Minichromosome maintenance (Mcm) proteins 2–7 are highly conserved in eukaryotes and play an essential role in DNA replication. Here, we describe the reconstitution of the various complexes of the Mcm proteins of *Schizosaccharomyces pombe* using the baculovirus expression system. The simultaneous expression of all six of the Mcm proteins, as well as different combinations of these proteins, yielded several stable complexes that included the heterohexamer of Mcm2/3/4/5/6/7, the Mcm2/6/7 heterotrimer, the dimer of the Mcm4/6/7 heterotrimer, and the Mcm3/5 heterodimer. The purification and characterization of the biochemical properties of these complexes showed that only the dimeric complex of the Mcm4/6/7 heterotrimer possessed single stranded DNA-dependent ATPase, ATP-dependent single stranded DNA binding, and 3' to 5' DNA helicase activities. Consistent with these results, the interaction of either Mcm2 or Mcm3/5 with the Mcm4/6/7 complex resulted in the disassembly of the dimeric complex of Mcm4/6/7 and the loss of DNA helicase activity. These results suggest that the Mcm4/6/7 complex is a catalytic core of the Mcm complex and that Mcm2 and Mcm3/5 may be involved in the regulation of the activity of this complex.

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Minichromosome maintenance (*MCM*) genes were initially identified as genes that were required for the maintenance of minichromosomes in the yeast *Saccharomyces cerevisiae* (1). Among these genes, six *MCM* genes, consisting of *MCM2*, *MCM3*, *MCM4*, *MCM5*, *MCM6*, and *MCM7*, encode a family of proteins that are structurally related (2) and highly conserved in all eukaryotes (3). All six of these genes are essential for DNA replication (4–6). Genetic and physical interactions between these gene products and proteins involved in the initiation of DNA replication, such as the origin recognition complex (ORC), Cdc6, Cdc45, and Cdc7/Dh14 kinase, have been reported in *S. cerevisiae* (7–16). The Mcm2–7 proteins were shown to be components of the pre-replicative complex and the assembly of these proteins onto replication origins is required for the initiation of DNA replication (9, 17–21). A protein complex containing all six Mcm proteins was identified and purified as a factor required for one round of DNA replication in the *Xenopus* system (called licensing factor) (22–24). The formation of a heterohexameric complex containing all six Mcm proteins, as well as the formation of subcomplexes such as Mcm2/4/6/7, Mcm4/6/7, or Mcm3/5, have been also identified in extracts prepared from various organisms, including human (25–30), *Xenopus* (24, 31, 32), *Drosophila* (33), *S. cerevisiae* (34, 35), and *Schizosaccharomyces pombe* (36–38). Although the role of these complexes in DNA replication is not fully understood, *in vitro* studies showed that the dimeric complex of the human and mouse Mcm4/6/7, as well as the dodecameric complex of the single archaeon *Methanobacterium thermoautotrophicum* (mith) Mcm protein, contained DNA helicase, single stranded (ss) DNA binding, and DNA-dependent ATPase activities (27, 29, 39, 40). It has been also demonstrated that Mcm2 interacted with the Mcm4/6/7 complex and inhibited the helicase activity of this complex (41). These biochemical properties, taken together with the genetic data obtained from yeast, suggest that the Mcm proteins may play a role as the replicative helicase in eukaryotes, similar to that of the bacterial DnaB protein or the large T antigen (T Ag) of simian virus 40 (SV40) (42–44).

In this study, various complexes of the Mcm proteins of *S. pombe* containing different Mcm subunits were reconstituted and purified to near homogeneity using the baculovirus expression system. The characterization of the biochemical properties of these complexes showed that only the Mcm4/6/7 complex contained activities similar to the human and mouse Mcm4/6/7 complex and the mthMcm protein (multimer complex). We also describe the direct interaction between the *S. pombe* Mcm4/6/7 complex and Mcm2 or the Mcm3/5 heterodimer. Such interactions resulted in the alteration of the dimeric structure of the Mcm4/6/7 complex and the loss of DNA helicase activity.

**MATERIALS AND METHODS**

**Reagents**—Labeled and unlabeled dNTPs and rNTPs were obtained from Amersham Pharmacia Biotech. Single stranded M13mp18 and φX174 DNAs and pUC19 plasmid DNA were from New England Biolabs. Anti-FLAG M2 Ab-agarose and FLAG peptide were from Sigma.

