpoint and Chk1 to damage (reviewed in Rhind and Russell 2000). We could not recover double mutants of *mcm7-98* with any of the checkpoint mutants, despite screening several hundred spores by random spore analysis or by tetrad analysis and plating on minimal plates at 25°, because of the *mcm7-98* media sensitivity. Thus, *mcm7-98* is synthetically lethal with Δ*rad3*, Δ*chk1*, and Δ*cds1* (Table 2). The inability to recover double mutants of *mcm7-98* with the checkpoint mutants suggests that both the replication and damage checkpoint pathways are required to maintain the viability of *mcm7-98* cells under all conditions, suggesting *mcm7-98* has significant defects even at 25°. Interestingly, no synthetic defect was observed in a double mutant Δ*cds1 mcm4ts* (Table 2) or Δ*cds1 mcm2ts* (Liang *et al.* 1999), so this defect is not common to other MCMs.

To investigate whether the Cds1 kinase is activated in the *mcm7-98* mutant, we compared Cds1 kinase activity in wild-type, *mcm2ts (cdc19-P1)*, and *mcm7-98* cells. We assayed Cds1 activity by monitoring phosphorylation of the amino terminus of bacterially produced Wee1p (Boddy *et al.* 1998). Cds1 was activated as expected in a hydroxyurea-treated wild-type control, but kinase activity was not observed in *mcm2ts (cdc19-P1)* or *mcm7-98* at either the permissive or restrictive temperature (Figure 4).

In contrast to *mcm7-98*, we were able to recover double

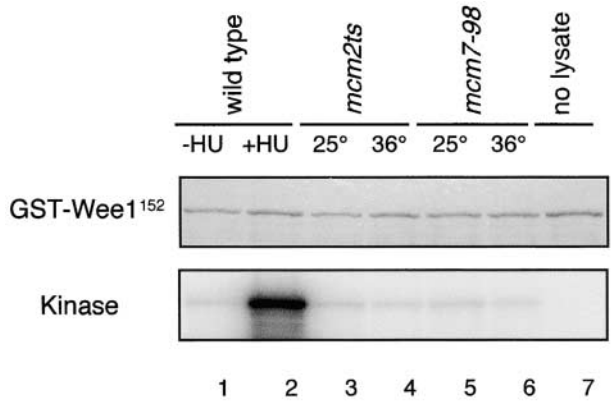

Figure 4.—Cds1 kinase activity. Cds1 activity was assayed by its ability to bind and phosphorylate the amino terminus of Wee1 (Boddy *et al.* 1998). Total lysate (275 μg) was incubated with bacterially produced GST:Wee1^{152} bound to GSH-Sepharose. The GSH-Sepharose was washed and assayed for associated kinase activity. Reaction products were fractionated by SDS-PAGE, stained with Coomassie to visualize GST-Wee1^{152} substrate (top), and exposed to a PhosphorImager screen (bottom). Lysates were prepared from wild-type cells (lane 1), wild-type cells treated with 12 mm hydroxyurea for 3 hr (lane 2), *mcm2ts (cdc19-P1)* cells grown at 25° and 36° for 6 hr (lanes 3 and 4), and *mcm7-98* cells grown at 25° and 36° for 6 hr (lanes 5 and 6). Lane 7 is no lysate control.

mutants of *cdc23-M36* with each of the checkpoint mutants. The *cdc23-M36* Δ*cds1* double mutants arrested at the restrictive temperature as elongated cells, similar to the parent *cdc23-M36* strain (data not shown). However, when *cdc23-M36* was combined with either Δ*rad3* or Δ*chk1* and shifted to the restrictive temperature, the cells no longer arrested as elongated cells but underwent aberrant divisions (data not shown). This is typical of late S phase mutants that require the damage checkpoint for arrest (*e.g.*, Maiorano *et al.* 1996; Gould *et al.* 1998; Liang *et al.* 1999). Thus, the arrest of *cdc23-M36* at the restrictive temperature requires the Rad3 and Chk1 kinases, both part of the damage checkpoint pathway. Interestingly, the *cdc23-M36* Δ*rad3* double mutants had a reduced restrictive temperature compared to the parent strains.

Previously we observed that temperature-sensitive alleles of *cdc* genes involved in replication initiation lost viability when grown at the restrictive temperature, indicating that defects in the initiation of DNA replication may lead to abnormal DNA synthesis and irreparable damage to cells (Liang *et al.* 1999). We compared the ability of *mcm7-98*, *cdc23-M36*, wild-type, and *mcm4ts (cdc21-M68)* cells to form colonies after being incubated at the restrictive temperature. Typical results of a viability experiment are shown in Figure 5A. Wild-type cells continue to grow and increase in number at the restrictive temperature. *cdc23-M36* cells did not increase in number or lose viability through 4 hr but after 6 hr at the restrictive temperature decreased to ~30% relative viability. Loss of viability was more pronounced in the

