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Reconstitution of the Mcm2-7p Heterohexamer, Subunit Arrangement, and ATP Site Architecture*

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The Mcm2-7p heterohexamer is the presumed replicative helicase in eukaryotic cells. Each of the six subunits is required for replication. We have purified the six *Saccharomyces cerevisiae* MCM proteins as recombinant proteins in *Escherichia coli* and have reconstituted the Mcm2-7p complex from individual subunits. Study of MCM ATPase activity demonstrates that no MCM protein hydrolyzes ATP efficiently. ATP hydrolysis requires a combination of two MCM proteins. The fifteen possible pairwise mixtures of MCM proteins yield only three pairs of MCM proteins that produce ATPase activity. Study of the Mcm3/7p ATPase shows that an essential arginine in Mcm3p is required for hydrolysis of the ATP bound to Mcm7p. Study of the pairwise interactions between MCM proteins connects the remaining MCM proteins to the Mcm3/7p pair. The data predict which subunits in the ATPase pairs bind the ATP that is hydrolyzed and indicate the arrangement of subunits in the Mcm2-7p heterohexamer.

The minichromosome maintenance proteins (MCM)[1] are components of the prereplicative complex (preRC) that assembles on replication origins prior to S phase (1, 2). The six proteins (Mcm2-7p) form a heterohexamer that is thought to be the replicative helicase in eukaryotic cells. Consistent with this idea, MCM proteins are required for replication initiation and elongation (3, 4). In addition, they appear to travel with the replication fork *in vivo* (5). Although helicase activity with all six MCM proteins has remained elusive, a subcomplex of Mcm4/6/7p is a helicase (6–8). Similar to replicative helicases in other systems, Mcm2-7p appears to be ring shaped, consistent with electron microscope studies of Mcm2-7p and Mcm4/6/7p (9, 10). An archaeal MCM protein also has helicase activity and is an oligomeric ring (11–14).

MCM proteins are members of the AAA+ family of proteins (15). As their name implies (ATPases associated with a variety of cellular activities), members of this family are usually ATPases and are involved in many different cellular processes. The structures of some AAA+ family members have been solved, and provide insight into Mcm2-7p structure and function. These structures include the heteropentameric *Escherichia coli* γ complex clamp loader (16), the homohexameric membrane trafficking proteins, *N*-ethylmaleimide sensitive factor (NSF) and p97 (17–19), and the homohexameric branch migration protein, RuvB (20).

A striking feature of these AAA+ machines is the location of ATP sites at the interface of subunits. Residues from both subunits are thought to be required for ATP hydrolysis. An arginine residue of one subunit is required for hydrolysis of ATP bound to a neighboring subunit. A catalytic Arg residue that reaches across a subunit interface to promote nucleotide hydrolysis is referred to as an arginine finger, first described in the Ras GTP-binding protein and its cognate GAP (GTPase activating protein) (21). These signaling proteins are important regulators of cell growth and differentiation (22). When bound to GTP, Ras is active but is inactive when bound to GDP. Ras hydrolyzes GTP very slowly and requires an arginine residue from GAP to promote rapid hydrolysis. Structural and biochemical studies have suggested that the Arg finger in GAP stabilizes the transition state thereby greatly increasing the catalytic rate of the Ras GTPase (21).

In this study, we report the purification of Mcm2-7p as individual subunits. We find that the Mcm2-7p heterohexamer can be reconstituted from these individual subunits. MCM proteins have been studied alone and in combinations for intrinsic ATPase activity. We find that no individual MCM protein contains significant ATPase activity even though they each have an ATP binding site. ATPase activity is produced by a combination of at least two MCM proteins, implying that ATPase activity requires residues from both subunits (*i.e.* a catalytic arginine from one subunit and an ATP binding site in another). Although one may anticipate six ATPase pairs, we find that only three different pairwise combinations of MCM proteins have ATPase activity. In-depth study of one of these combinations, Mcm3/7p, demonstrates that Mcm3p contributes a catalytic arginine residue for hydrolysis of ATP bound to Mcm7p. Examination of the fifteen different pairwise combinations of MCM proteins for physical interaction, along with the ATPase data, indicates which subunits contribute catalytic arginine residues and which bind ATP in the other two ATPase pairs. The results also suggest a unique arrangement of MCM proteins in a heterohexameric ring.

EXPERIMENTAL PROCEDURES

Buffers—Tris/sucrose (50 mM Tris-HCl pH 7.5, 10% (w/v) sucrose); Buffer A is 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, and 10% (v/v) glycerol. Buffer B is 20 mM HEPES-NaOH, pH 7.5, 0.1 mM EDTA, 2 mM DTT, and 10% (v/v, glycerol). Buffer C is 20 mM Tris, pH 7.9 and 0.5 M NaCl.