**Cloning of MCM Genes into Baculovirus Transfer Vectors and Preparation of Recombinant Viruses—**DNAs containing spmcm2* (nda1*, cdc19*) (pREP1-nda1), spmcm5* (nda4*) (pREP1-nda4), spmcm6* (nda5*) (pREP3s-cdc21), kindly provided by Dr. M. Yanagida, Kyoto University, Japan (46, 47), and spmcm4* (pREP3s-cdc21), kindly provided by Dr. S. E. Kearsey, University of Oxford, United Kingdom (48), were

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used for the preparation of baculovirus transfer vectors for Mcm2, 5, 6, and 4, respectively. The DNA linker containing the BssHII (5'-TATT- GGGCCGGCCCAA-3') site was added to the NdeI site of pREPI-ndal or pREPI-nd4, and BssHII-Smal fragments containing the spmc2- or the spmcn7- gene were subcloned into the BssHII and SmaI sites of pFastBac1 plasmid (Life Technologies, Inc.). A portion of the intron of the spmc5- gene, the EcoRI-MluI fragment was replaced with the cdNA fragment obtained by PCR. The constructs encoding the spmcn2- or spmcn7- genes that contain six histidines and FLAG (His6)- FLAG tags at the N terminus were made as above except for the use of BssHII linker encoding the His6-FLAG epitope (5'-TAGCCGGCCAGCTAGTCTCTATCATCGTGATGTCGGG-3') and 5'-TACGTTGATGCTTGTCATCGTCATCTTTATAATCA-3' were used for the amplification of the spmcn3- gene and oligonucleotides 5'-CTCACGCCGCACTACTAGGAGAATGTCGACCTCGAATTC-3' and 5'-GTTTCTGCGGTATGCAGTG-3' were used for the amplification of the spmcn3- gene. The FLAG/His6 fusion proteins were expressed in the cytosol of S. pombe Mcm Complexes

**DNA Helicase Assay—**For the preparation of substrate for the assay of helicase activity, a 17-mer oligonucleotide (5'-GATTTCCTCAG- CACGAC-3'), 40 sequencing primer for M13) was synthesized and annealed to M13mp18 ssDNA. After labeling of the 3' end of the 3' terminus of the M13mp18 substrate, a 36-mer oligonucleotide (5'-GACTC TAGAGGATCCCCGGGTACCGAGCTCGAATTC-3') was synthesized, labeled at its 3' end with [32P]ATP and T4 polynucleotide kinase, and annealed to M13mp18 ssDNA. After digestion of this annealed DNA with Smal, DNA was purified by Sephadex G-50 column chromatography. For the preparation of the 5'-tailed substrate, a 36-mer oligonucleotide (5'-GACTC TAGAGGATCCCCGGGTACCGAGCTCGAATTC-3') was synthesized, labeled at its 5' end with [32P]ATP and T4 polynucleotide kinase, and annealed to M13mp18 ssDNA. After digestion of this annealed DNA with Smal, DNA was purified by Sephadex G-50 column chromatography. To determine the maximal length of duplex DNA displaced by the Mcm4/6/7 helicase, a substrate containing longer duplex regions was prepared by elongating singly primed M13mp18 ssDNA using Sequenase (U. S. Biochemical Corp.). For this purpose, the M13 forward sequencing primer was used as a primer for the elongation. The 3' end of this oligonucleotide was labeled with [32P]dGTP and [32P]dATP in the presence of Sequenase and then eluted in the presence of ddCTP and all four dNTPs according to the manufacturer's protocol. The DNA product was purified by Sephadex G-50 column chromatography. The resulting substrate (25,000 cpm/fmol) contained duplex regions that varied in length between 22 and 600 base pairs.

**DNA helicase activity was measured in reaction mixtures (15 µl) containing 25 µM Hespero-NaOH (pH 7.5), 25 mM sodium acetate, 12.5 mM magnesium acetate, 4 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 5 mol of 32P-labeled substrate (4,000 cpm/mole), and enzyme fraction. After incubation at 32 °C for 1 h, 4 µl of 50 × loading buffer (100 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromphenol blue, and 25% glycerol) was added, and 7-µl aliquots were loaded onto a 15% polyacrylamide gel containing 0.1% SDS in 1 x TBE (90 mM Tris, 90 mM boric acid, 1 mM EDTA) and electrophoresed for 1 h at 150 V.**