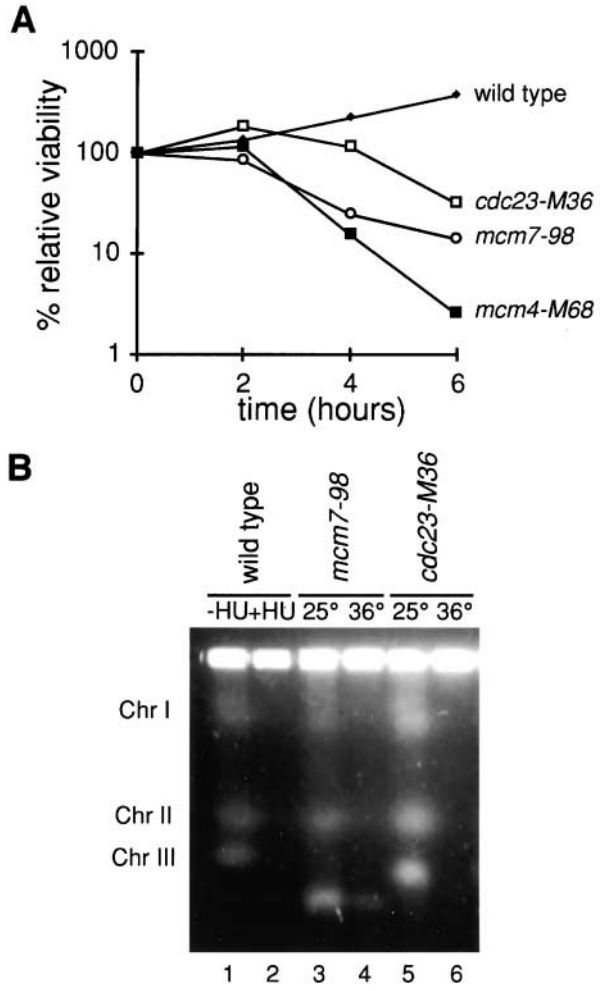

FIGURE 5.—*mcm7-98* and *cdc23-M36* viability and chromosome gel. (A) *mcm7-98* and *cdc23-M36* lose viability at the restrictive temperature. Wild-type (solid diamonds), *mcm7-98* (open circles), *cdc23-M36* (open squares), and *mcm4ts (cdc21-M68)* (solid square) cells were grown in minimal media and shifted from 25° to 36° at time zero. Aliquots were taken and plated onto minimal medium at 25° to determine percentage of relative viability. Percentage of relative viability was calculated as (number of colonies at time *t*) × 100/(no. of colonies at time zero). (B) Chromosomes from *mcm7-98* and *cdc23-M36* cells do not enter a pulsed-field gel at 25° or 36°. Chromosomes (Chr) were prepared and fractionated by pulsed-field gel electrophoresis as described in MATERIALS AND METHODS. Lanes 1 and 2, wild-type cells treated without and with 20 mM hydroxyurea (HU) for 6 hr at 32°. Lanes 3 and 4, *mcm7-98* cells grown at 25° and 36° for 6 hr. Lanes 5 and 6, *cdc23-M36* cells grown at 25° and 36° for 6 hr.

mcm mutants. After 4 hr at the restrictive temperature, the *mcm4ts (cdc21-M68)* and *mcm7-98* strains were similar. However, after 6 hr, *mcm4ts (cdc21-M68)* dropped to ~3% relative viability; *mcm7-98* only decreased to 14% relative viability.

We used pulsed-field gel analysis to examine the state of the chromosomes in our strains. We prepared chromosomes from wild-type cells, wild-type cells treated with hydroxyurea, and from *mcm7-98* and *cdc23-M36* cells grown at 25° or shifted to 36° for 6 hr (Figure 5B). In wild-type cells, all three of the chromosomes were able to migrate into the gel. In contrast, the chromosomes from wild-type cells treated with hydroxyurea did not enter the gel, consistent with the observation that hydroxyurea causes paused replication forks, resulting in unresolved replication intermediates (KELLY *et al.* 1993; LIANG *et al.* 1999). Chromosomes prepared from each of the temperature-sensitive strains grown at the permissive temperature were able to migrate into the pulsed-field gel as well as wild type. Notably, chromosome III has an increased mobility in the *mcm7-98* strain at the permissive temperature. This may reflect recombination in the rDNA region and is typical of a number of early replication mutants including *mcm2ts* (LIANG *et al.* 1999) and *hsk1ts* (SNAITH *et al.* 2000). At the restrictive temperature, the chromosomes prepared from *mcm7* cells did not migrate into the gel, similar to chromosomes prepared from hydroxyurea-treated wild-type cells. Thus the chromosomes from *mcm7-98*- or *cdc23-M36*-arrested cells likely contain replication intermediates; this result is consistent with the observation that the cells arrest with an S phase delay.