Cloning of the MCM2-7 Genes—Each of the *MCM* genes was amplified from *Saccharomyces cerevisiae* genomic DNA (S288C, MATα SUC2 mal mel gal2 CUP1 flo1 flo8–1; from ATCC) using either Vent polymerase (New England Biolabs) or Elongase enzyme mix (Invitrogen). Yeast genomic DNA was obtained as described previously (23). Amplified genes were ligated, as described below, into various pET plasmids so that expression of the *MCM* genes is under control of the T7 RNA

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[1] The abbreviations used are: MCM, minichromosome maintenance; pre-RC, pre-replicative complex; AAA+, ATPases associated with a variety of cellular activities; NSF, *N*-ethylmaleimide-sensitive fusion protein; GAP, GTPase-activating protein; DTT, dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

polymerase promoter and inducible by IPTG. All genes were sequenced to ensure that there were no mutations introduced by PCR.

The *MCM2* gene was amplified using primer 2D, 5′-d(CTACAATATAACATATGTCTGATAATAGCAGCCGTAGCCGTGAGGAAGATG)-3′, which inserts a *Nde*I site at the *MCM2* start codon, and primer 2U, 5′-(dTAGGAATGGATCCGTTTTAGTGACCCAAGG-3′), which inserts a *Bam*HI site 4-bp downstream of the *MCM2* stop codon. The resulting 2632-bp PCR fragment was digested with *Nde*I and *Bam*HI and was inserted into the *Nde*I and *Bam*HI sites of pET11a (Novagen) to yield pET11a-MCM2.

The *MCM3* gene was amplified using primer 3D, 5′-d(GATACAAACGTCACATATGGAAGGCTCAACG)-3′ which inserts an *Nde*I restriction site at the *MCM3* start codon, and primer 3U, 5′-d(TTAGTAAACAGAGCTCTGACATCAGAC)-3′, which inserts a *Sac*I restriction site 6-bp downstream of the *MCM3* stop codon. The PCR fragment was ligated into the pCR2.1-TOPO vector (Invitrogen) using the manufacturer's instructions to yield pMD132. The 2923-bp *Nde*I-*Sac*I fragment from pMD132, containing *MCM3*, was inserted into the *Nde*I-*Sac*I sites of pET21b (Novagen) to yield pET21b-MCM3.

The *MCM4* gene was amplified using the primers, 4D, 5′-d(CTCAAGAACTTCATATGTCTCAACAGTC)-3′, which inserts an *Nde*I restriction site at the *MCM4* start codon and 4U, 5′-d(TGATTGTAGTAGATCTCATCAGACACG)-3′, which inserts a *Bgl*II site 3-bp downstream of the *MCM4* stop codon. The 2829-bp PCR fragment was ligated into pCR2.1-TOPO (Invitrogen) to create pMD134. The 2809-bp *Nde*I-*Bgl*II fragment from pMD134, containing *MCM4*, was inserted into the *Nde*I and *Bgl*II sites of pET11a to yield pET11a-MCM4.

The primers used to amplify *MCM5* from the yeast genome were 5D, 5′-d(TAACTGCATATGTCATTTGATAGACCG)-3′, which inserts an *Nde*I restriction site at the *MCM5* start codon and 5U, 5′-d(GCTAAGACTTAGATCTTGTCATACACCAC)-3′, which inserts a *Bgl*II restriction site 3 bp downstream of the *MCM5* stop codon. The-2535 bp PCR fragment was amplified, digested with *Nde*I and *Bgl*II, and then inserted into the *Nde*I and *Bam*HI sites of pET16b (Novagen) to yield pET16b-MCM5.

The *MCM6* gene was amplified using the primers 6D, 5′-d(CTGGTTTTTTCATATGTCATCCCCTTTTCC)-3′, which inserts an *Nde*I site at the start codon of *MCM6* and 6U, 5′-d(GAAAATCCGCAAGGATCCACTGAATTAGC)-3′, which inserts a *Bam*HI site 8-bp downstream of the *MCM6* stop codon. The 3086-bp PCR fragment was digested with *Nde*I and *Bam*HI and inserted into the *Nde*I and *Bam*HI sites of pET16b to yield pET16b-*MCM6*.