**Gel Mobility Shift Assay—**The 41-mer oligonucleotide (5'-ATTACATA GATAGTCTCTCGGAAATACACGAGTATC-3') or oligo(dT)20 was labeled at the 5'-end with [32P]ATP and T4 polynucleotide kinase and used as the substrate for gel mobility shift assays. EcoRI-MluI fragments were subcloned into the BamHI site of pBluescript II KS(+), and linker encoding the His 6/FLAG epitope (5'-CAGAAAAGTAGTTTGACGTTCGACCTCC-3') was used for the amplification of the spmcn3- gene and oligonucleotides 5'-GTCAGCGCGCACTACACTTGAAGATGTCCGGG-3' and 5'-GTCAGCGCGCACTACACTTGAAGATGTCCGGG-3' were used for the amplification of the spmcn3- gene. The FLAG/His6 fusion proteins were expressed in the cytosol of S. pombe
coli BL21(DE3) cells by Ni²⁺ (150 μl) containing 5% serum were added to various mixtures of Mcm complexes in buffer C and centrifuged for 13 h at 45,000 rpm in a Beckman SW50.1 rotor at 4 °C (Panel A, Mcm2/3/4/5/6/7; B, Mcm2/4/6/7; C, Mcm3/5; D, Mcm2). After collection of 20 fractions from the bottom of the tubes, aliquots (15 μl) of each fraction were subjected to SDS–10% PAGE analysis and stained with Coomassie Blue. Arrows indicate the positions of marker proteins that were run in a separate gradient. The marker proteins used in these experiments were thyroglobulin (Thy, 19.0 S), catalase (Cat, 11.3 S), and bovine serum albumin (4.3 S). Lanes M, molecular weight marker proteins; LO, material loaded onto the glycerol gradient.

and 5% glycerol in 0.5 × TBE at 4 °C.

Nitrocellulose Filter Binding Assay—Nitrocellulose filter binding assays were carried out in reaction mixtures (15 μl) containing 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 20 fmol of 5’-labeled ssDNA (4,000 cpm/fmol), and enzyme fractions in the presence or absence of ATP or ATP analogues. After incubation at 25 °C for 20 min, mixtures were filtered through an alkaline-washed nitrocellulose filter (Millipore, HA 0.45 μm) (49), and then washed with buffer containing 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 10 mM magnesium acetate, and 1 mM DTT. The radioactivity adsorbed to the filter was determined by liquid scintillation counting.

ATPase Assay—ATPase activity was measured in reaction mixtures (15 μl) containing 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 5 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 1.5 nmol of [γ-32P]ATP (1.5 × 10⁴ cpm/fmol), indicated amounts of polynucleotide, and the enzyme fraction. After incubation at 30 °C for 1 h, aliquots (1 μl) were spotted onto a polyethyleneimine-cellulose thin-layer plate, and ATP and P₃ were separated by chromatography in 1 M formic acid, 0.5 M LiCl. The extent of ATP hydrolysis was quantitated by PhosphorImager (Fuji) analysis. The 25-mer oligonucleotide used in this study contained the sequence 5’-GCAAGCACATCACCTGAATGCCAC-3’.

Preparation of Antibodies against Mcm Proteins and Immunoprecipitation Studies—For the preparation of polyclonal antibodies against the different Mcm proteins, regions of the Mcm genes (encoding amino acids 22 to 460 for mcm2, 3 to 399 for mcm6, 4 to 371 for mcm4, 5 to 313 for mcm3, 220 to 758 for mcm7, and 77 to 350 for mcm5) were cloned into the BamHI site of pET28-a (Novagen), expressed, and purified from E. coli BL21(DE3) cells by Ni²⁺-agarose chromatography using denaturing conditions as described by the manufacturer’s protocols (Novagen). After SDS-10% PAGE, protein bands were excised from the gel and used for immunization of rabbits.

In order to determine the interactions between the different Mcm complexes, polyclonal antibodies against Mcm4 or Mcm2 (0.5 μl of serum) were added to various mixtures of Mcm complexes in buffer C (150 μl) containing 0.1 mM sodium glutamate and 0.1 mg/ml BSA. After incubation at 4 °C for 30 min, protein A-agarose beads (15 μl) were added and the mixture was incubated for an additional 30 min. The beads were washed three times with 1 ml of buffer C containing 0.1 mM sodium glutamate and 0.1 mg/ml BSA and then once with buffer C containing 0.1 mM sodium glutamate. Proteins bound to the beads were then analyzed by SDS-8% PAGE followed by staining with silver.

RESULTS

Reconstitution and Purification of Various Recombinant S. pombe Mcm Protein Complexes—In order to determine the biochemical properties of S. pombe Mcm protein complexes containing various subunits, Mcm complexes were reconstituted using the baculovirus expression system. To facilitate the purification of proteins and complexes, a Histidine/FLAG-tag was added to the N terminus of the Mcm2 or Mcm5 proteins, or to the C terminus of the Mcm7 protein. The addition of the Histidine/FLAG-tag at the N terminus of Mcm2 or Mcm5 proteins did not appear to affect their biological activity. The expression of these tagged proteins under the control of nmt1 promoter in the presence of thiamine complemented the cold-sensitive phenotypes of nda1–376 (mcm2) or nda4–108 (mcm5) mutant strain. After co-infection of baculoviruses encoding each of the Mcm proteins in various combinations, we obtained stable complexes that included Mcm2/3/4/5/6/7, Mcm2/4/6/7, Mcm4/6/7, Mcm3/5, as well as Mcm2. These proteins were purified to near homogeneity by Ni²⁺-agarose chromatography, anti-FLAG M2 Ab-agarose chromatography, and glycerol gradient centrifugation as described under “Materials and Methods.” All of the purified Mcm complexes were more than 80% homogeneous after the anti-FLAG Ab affinity-pellet elution step.