Mcm7p localization: Fission yeast MCM proteins, like metazoan MCM proteins, have been shown to be localized in the nucleus throughout the cell cycle, and their localization is interdependent (PASION and FORSBURG 1999). We determined the cellular localization of fission yeast Mcm7p by performing indirect immunofluorescence microscopy, using polyclonal antibodies to Mcm7p (see below). In an asynchronous population of wild-type cells, cells in all stages of the cell cycle show nuclear localization of Mcm7p (Figure 6, A and B). To see whether wild-type Mcm7p localization depends on the activity of other MCM proteins, we examined temperature-sensitive strains of *mcm2ts (cdc19-P1)*, *mcm4ts (cdc21-M68)*, or *mcm6ts (mis5-268)* grown at 36°. In each of the *mcmts* backgrounds, Mcm7p showed increased cytoplasmic staining at the restrictive temperature compared to the permissive temperature (Figure 6, D, F, and H). Mcm7^{ts}p also shows reduced nuclear staining when the *mcm7-98* strain is shifted to the restrictive temperature (Figure 6J). In contrast, mutation of *cdc23* does not affect Mcm7p localization (data not shown).

Mcm7p protein levels and complex interactions: To characterize the Mcm7 protein, we raised polyclonal antibodies to a bacterially produced protein fragment corresponding to the N-terminal half of Mcm7p, a region that shares little homology with the other fission yeast MCM proteins. Affinity-purified antibodies recognize a band of 97 kD, slightly larger than the predicted molecular weight of 83.6 kD (Figure 7A). To confirm the specificity of the antibody on Western blots, we compared lysates from wild-type cells transformed with pSLF173 (vector control), wild-type cells transformed with pDTL87 (*nmt-mcm7HA*) and grown in thiamine to repress the plasmid promoter to a low level of expres-

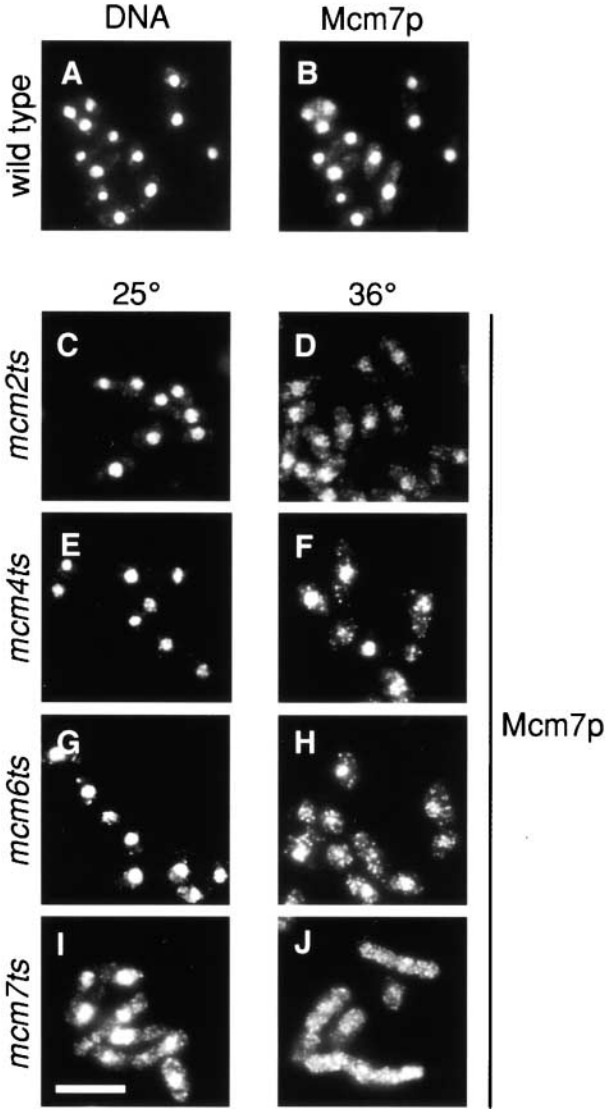

FIGURE 6.—Localization of Mcm7p. Cells were prepared for indirect immunofluorescence and stained with DAPI to detect DNA or with anti-Mcm7p antibody. (A and B) Mcm7p is nuclear throughout the cell cycle. Asynchronous wild-type cells were stained with (A) DAPI or (B) anti-Mcm7p antibody. (C–J) Mcm7p is more cytoplasmic in *mcmts* strains at 36°. Cells were grown at 25° (C, E, G, I) or 36° (D, F, H, J) for 4 hr and stained with anti-Mcm7p antibody. (C and D) *mcm2ts (cdc19-P1)*. (E and F) *mcm4ts (cdc21-M68)*. (G and H) *mcm6ts (mis5-268)*. (I and J) *mcm7-98*. Bar, 10 μm.