The *MCM7* gene was amplified using primer 7D, 5′-d(GATAGACCAGATCATGAGTGCGGCACTTC)-3′, which inserts a *Bsp*HI restriction site at the MCM7 start codon, and primer 7U, 5′-d(GTTGCTTGACGTCGCTGAGCTTTCAAGCGTC)-3′, which inserts a *Blp*I restriction site 5 bp downstream of the *MCM7* stop codon. The resulting 2545-bp PCR fragment was inserted into pCR2.10-TOPO to yield pMD131. The 1491-bp *Bsp*HI fragment from pMD131, encoding an N-terminal fragment of Mcm7p, was inserted into the *Nco*I site of pET16b to yield pMD135. A plasmid encoding full-length Mcm7p (pET16b-MCM7) was produced by inserting the 2075-bp *Bam*HI-*Blp*I fragment from pMD131 into the *Bam*HI-*Blp*I sites of pMD135.

SRF and P-loop Mutations of MCM3 and MCM7—For the Mcm3p SRF mutation, the Arg at residue 542 was changed to alanine so that nucleotides 1761–1765 (numbering begins at the start codon) were changed from 5′-d(TCTCG)-3′ to 5′-d(AGCGC)-3′. The Arg of the Mcm7p SRF motif (R593) was changed to alanine by altering nucleotides 1924–1929 from 5′-d(TCCAGA)-3′ to 5′d(AGCGCA)-3′, also introduces a *Hae*II restriction site. The lysine at residue 415 in Mcm3p was replaced with alanine by changing nucleotides 1373–1378 from 5′-d(AAGTCC)-3′ to 5′-d(GCTAGC)-3′, which also introduces a *Nhe*I restriction site. Lysine 466 in Mcm7p was changed to alanine by PCR. A portion of the *MCM7* gene was amplified from an internal *Bam*HI site to a *Bsu*36I restriction site using the primers 7BAM, 5′-d(CACGATGGATCCACCCTCTTC)-3′, which anneals across the *Bam*HI site and 7KA, 5′-d(GACCAACGCCTGAGGAACCCTTACCAGTGGTATACACTCCTCGAGGTGATATTTTGCAAATGGCCTTCAGCAGTTGAGATGCGGCAACACCGGGATCACC)-3′, which changes the lysine codon to alanine and anneals across the *Bsu*36I site. The amplified fragment was inserted into pET16b-MCM7 digested with the same restriction enzymes. All mutant genes were sequenced to confirm the changes and mutant proteins were purified in the same manner as their wild type counterparts (see below).

Cell Growth and Lysis—Expression plasmids were transformed into BL21 DE3 Codon+ RIL cells (Strategene, *E. coli* B F$^-$ *ompT hsdS* (r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* (λDE3) *endA* Hte (*argU ileY leuW* Cam^r)). Fresh transformants were grown in 24 liters of LB containing 100 μg of ampicillin and 25 μg of chloramphenicol per ml to a density of OD_{600} = 0.5–0.8. Cells were then chilled to 15 °C on ice, and then IPTG was added to 1 mM. Cells were incubated another 20 h with shaking at 15 °C, then pelleted and resuspended in Tris/sucrose (total volume, ~200 ml).

For cell lysis, cells were brought to a final volume of 250–300 ml with a final concentration of 30 mM spermidine, 1 M NaCl, and 2 mM DTT in Tris/sucrose. Cells were lysed by two passages through a French Press at 17,000 psi, and insoluble material was removed by centrifugation at 10,000 rpm for 40 min at 4 °C in an SLA1500 rotor. The supernatant was decanted and referred to below as the cell lysate or FrI (Fraction I).

Mcm2p Purification—FrI of the Mcm2p expression strain was treated with 0.25 g/ml of ammonium sulfate. After stirring at 4 °C, the mixture was centrifuged at 10,000 rpm for 30 min in a SLA1500 rotor. The resulting supernatant was removed, and the pellet was resuspended in Buffer A containing 0.2 g/ml ammonium sulfate and then re-pelleted. After repeating this step, the pellet was resuspended in 100 ml of Buffer A and dialyzed against Buffer A for ~3 h. The protein was diluted to a conductivity equal to 60 mM NaCl with Buffer A (final volume, 220 ml) and then applied to a 225 ml of Fast Flow Q Sepharose column equilibrated in Buffer A. Protein was eluted with a 2250 ml, 0–600 mM NaCl gradient in Buffer A. Fractions containing Mcm2p, as determined by Coomassie-stained gels, were pooled (FrIII, 200 ml, 1.6 mg/ml) and then precipitated with 0.25 g/ml ammonium sulfate. After centrifugation, the pellet was resuspended in 50 ml of Buffer B and dialyzed against Buffer B for 2.5 h. The protein was diluted with Buffer B before being applied to a 200-ml heparin-agarose column (BioRad) equilibrated in Buffer B. The protein was eluted with a 1750 ml, 0–500 mM NaCl gradient in Buffer B. Fractions containing Mcm2p were pooled (FrIV, 500 ml, 0.47 mg/ml) and precipitated with 0.25 g/ml ammonium sulfate. After centrifugation, the pellet was resuspended in 50 ml of Buffer A and then dialyzed against Buffer A for 2.5 h. The protein was diluted with Buffer A to a conductivity equivalent to 100 mM NaCl. Particulate matter was removed by centrifugation at 10,000 rpm for 10 min at 4 °C in an SS34 rotor. The supernatant was applied to a 20-ml Mono Q column equilibrated in Buffer A. The column was then washed with Buffer A containing 200 mM NaCl before eluting the protein with a 400 ml, 200 mM to 600 mM NaCl gradient in Buffer A. Fractions containing Mcm2p were pooled (FrV, 50 ml, 2.7 mg/ml) and then stored at −80 °C.