The purity and stoichiometry of the various subunits present in the different complexes were examined following glycerol gradient sedimentation followed by SDS-PAGE and Coomassie Blue staining. The peak glycerol gradient fractions included each protein expected in the complex (Fig. 1, A–D, and Fig. 2A) and contained subunits that stained with similar intensity. This suggests that the subunits that constituted each complex were present in stoichiometric amounts.

The sedimentation coefficients (S) of these complexes, based...
and centrifuged for 13 h at 45,000 rpm in a Beckman SW50.1 rotor at 4 °C. After collection of 20 fractions from the bottom of the gradient, aliquots (15 μl) of each fraction were subjected to SDS-10% PAGE analysis and stained with Coomassie Blue. B, distribution of DNA-dependent ATPase activity in glycerol gradient fractions after sedimentation. The ATPase assays were carried out in the presence of α-32P dATP (450 ng) and 1-μl aliquots of each fraction. The numbers at the bottom of the autoradiogram denote the amount of 32Pi released (in picomole) quantitated on the various markers shown in Fig. 1, A-D, and Fig. 2A, and the Stokes radii of the various preparations deduced from the Superdex-200 gel filtration chromatography analysis (data not shown), are summarized in Table I. The molecular masses (in kDa) of these complexes were calculated by the method of Siegel and Monty (50), using the Stokes radii, the S values, and assuming a partial specific volume of 0.725 ml/g for each preparation. The deduced molecular masses of Mcm2/3/4/5/6/7, Mcm2/4/6/7, Mcm3/5, and Mcm2 were in fair agreement with the molecular weights calculated by summing the molecular weights of the protein subunits of the complexes (Table I). These results suggest that Mcm2/3/4/5/6/7, Mcm2/4/6/7, Mcm3/5, and Mcm2 were monomeric in structure. In contrast, the molecular mass of the Mcm4/6/7 complex (542 kDa) was about 2-fold greater than the calculated molecular mass of the heterotrimeric complex (284 kDa), suggesting that the structure of this complex was a dimer of the heterotrimer.

In addition to the complexes described above, we also attempted to isolate the Mcm3/3/4/5/6/7 and Mcm2/3/5 complexes. Coexpression of the Mcm3, 4, 5, 6, and 7 proteins in Sf9 cells, followed by the purification procedure described above, yielded a complex containing stoichiometric amounts of each of these subunits after Ni2+-agarose chromatography. However, after further purification, either Mcm4/6/7 or Mcm3/5 was isolated, depending upon the Mcm subunit that contained the His6-FLAG tag. These observations suggest that the interaction between Mcm4/6/7 and Mcm3/5 complexes leads to a product that is relatively unstable. We also attempted to coexpress and isolate the Mcm2/3/5 complex, but failed to detect a stable interaction between Mcm2 and the Mcm3/5 complex.

The Mcm4/6/7 Complex Contains ATPase and DNA Helicase Activities—The biochemical activities associated with the isolated complexes were examined. We determined whether these complexes contained DNA-dependent ATPase, ssDNA binding, and DNA helicase activities as described under “Materials and Methods.” Among the Mcm complexes, Mcm2/3/4/5/6/7, Mcm2/4/6/7, Mcm3/5, and Mcm2 were devoid of ssDNA binding and DNA helicase activities (data not shown). We also failed to detect significant ATPase activity with Mcm3/5 and Mcm2. Mcm2/3/4/5/6/7 and Mcm2/4/6/7 complexes possessed weak ATPase activity. These complexes catalyzed the hydrolysis of 0.8 and 1.5 pmol of ATP/min/μmol of protein, respectively. The ATPase activity associated with these complexes, however, was unaffected by DNA and the possibility that this low activity was due to contaminants could not be ruled out (data not shown). On the other hand, the Mcm4/6/7 complex contained DNA-dependent ATPase, ssDNA binding, and DNA helicase activities (Figs. 2–4). When the ATPase and the DNA helicase activities present in the glycerol gradient fractions were determined, both activities cosedimented with the heterotrimeric complex (Fig. 2, A-C, peaking at fraction 8) suggesting that these are intrinsic activities of this complex. The Mcm4/6/7 complex hydrolyzed about 24 pmol of ATP/min/μmol of the dimer of the heterotrimer in the presence of α-32P dATP under the assay conditions described in Fig. 2B.