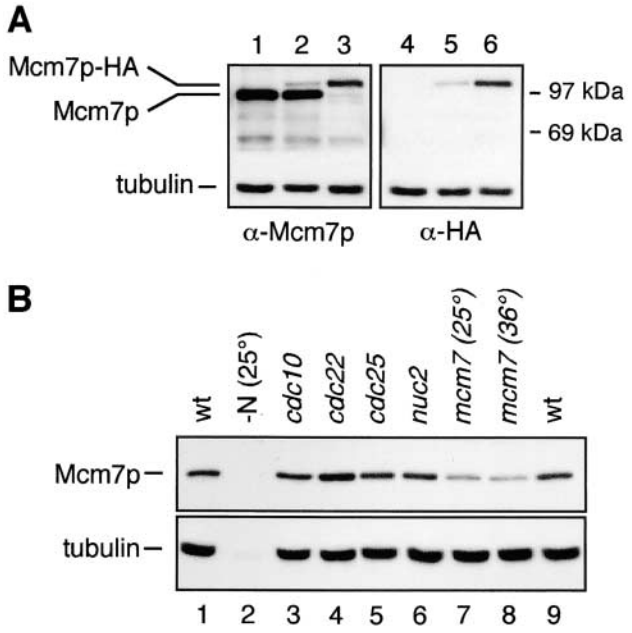

FIGURE 7.—Mcm7p characterization. (A) Characterization of antibodies to Mcm7p. Cell lysates were prepared and 10 μg of total protein was separated by SDS-PAGE. Lanes 1 and 4, lysate from wild-type cells transformed with vector control (pSLF173). Lanes 2 and 5, lysate from wild-type cells transformed with *nmt-mcm7HA* grown in the presence of thiamine (pDTL87). Lanes 3 and 6, lysate from wild-type strain with endogenous copy of *mcm7*$^+$ replaced with *mcm7HA* (FY1021). Duplicate filters were immunoblotted with anti-Mcm7p (lanes 1–3) or anti-HA (lanes 4–6) antibodies. All filters were immunoblotted with anti-tubulin as a loading control. (B) Mcm7p levels do not vary with the cell cycle but are decreased in *mcm7-98*. Cell lysates were prepared and 10 μg of total protein was separated by SDS-PAGE. Strains were grown at 36° for 4 hr unless otherwise indicated. Lane 1, wild-type asynchronous cells; lane 2, wild-type cells starved in minimal media lacking nitrogen overnight at 25°; lane 3, *cdc10-V50* (G_1 arrest); lane 4, *cdc22-M45* (early S phase arrest); lane 5, *cdc25-22* (G_2/M arrest); lane 6, *nuc2-663* (M phase arrest); lane 7, *mcm7-98* at 25°; lane 8, *mcm7-98*; lane 9, wild-type asynchronous cells. Filters were immunoblotted with anti-Mcm7p and anti-tubulin antibodies.

sion, and a strain in which the endogenous copy of *mcm7*$^+$ has been replaced with an epitope-tagged version of the gene (FY1021). The Mcm7p antibodies recognize a single band in vector control lysate, a major band and a weaker slower-migrating band in the *nmt-mcm7HA* lysate, and a single slower-migrating band in the integrated *mcm7HA* lysate. The slower-migrating band was confirmed to be the HA-tagged Mcm7p by immunoblotting with monoclonal antibodies to the HA epitope; its mobility is reduced due to the triple HA tag (Figure 7A).

We investigated the levels of Mcm7p throughout the cell cycle by preparing lysates from cells arrested at various points of the cell cycle (Figure 7B). Crude lysates were prepared from asynchronous wild-type cells, wild-type cells starved for nitrogen, and the following temperature-sensitive strains grown at the restrictive temperature (NURSE *et al.* 1976; FANTES 1979; NASMYTH and NURSE 1981; HIRANO *et al.* 1988): *cdc10-V50* (transcription factor, arrests at G_1), *cdc22-M45* (ribonucleotide reductase, arrests early in S phase), *cdc25-22* (CDK phosphatase, arrests at G_2/M), and *nuc2-663* (APC component, arrests in M phase). Mcm7p is not detectable in cells starved for nitrogen, but levels remain constant in the other cell cycle arrests. We also examined *mcm7-98* cells to see whether Mcm7p levels change upon the shift to the restrictive temperature (Figure 7B, lanes 7 and 8). Mcm7p levels in *mcm7-98* are reduced compared to

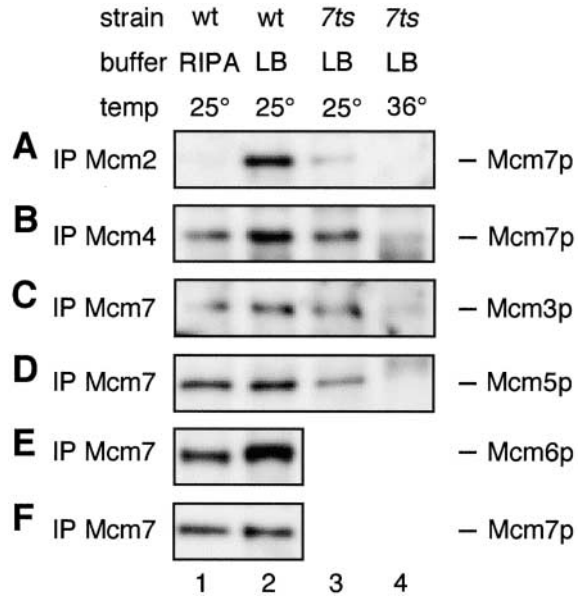

FIGURE 8.—Mcm7p coimmunoprecipitations. Mcm7p interacts with each of the other MCM proteins. Lysates were prepared from wild-type asynchronous cells (lanes 1 and 2) and *mcm7-98* cells grown at 25° (lane 3) and 36° (lane 4). Identical amounts of lysate were immunoprecipitated with the antibodies shown. Immunoprecipitates were washed stringently with modified RIPA buffer (lane 1) or nonstringently with lysis buffer (LB; lanes 2, 3, and 4). Samples were separated by SDS-PAGE. (A) Immunoprecipitation with anti-Mcm2p and immunoblot with anti-Mcm7p. (B) Immunoprecipitation with anti-Mcm4p and immunoblot with anti-Mcm7p. (C) Immunoprecipitation with anti-Mcm7p and immunoblot with anti-Mcm3p. (D) Immunoprecipitation with anti-Mcm7p and immunoblot with anti-Mcm5p. (E) Immunoprecipitation with anti-Mcm7p and immunoblot with anti-Mcm6p. (F) Immunoprecipitation with anti-Mcm7p and immunoblot with anti-Mcm7p.

wild type at the permissive temperature but do not change significantly at the restrictive temperature.

The MCM proteins form a multisubunit complex and associate with each other with different relative affinities. In fission yeast, as in other systems, Mcm4p and Mcm6p are tightly associated with each other, whereas Mcm2p is peripherally associated with Mcm4p and Mcm6p; Mcm3p and Mcm5p form a tight dimer and are also peripherally associated with Mcm4p and Mcm6p (SHERMAN and FORSBURG 1998; SHERMAN *et al.* 1998; LEE and HURWITZ 2000). The relative affinities can be distinguished from one another by using an immunoprecipitation protocol and washing with gentle (lysis buffer: low salt, low detergent) or harsh (modified RIPA buffer: moderate salt, high detergent) buffers. We used a coimmunoprecipitation strategy to place Mcm7p within the fission yeast MCM complex. We used antibodies against Mcm2p, Mcm4p, and Mcm7p to immunoprecipitate the cognate proteins from a wild-type lysate and then washed duplicate immunoprecipitates with either the gentle buffer or the harsh buffer (Figure 8). When the immunoprecipitates were washed with the gentle buffer, each of the other MCM proteins coimmunoprecipitated with Mcm7p. When the immunoprecipitates were washed with the stringent buffer, Mcm2p and Mcm7p no longer coimmunoprecipitated, but the other four MCM proteins (Mcm3p, Mcm4p, Mcm5p, and Mcm6p) still coimmunoprecipitated with Mcm7p. These results suggest that, as expected, Mcm7p interacts with each of the other members of the MCM complex but that the interaction with Mcm2p may be indirect. The interactions of Mcm7p with the other members of the MCM complex are relatively strong. We also looked at the interactions in lysates from the *mcm7-98* strain grown at 25° and 36° to see if Mcm7^{ts}p still interacts with the rest of the MCM complex. Not surprisingly, the interactions between Mcm7^{ts}p and the other MCM proteins were reduced at 36° (Figure 8, lane 4).

Cdc23p is associated with insoluble fraction: In budding yeast, the initiation factor MCM10 has been shown to interact physically with members of the MCM complex using either glutathione S-transferase (GST) precipitation experiments or yeast two-hybrid assays (MERCHANT *et al.* 1997). To see whether similar interactions exist with the fission yeast homolog Cdc23p, we used a strain in which Cdc23p was tagged with HA and integrated into the chromosomal locus; ectopic expression of Cdc23p-HA is able to complement the temperature sensitivity of cdc23-M36 and thus Cdc23p-HA is functional (data not shown). We planned to use a coimmunoprecipitation assay to determine Cdc23p-HA interactions with the MCM proteins; however, Cdc23p-HA was not detectable in the soluble fraction. Because MCM10 in budding yeast and mammalian cells is insoluble (HOMESLEY *et al.* 2000; IZUMI *et al.* 2000; KAWASAKI *et al.* 2000), we investigated whether *S. pombe* Cdc23p is also insoluble. We compared the solubility of Cdc23p against a well-characterized chromatin-bound protein, Orp1p, in a strain in which both Cdc23p and Orp1p are HA tagged. Multiple methods have been used to show that Orp1 is detected in both soluble and insoluble fractions of protein extracts and can be released from the insoluble fraction upon treatment with DNase I, salt, or micrococcal nuclease (GRALLERT and NURSE 1996; LYGEROU and NURSE 1999; OGAWA *et al.* 1999; NISHITANI *et al.* 2000). Figure 9A diagrams the soluble/insoluble fractionation that we used. Briefly, crude lysates were prepared and a short spin was used to remove whole cells. Insoluble proteins (P_1) were separated from the soluble proteins (S) by centrifugation. The insoluble proteins were then either analyzed or treated and extractable proteins (E) were separated from insoluble proteins (P_2) by centrifugation. Figure 9B shows that Orp1p-HA and Cdc23p-HA are each detectable in the insoluble fractions of the parent and *cdc23HA orp1HA* cells. We immunoblotted fractions prepared from asynchronous *cdc23HA orp1HA* cells with antibodies to HA, tubulin, Mcm5p, and Mcm7p (Figure 9C). Cdc23p-HA