Stimulation of the ATPase Activity of the Mcm4/6/7 Complex by ssDNA—The influence of various DNA preparations on the ATPase activity of the glycerol gradient peak fraction (fraction 8) was determined. As shown in Fig. 3, the ATPase activity of the Mcm4/6/7 complex was stimulated about 3.5-fold by X174 ssDNA. Short ss oligonucleotides (25-mer or oligo(dT)25) also stimulated the ATPase activity about 5-fold. In contrast, double stranded DNA (pUC19 plasmid DNA) did not affect the ATPase activity of the complex. We also examined the effects of other double stranded DNAs including DNAs containing S.
Materials and Methods.

The ATPase activity of Mcm4/6/7 complex was determined in the absence or presence of the indicated amounts of ATP or ATPγS. After incubation at 25 °C for 20 min, formation of MCM-ssDNA complexes were analyzed by gel mobility shift assay as described under “Materials and Methods.”

![Figure 3](image)

**Fig. 3.** The influence of various DNAs on the ATPase activity of the Mcm4/6/7 complex. The ATPase activity of Mcm4/6/7 complex (glycerol gradient peak fraction), as a function of protein concentration, was determined in the absence or presence of the various DNAs (30 ng/ml) as indicated. ○, reactions carried out in the absence of DNA; □, in the presence of pUC19 plasmid DNA; △, oligo(dT)25; ■, 25-mer ssDNA. The assays were as described under “Materials and Methods.”

![Figure 4](image)

**Fig. 4.** Single stranded DNA binding activity of the Mcm4/6/7 complex. Indicated amounts of Mcm4/6/7 complex (glycerol gradient peak fraction) were incubated with 20 fmol of 32P-labeled 41-mer oligonucleotide in the presence or absence of the indicated amounts of ATP or ATPγS. After incubation at 25 °C for 20 min, formation of MCM-ssDNA complexes were analyzed by gel mobility shift assay as described under “Materials and Methods.”

S. pombe autonomous replicating sequences (ars 3002 and ars 1), but failed to detect any stimulation (data not shown).

Mcm4/6/7 Complex Binding to ssDNA Is ATP-dependent—

We examined the interaction between the Mcm4/6/7 complex and ssDNA. For this purpose, the Mcm4/6/7 complex (glycerol gradient peak fraction, Fig. 2A) was incubated with a 32P-labeled 41-mer ssDNA in the presence or absence of ATP or an ATP analogue, and the Mcm complexes bound to DNA were analyzed by the gel mobility shift assay described under “Materials and Methods.” As shown in Fig. 4, the Mcm4/6/7 complex bound to ssDNA and this interaction required ATP. Under the conditions used, the optimal concentration of ATP required for this interaction was about 1 mM and higher concentrations of ATP decreased the binding efficiency, presumably by reducing the level of free Mg2+ ions. In support of this notion, when the Mg2+ ion concentration was increased to 15 mM (in contrast to the 10 mM Mg2+ ion concentration used in the experiment described in Fig. 4), no inhibition of binding was observed up to 5 mM ATP (data not presented). The non-hydrolyzable ATP analogue, ATPγS, also supported the binding of the Mcm4/6/7 complex to ssDNA, suggesting that this interaction does not require ATP hydrolysis. These results were also confirmed using a nitrocellulose filter binding assay (data not shown). To rule out the possibility that these binding properties were caused by the secondary structures present in the ssDNA, these experiments were repeated with 32P-labeled oligo(dT)25 as substrate. Results identical to those described with the 32P-labeled 41-mer ssDNA were obtained (data not presented).

Biochemical Properties of Mcm4/6/7 Helicase Activity—

The biochemical characteristics of the Mcm4/6/7 DNA helicase activity were examined (Fig. 5). When increasing levels of the Mcm4/6/7 complex (glycerol gradient peak fraction) were incubated with 5 fmol of a helicase substrate DNA (18-mer oligodeoxynucleotide hybridized to M13 mp18 s DNA) described under “Materials and Methods,” about 60 ng of protein resulted in the displacement of approximately 40% of the labeled 18-mer oligomer. The helicase activity associated with the Mcm4/6/7 complex in the absence of ATP. Therefore, these conditions were used in the following experiments. As shown in Fig. 5B, DNA helicase activity of the Mcm4/6/7 complex required ATP or dATP, and ATP hydrolysis was essential for this activity since the non-nonhydrolyzable ATP analogue, ATPγS, did not support strand displacement (Fig. 5B).
other rNTPs or dNTPs supported helicase activity, and the optimal concentration of ATP was about 4 mM under the conditions used.

In order to determine the direction of translocation of the Mcm4/6/7 helicase on DNA, two different substrates containing either a 3′-ssDNA tail (substrate A, Fig. 5C) or a 5′-ssDNA tail (substrate B, Fig. 5C) were prepared as described under “Materials and Methods.” Helicase activity was only observed with the 3′-ssDNA-tailed substrate, indicating that the polarity of translocation is in the 3′ to 5′ direction.