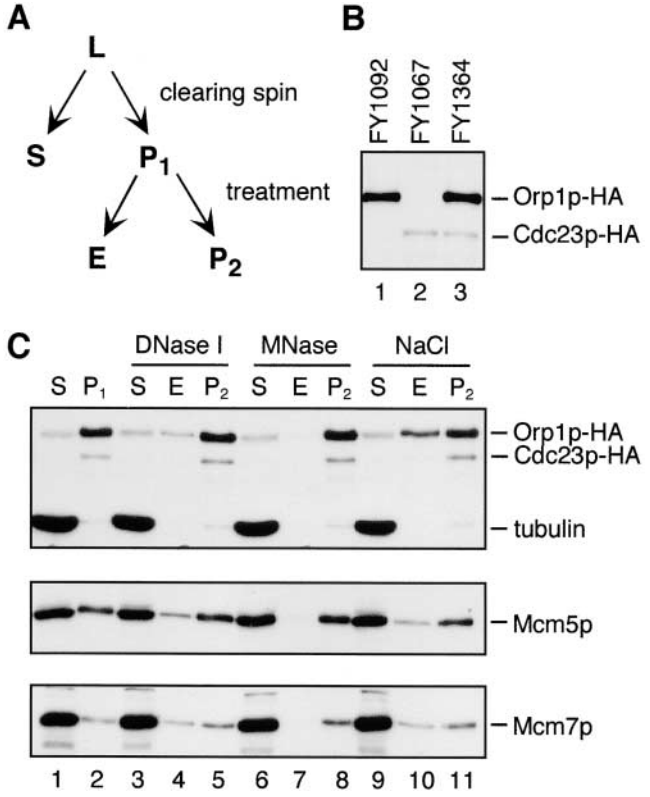

FIGURE 9.—Solubilization of Cdc23p-HA. (A) Schematic of soluble/insoluble fractionation. After a short spin to remove whole cells, crude lysate (L) was cleared by centrifugation. The soluble fraction (S) was removed (1× volume) and the pellet was washed. The pellet (P_1) was then either resuspended in 1× volume 8 M urea or treated. After treatment, the extractable fraction (E) was removed after centrifugation and the pellet (P_2) was washed and resuspended in 1× volume 8 M urea. (B) The pellet (P_1) fraction was prepared from *Orp1pHA* (lane 1), *Cdc23HA* (lane 2), and *Orp1HA Cdc23HA* (lane 3) cells and separated by SDS-PAGE and filters were immunoblotted with anti-HA antibodies. (C) Lysate was prepared from FY1364 (*Cdc23HA Orp1HA*), fractionated as in A, and not treated (lanes 1 and 2), treated with DNase I (lanes 4 and 5), treated with micrococcal nuclease (MNase; lanes 7 and 8), or with 1 M NaCl (lanes 10 and 11). Fractions were separated by SDS-PAGE and filters were immunoblotted with anti-HA, anti-tubulin, anti-Mcm5p, or anti-Mcm7p antibodies.

was detected exclusively in the insoluble pellet fraction. Mcm7p and Mcm5p were detected in both the supernatant and insoluble pellet fractions but were mostly soluble. Orp1p-HA was found in both fractions slightly enriched in the insoluble pellet fraction, whereas tubulin was detected mostly in the supernatant fraction. The pellet fraction in this simple procedure contains insoluble material from the cytoplasm and nucleus. We treated the insoluble pellet fractions with DNase I (lanes 4 and 5), micrococcal nuclease (lanes 7 and 8), or 1 M NaCl (lanes 10 and 11) to compare release of Orp1-HA and Cdc23-HA from the insoluble fraction. Using our conditions, some of the Orp1p-HA was extracted from the pellet fraction with DNase I or salt, but none of the treatments released Cdc23p-HA from the insoluble fraction. These results suggest that Cdc23p may be attached to DNA or assembled into a larger order complex that is more rigidly attached to nuclear structures than the MCMs or Orp1p; alternatively, we could simply be observing protein aggregrates of some sort.

DISCUSSION

Many helicases form hexameric rings of identical subunits (PATEL and PICHA 2000). However, the MCM protein complex, which is proposed to be a major replicative helicase (reviewed in LABIB *et al.* 2000; TYE and SAWYER 2000), consists of six different subunits. We set out to understand how the MCM7 component contributes to the functioning of the MCM complex by cloning and characterizing fission yeast *mcm7*$^+$ and isolating a novel temperature-sensitive allele, *mcm7-98*.

The temperature-sensitive phenotype of *mcm7-98* is due to a change in the conserved arginine 644 to histidine. The mutation confers a tight temperature sensitivity; *mcm7-98* cells form colonies at 25° but not at 29°. The flow cytometry profile of *mcm7-98*-arrested cells is similar to that of the *mcm6ts (mis5-268)* and distinct from that of the *mcm2ts (cdc19-P1)* and *mcm4ts (cdc21-M68)* alleles (NASMYTH and NURSE 1981; TAKAHASHI *et al.* 1994). Both *mcm2ts (cdc19-P1)* and *mcm4ts (cdc21-M68)* arrest as elongated cells with apparent 2C DNA content, a classic *cdc* phenotype (COXON *et al.* 1992; FORSBURG and NURSE 1994). In contrast, both *mcm6ts (mis6-268)* and *mcm7-98* arrest as a mixture of normal and elongated cells with a flow cytometry profile consisting of cells with apparent 2C DNA content and cells with 1C or S phase DNA content (TAKAHASHI *et al.* 1994; Figure 3). *mcm2ts (cdc19-P1)* and *mcm4ts (cdc21-M68)* show a dramatic loss of viability at the restrictive temperature (LIANG *et al.* 1999), while both *mcm6ts (mis5-268)* and *mcm7-98* display an intermediate loss of viability at the restrictive temperature (data not shown; Figure 5). Data from many systems suggest that the MCM proteins play a role in both the initiation and elongation phases of DNA replication. We previously showed that relatively little MCM protein is required for initiation, while more is required for completion of S phase (LIANG *et al.* 1999). The mixed *mcm7-98* phenotype suggests that *mcm7*$^+$ may affect both of these functions. Heterogeneity in both the cell morphology and DNA content of arrested *mcm7-98* cells may account for the intermediate loss of viability. The greater viability of *mcm7-98* compared to other *mcmts* mutants could indicate that cells blocked earlier in S phase undergo less damage and can recover activity or reload MCM proteins more successfully when returned to the permissive temperature.

As is the case with all yeast *mcm* genes, *mcm7*$^+$ is essential for viability. Replication initiation in Δ*mcm7* spores is delayed relative to wild type but cells eventually achieve an apparent 2C DNA content and arrest with a

single nucleus. This delayed S phase is probably due to maternal carryover of wild-type Mcm7p from the parent diploid, as we showed with other Δ*mcm* strains (Liang *et al.* 1999). Neither Mcm7p levels nor localization change with the cell cycle. This has also been observed with each of the other fission yeast MCM proteins (Maiorano *et al.* 1996; Okishio *et al.* 1996; Forsburg *et al.* 1997; Sherman and Forsburg 1998; Sherman *et al.* 1998; Pasion and Forsburg 1999). Mcm7p does not contain any characterized nuclear localization signals but is nuclear throughout the cell cycle. We determined that Mcm7p localization depends on the other MCM proteins, because Mcm7p cytoplasmic staining increases in *mcm2ts (cdc19-P1)*, *mcm4ts (cdc21-M68)*, and *mcm6ts (mis6-268)* cells grown at the restrictive temperature. This is consistent with previous studies showing that localization of all the MCM proteins depends on MCM complex formation (Pasion and Forsburg 1999).

We used a coimmunoprecipitation strategy to investigate the physical interactions of Mcm7p with the rest of the MCM complex. Data from many systems suggest that the MCM proteins form a hexameric complex with each one of the MCM protein subclasses (reviewed in Kearsey and Labib 1998). Our results show that Mcm7p interacts weakly with Mcm2p and strongly with the other four members of the complex. Interactions are disrupted in *mcm7-98*. These results are consistent with the model that MCM4-6-7 forms a core complex with helicase activity and MCM2 and MCM3-5 are more loosely associated (Ishimi 1997; Ishimi *et al.* 1998; Lee and Hurwitz 2000; Sato *et al.* 2000). Because the association of Mcm3p or Mcm5p with Mcm7p is stronger than with Mcm4p or Mcm6p (Sherman and Forsburg 1998; Sherman *et al.* 1998), we suggest that the MCM3-5 dimer interacts with the core complex via MCM7. Interactions between MCM3 and MCM7 have previously been reported in Xenopus (Romanowski *et al.* 1996; Prokhorova and Blow 2000) and in budding yeast (Dalton and Hopwood 1997). We envision a model where MCM2 or MCM3-5 can regulate the MCM4-6-7 helicase by interactions with MCM4 or MCM7, respectively.

Other factors modulating these interactions are suggested by numerous genetic interactions between the *mcm*'s and other mutants. In this study we analyzed additional mutants in the network regulating MCM function, with particular focus on *cdc23*$^{+}$ (Aves *et al.* 1998), the fission yeast homolog of budding yeast *MCM10*, which interacts specifically with *MCM7* in budding yeast (Merchant *et al.* 1997; Homesley *et al.* 2000). We found that *cdc23-M30* and *cdc23-M36* alleles cause a first cell cycle arrest after a slow S phase (Nasmyth and Nurse 1981; Figure 3 and data not shown). Both *cdc23ts* alleles have an S phase delay at the restrictive temperature but eventually arrest with 2C DNA content with a mixed elongated *cdc* phenotype. In a hydroxyurea block and release experiment, *cdc23ts* mutants go on to complete S phase, albeit slower than wild type, indicating an execution point at or prior to the hydroxyurea block point.

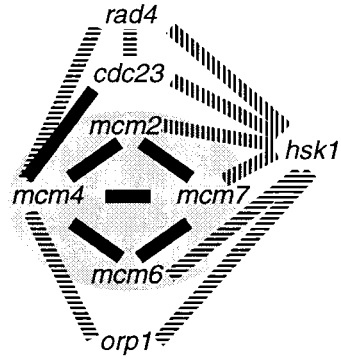

Figure 10.—Mcm7 is part of an extensive genetic network that regulates S phase. Interactions between temperature-sensitive alleles are diagrammed here with data from Table 2 and references therein. Shaded oval, MCM complex; solid rectangles, synthetic lethal; hatched lines, reduced restrictive temperature.