The processivity of helicase activity was also determined using a M13mp18 DNA substrate that contained duplex DNA regions that varied between 22 and 600 base pairs in length. In the presence of 250 ng of the Mcm4/6/7 complex, the maximum size of the DNA displaced was about 50 base pairs, suggesting that the processivity of this helicase activity is relatively low (data not shown). We also examined whether ssDNA-binding proteins influenced the DNA helicase activity. The addition of S. pombe SSB or E. coli SSB did not stimulate the helicase activity. The addition of excess amounts of E. coli SSB markedly inhibited the helicase activity whereas apSSB did not.

Interaction of Mcm2 or Mcm3/5 with the Mcm4/6/7 Complex—The interactions between the isolated Mcm subcomplexes were examined by immunoprecipitation and glycerol gradient sedimentation analyses. Various combinations of the purified Mcm preparations were mixed together and incubated on ice for 8 h. Interactions between these complexes were analyzed by immunoprecipitations using anti-Mcm4 or anti-Mcm2 antibodies as described under “Materials and Methods.” As shown in Fig. 6A, the Mcm4/6/7 complex interacted with Mcm2 (lanes 1–3) as well as with Mcm3/5 (lanes 4 and 5). When all three protein preparations were incubated together, a complex containing all six subunits was detected following precipitation with either anti-Mcm4 or anti-Mcm2 antibodies (lanes 6–8). However, significant interaction between Mcm2 and the Mcm3/5 complex was not detected (lanes 9 and 10).

Glycerol gradient sedimentation analyses were also performed to examine the interactions between these complexes. For this purpose, various combinations of Mcm4/6/7, Mcm2, or Mcm3/5 complexes were mixed as indicated and the complexes formed were analyzed by glycerol gradient centrifugation (Fig. 6B). As shown in Fig. 6B, panel a, incubation of Mcm4/6/7 with Mcm2 resulted in the formation of a complex containing Mcm2/4/6/7 subunits that peaked at fractions 10 and 11 after sedimentation. The sedimentation property of this four-subunit complex was identical to the Mcm2/4/6/7 complex isolated from Sf9 cells after co-expression of these four subunits (Fig. 1B). This observation suggests that the interaction of Mcm2 with the Mcm4/6/7 complex resulted in the dissociation of the dimeric structure of Mcm4/6/7 and formation of a monomeric structure of the heterotetramer.

Incubation of Mcm4/6/7 with Mcm3/5 also resulted in the formation of a complex containing Mcm3/4/5/6/7 subunits (Fig. 6B, panel b). However, only small amounts of the added Mcm3/5 complex cosedimented with Mcm4/6/7 in the high molecular weight region (fractions 6 to 9). Substantial levels of Mcm3/5 and Mcm4/6/7 proteins were detected in the middle of the gradient, in the vicinity of the catalase marker (232 kDa, 113 S). This heterogeneous distribution of Mcm proteins may have been caused by the dissociation of the Mcm3/4/5/6/7 complex during the 13-h glycerol gradient centrifugation, which yielded Mcm3/5 and Mcm4/6/7 complexes that sedimented more slowly than the heteropentameric complex. Although interaction between Mcm4/6/7 and Mcm3/5 complexes was detected in the immunoprecipitation experiment (Fig. 6A), the failure to obtain substantial levels of the five-subunit complex after glycerol gradient sedimentation suggests that interaction between Mcm4/6/7 and Mcm3/5 is relatively weak. This notion is in keeping with our failure to purify the Mcm3/4/5/6/7 complex from Sf9 cells. As described above, the Mcm3/4/5/6/7 complex obtained after co-expression and purification using Ni2+-agarose chromatography, completely dissociated to the Mcm3/5 and 4/6/7 complex following further purification steps.

The interaction of the Mcm3/5 complex with the Mcm4/6/7 complex appeared to convert the dimeric Mcm4/6/7 complex to the monomer structure. This conclusion is based on two findings. First, the largest complex formed containing all five subunits sedimented slower than thyroglobulin. If Mcm3/5 formed a complex with the dimeric Mcm4/6/7 complex, the size of the dimeric Mcm3/4/5/6/7 complex should be about 924 kDa, which would be expected to sediment faster than thyroglobulin. As shown (Fig. 6B, panel b), no Mcm proteins sedimenting faster than thyroglobulin were observed. Second, the presence of
Mcm4/6/7 proteins in the catalase region (panel B, fractions 10–12) also support this suggestion. This Mcm4/6/7 complex, which appeared to have dissociated from the pentameric Mcm3/4/5/6/7 complex during glycerol gradient centrifugation, sedimented slower than the dimeric Mcm4/6/7 complex isolated from Sf9 cells (Fig. 2A). These observations indicated that analogous to Mcm2, the interaction of Mcm3/5 with Mcm4/6/7 dissociated the dimeric Mcm4/6/7 complex to a monomeric structure. In the case of Mcm2, the interaction resulted in the formation of the stable heterotetramer whereas incubation with Mcm3/5 resulted in the production of the unstable heterotetramer.

Incubation of Mcm4/6/7 with Mcm2 and Mcm3/5 complex resulted in the formation of a complex containing all six subunits (Fig. 6B, panel c, peaking at fractions 7 and 8) that sedimented to the same position observed with the complex purified from Sf9 cells after co-expression of all six subunits (Fig. 1A). In accord with the immunoprecipitation experiment described in Fig. 6A, no stable interaction between Mcm2 and Mcm3/5 was detected after glycerol gradient centrifugation analysis (Fig. 6B, panel D). Although Mcm2 alone did not interact with Mcm3/5, the addition of Mcm2 with Mcm4/6/7 and Mcm3/5 complexes yielded a stable six-subunit complex. The interaction of Mcm2 protein with the Mcm4/6/7 complex may stabilize the interaction between Mcm4/6/7 and Mcm3/5 complexes. These results suggest that Mcm2 may be important for the formation and regulation of the six-subunit Mcm complex.

**Influence of Mcm2 and Mcm3/5 on the DNA Helicase Activity of the Mcm4/6/7 Complex**—As described above, only the Mcm4/6/7 complex contained DNA helicase activity. Because Mcm2 and the Mcm3/5 complex interacted with the Mcm4/6/7 complex, the influence of these proteins on the Mcm4/6/7 helicase activity was examined (Fig. 7). For this purpose, the Mcm2 and/or Mcm3/5 complexes were added to the DNA helicase reaction mixtures devoid of the helicase DNA substrate. After incubation at 25 °C for 20 min, DNA helicase activity was determined after the addition of 5 fmol of M13 ssDNA/18-mer substrate and further incubation at 32 °C for 60 min. The lane B indicates that the DNA substrate was boiled. The percent displacement of the 18-mer from the duplex DNA substrate is indicated as %.

The inhibitory effects correlated with the efficiency of interaction of the Mcm4/6/7 complex with Mcm3/5 resulted in the loss of DNA helicase activity. Because Mcm2 alone did not inhibit the Mcm4/6/7 helicase activity, Mcm2 (lanes 3 and 4) was somewhat more effective as an inhibitor than Mcm3/5 (lanes 5 and 6) and the addition of both proteins (lanes 7 and 8) showed the highest level of inhibition. The inhibitory effects correlated with the efficiency of interaction between these complexes. They support the notion that the interaction of the Mcm4/6/7 complex with Mcm3/5 was less stable than the interaction between Mcm4/6/7 and Mcm2 (described in Fig. 6B). These findings further support the suggestion that Mcm2 is required to stabilize the interaction between Mcm4/6/7 and Mcm3/5.

**DISCUSSION**

The initiation of DNA replication in eukaryotes is a multi-step process that requires a number of proteins. It involves the binding of ORC to replication origins, the recruitment of Cdc6/Cdc18, the Mcm complex, and Cdc45 to form the pre-replicative complex, and the activation of the pre-replicative complex by Cdc7 and Cdc28 protein kinases to initiate DNA synthesis (6, 11, 51, 52).

In this study, we have reconstituted the *S. pombe* Mcm complex containing all six Mcm proteins as well as several subcomplexes with different Mcm subunits using the baculovirus expression system. The coexpression of various combinations of the Mcm proteins yielded several stable complexes including the heterohexameric complex of Mcm2/3/4/5/6/7 and the subcomplexes Mcm2/4/6/7, Mcm4/6/7, and Mcm3/5. Although the biological role of these subcomplexes is not fully understood, the subunit composition of these stable complexes are consistent with the Mcm complexes previously observed from a number of immunoprecipitation studies carried out with crude extracts prepared from several organisms from yeast to human (25, 26, 28, 33, 37, 38). The direct interaction between purified subcomplexes and the stability of these interactions observed in this study are consistent with these earlier observations. They suggest that these interactions are intrinsic properties of these proteins and not in vitro artifacts.

As described above, only the dimeric form of the Mcm4/6/7 complex possesses biochemical properties similar to those found with the human and mouse Mcm4/6/7 complexes (27, 39). None of the other Mcm complexes contained detectable ATPase, DNA binding, or DNA helicase activities. Consistent with these results, the interaction of either Mcm2 or Mcm3/5 with the Mcm4/6/7 complex resulted in the loss of DNA helicase activity.