In contrast to observations in budding yeast, *S. pombe cdc23-M36* and *mcm7-98* do not interact genetically. However, *cdc23-M36* is synthetically lethal with *mcm4ts (cdc21-M68)*. Our analysis also revealed genetic interactions between *cdc23ts* and *mcm4ts* with *rad4ts/cut5ts*. Rad4p/Cut5p is required for both replication initiation and checkpoint control (Saka and Yanagida 1993; Saka *et al.* 1994); it is homologous to budding yeast DPB11, a DNA polymerase epsilon-associated factor (Araki *et al.* 1995) that interacts genetically with *mcm10* (Kawasaki *et al.* 2000). Genetic interactions also occur between *hsk1-1312* and *rad4-116*, *mcm2ts (cdc19-P1)*, or *cdc23-M36* (Snaith *et al.* 2000) and we observed a slow growth in the *mcm7-98 hsk1-1312* double mutant. Hsk1p is homologous to budding yeast CDC7 (Masai *et al.* 1995) and phosphorylates Mcm2p *in vitro* (Brown and Kelly 1998). It is possible that this phosphorylation may remodel the heterohexameric MCM complex (Tye and Sawyer 2000). These interactions suggest that MCM phosphorylation may affect the interactions of Rad4p/Cut5p and MCM10/Cdc23p with the MCM complex as well. A map of these interactions is presented in Figure 10.

We could not examine physical interactions between Cdc23p and the MCM proteins using coimmunoprecipitation because Cdc23p was not present in soluble lysates. However, the protein was easily detected in total lysates, and crude fractionation revealed it to be insoluble although the bulk of MCM proteins is soluble. This suggests that Cdc23p is tightly associated with chromatin or nuclear matrix components or forms aggregates. Both human and budding yeast MCM10 are also insoluble and have been shown to be chromatin bound (Homesley *et al.* 2000; Izumi *et al.* 2000; Kawasaki *et al.* 2000).

Defects in DNA replication result in activation of the Rad3 kinase and two downstream kinases: Cds1, which

is required for replication fork recovery in early S phase arrest; and Chk1, which is required to arrest mitotic progress when DNA is damaged (reviewed in O'Connell *et al.* 2000; Rhind and Russell 2000). Mutants in DNA polymerase alpha (Bhaumik and Wang 1998) or the primase subunits *spp1* and *spp2* (Tan and Wang 2000; Griffiths *et al.* 2001) are synthetically lethal (or have reduced restrictive temperature) with Δ*rad3* and Δ*cds1*, suggesting that these early mutants are particularly sensitive to the loss of Cds1p. Consistent with this, mutants blocked early in S phase by mutants of *polα ts* (DNA polymerase alpha), *cdc20* (DNA polymerase epsilon), or *cdc22* (ribonucleotide reductase) activate Cds1p kinase activity to the same degree as hydroxyurea treatment (Lindsay *et al.* 1998). In contrast, mutants blocked later (*mcm4*, *cdc6/polδ ts*) in S phase show no activation of Cds1 kinase activity (Lindsay *et al.* 1998). The synthetic lethality between *mcm7* and Δ*rad3* or Δ*cds1* suggests that *mcm7-98* is affecting an early stage of S phase, similar to *polα ts* and distinct from other *mcm* alleles. However, we observed no strong activation of the Cds1 kinase at 25° or 36° in *mcm7-98*. It may be that our assay is insensitive to modest changes in Cds1p activity; alternatively, the requirement for Cds1p may be structural rather than catalytic. In either case, the nature of the requirement for Cds1p in *mcm7-98* remains unclear.

Interestingly, *mcm7-98* is also synthetically lethal with Δ*chk1* on the damage arm of the pathway. This is reminiscent of mutants such as *hsk1ts*, which also require *chk1*$^+$ for viability, presumably because of low levels of constitutive DNA damage (Snaith *et al.* 2000). However, *chk1*$^+$ is not required for viability in early S phase mutants such as *polα* (Bhaumik and Wang 1998) or other MCMs such as *mcm2ts* (Liang *et al.* 1999), indicating that these mutants do not generate damage at the permissive temperature. It is uncommon for S phase mutants to require both arms of the pathway. That *mcm7-98* requires both arms of the checkpoint pathway suggests it has multiple, perhaps independent, defects even at the permissive temperature. Whether this reflects an allele-specific effect, or a difference between Mcm7p and the other MCMs, remains to be determined. Synthetic lethality with all three checkpoint kinases has been previously observed only with a mutant in the fission yeast signalosome complex (Mundt *et al.* 1999), which is not essential, but affects the rate of DNA replication and the checkpoint response by an undetermined mechanism.

S. pombe Mcm7p behaves much as predicted for a core MCM subunit. However, the *mcm7-98* mutant phenotype, with its mixed arrest and unusual synthetic lethality with all three checkpoint kinases, hints at further complexity. A long-standing question in the field has been to understand why the MCMs are so abundant if their role is as a replicative helicase. Genetic analysis of *mcm7* and other replication mutants will help us to explore these questions *in vivo* to gain a complete description of their role in DNA replication and genome integrity.

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