Although all of the Mcm proteins contain conserved ATPase motifs, significant ATPase activity has been observed only with the Mcm4/6/7 complex. Similar to the observations made with the cloned *S. pombe* heterohexamer, the *S. pombe* Mcm2/3/4/5/6/7 complex isolated from asynchronous cultures and from cells synchronized with hydroxyurea at the beginning of S phase were devoid of enzymatic activities (36, 53). Possibly, the motifs responsible for the binding and hydrolysis of ATP are not exposed in this six-subunit complex. It is also possible that the activation of the cryptic ATPase activity of this complex may depend on its interaction with other components of the pre-replicative complex such as ORC, Cdc6/Cdc18, or Cdc45/Sna1. Studies carried out with the baculovirus-cloned mouse Mcm4/6/7, in which the conserved ATPase motifs of either Mcm6 and Mcm4 were mutated, resulted in the marked loss of DNA helicase and ssDNA binding activity, respectively (39). Thus, the ATPase motif of each Mcm protein in the Mcm4/6/7 complex may play distinct roles in the enzymatic activities associated with the active complex.

The biochemical properties of *S. pombe* Mcm4/6/7 complex are comparable to those observed with the human and mouse Mcm4/6/7 complexes with the exception that the ssDNA binding activity of the *S. pombe* preparation depended on the presence of ATP or ATPγS. This difference, however, may be due to the reaction conditions used. The DNA binding activity of the human and mouse Mcm4/6/7 complex was examined following cross-linking with glutaraldehyde. It is possible that weak and/or transient interactions between the Mcm proteins and DNA in the absence of ATP, which were not observed under our
assay conditions even in the nitrocellulose filter binding assay, may be detected using the cross-linking assay conditions.

The biochemical activities observed with the S. pombe and human Mcm4/6/7 complexes as well as with the single mthMcm protein are in keeping with the proposal that the Mcm complex may play a role as a DNA helicase at replication forks, analogous to that of the E. coli DnaB protein or the SV40 T Ag. This hypothesis is also consistent with chromatin immunoprecipitation studies that showed changes in the localization of Mcm proteins (Mcm4 and Mcm7) to inter-origin regions during S phase (19). However, the limited processivity of the S. pombe and human Mcm4/6/7 helicase activity (27), suggest that the Mcm proteins may be required only during the initial step of unwinding of replication origins. It is also possible that modifications of the Mcm proteins or their interactions with other replication factors may be required to increase the processivity of the helicase activity.

The presence of the Mcm proteins in the pre-replicative complex was demonstrated by chromatin binding experiments in Xenopus and yeast (17, 54) and in vivo cross-linking of the Mcm proteins to replication origins (19, 20). The Mcm proteins appear to bind chromatin as a multimeric complex containing all six Mcm proteins (29, 55), which contains no catalytic activities. If the helicase activity is a critical property of the Mcm complex, as discussed above, alterations of the six-subunit complex leading to the activation of the cryptic DNA helicase activity would be required. Possibly, the two S phase promoting kinases contribute to this alteration by directly phosphorylating the Mcm proteins. In support of this model, the extent of phosphorylation of the Mcm proteins has been shown to vary during the different phases of the cell cycle (18, 56–58) and some of the Mcm proteins are modified by these two kinases (16, 53, 57, 59, 60). In S. cerevisiae, a specific mutant allele of mcm5-cho1 has been shown to suppress all mutations in CDC7 or DBF4, suggesting that an alteration in MCM5 satisfies the essential function of Cdc7. In addition, a mutant allele of dbf4 has been isolated which suppresses a mutation in mcm2 (16). These findings suggest that the chief target of the Cdc7-Dbf4 kinase is the Mcm protein complex. Interestingly, Mcm2 appears to be a substrate of the Cdc7-Dbf4 kinase (53, 60). As shown here, Mcm2 appeared to be important for the formation of a complex containing all six subunits. The interaction between Mcm4/6/7 and Mcm3/5 was relatively weak, and Mcm2 was required for the formation of the stable six-subunit complex. Although Mcm2 alone did not interact with Mcm3/5, the binding of Mcm2 to the Mcm4/6/7 complex stabilized the interaction between Mcm4/6/7 and Mcm3/5. This suggests that the removal of Mcm2 from the hexameric complex or conformational changes caused by its modifications may lead to the release of the Mcm4/6/7, which is active as a helicase.

The reconstitution of Mcm complexes reported in this study should enable us to examine further the role and the regulation of these proteins in vitro. The modifications of these proteins by G1-S promoting kinases and the interactions of these proteins with other replication factors such as ORC, Cdc18, and Sna41/Cdc45 are currently under investigation.

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Isolation and Characterization of Various Complexes of the Minichromosome Maintenance Proteins of *Schizosaccharomyces pombe*

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