Purification of Mcm3p—Lysate from the Mcm3p expression strain was fractionated by adding 0.2 g/ml ammonium sulfate and stirred at 4 °C for 30 min, before centrifugation for 30 min at 10,000 rpm in an SLA1500 rotor. The pellet was resuspended in 200 ml of Buffer A containing 0.2 g/ml ammonium sulfate and then re-pelleted. The pellet was resuspended in 200 ml of Buffer A, then dialyzed against Buffer A for ~3 h. The preparation was diluted with Buffer A to a conductivity equal to 100 mM NaCl (final volume, 400 ml), then applied to a 200-ml Fast Flow Q Sepharose column equilibrated in Buffer A. The protein was eluted with a 2 liter, 0–500 mM NaCl gradient, in Buffer A. Peak fractions containing Mcm3p were pooled (Fr III, 130 ml, 1 mg/ml) and then dialyzed against Buffer B for 2 h. The protein was diluted with Buffer B before it was applied to a 100-ml heparin-agarose column equilibrated in Buffer B. The column was washed in Buffer B, then Mcm3p was eluted with a 1000-ml, 0–500 mM NaCl gradient in Buffer B. Fractions containing Mcm3p were pooled (FrIV, 280 ml, 0.25 mg/ml) and then 0.3 g/ml of ammonium sulfate was added. After centrifugation, the pellet was resuspended in 25 ml of Buffer A and dialyzed against Buffer A for 2 h. The preparation was diluted with Buffer A to a conductivity equivalent to 50 mM NaCl (final volume, 60 ml) before being applied to an 8-ml Mono Q column equilibrated in Buffer A. The column was washed, and then Mcm3p was eluted with a 160-ml gradient of 0–500 mM NaCl in Buffer A. Peak fractions containing Mcm3p were pooled (FrV, 40 ml, 3.3 mg/ml) and stored at −80 °C.

Purification of Mcm4p—FrI containing Mcm4p was treated with 0.3 g/ml ammonium sulfate. After centrifugation at 4 °C and 12,000 rpm in an SLA1500 rotor, the pellet was resuspended in Buffer A containing 100 mM NaCl. Then, 0.25 g/ml ammonium sulfate was added and re-pelleted. This step was repeated two more times. The resulting pellet was resuspended in 150 ml Buffer A, then dialyzed against Buffer A. The protein was applied to a 200-ml Fast Flow Q Sepharose column equilibrated in Buffer A + 100 mM NaCl, and the column was washed with the same buffer. Mcm4p was eluted with a 2-liter, 100–500 mM NaCl gradient in Buffer A. Peak fractions containing Mcm4p were pooled (FrIII, 300 ml, 0.51 mg/ml) and 0.3 g/ml ammonium sulfate was added. After centrifugation, the pellet was resuspended in 30 ml of Buffer B and dialyzed against Buffer B for 4 h. The protein was applied to a 70-ml heparin-agarose column equilibrated in Buffer B with 100

mM NaCl, and then the column was washed in Buffer B containing 100 mM NaCl. Mcm4p was eluted from the column with a 700 ml, 100–500 mM NaCl gradient in Buffer B. The peak fractions containing Mcm4p were pooled (FrIV, 180 ml, 0.12 mg/ml) and precipitated with 0.3 g/ml ammonium sulfate. After centrifugation, the pellet was resuspended in 10 ml of Buffer B and dialyzed against Buffer B for 2.5 h. The protein was applied to a 1-ml Mono S column equilibrated in Buffer B, and the column was washed with Buffer B + 100 mM NaCl. Mcm4p was eluted from the column with a 20 ml, 100–500 mM NaCl gradient in Buffer A. Peak fractions were pooled (FrV, 2.5 ml, 2.8 mg/ml) and stored at −80 °C.

Purification of His-Mcm5p—Lysis of induced cells harboring pET16b-MCM5 was performed as described above, except that DTT was omitted, the pH was adjusted to 7.9, and 5 mM imidazole was added. After centrifugation to remove cell debris, the supernatant was applied to a 50-ml Ni-charged chelating-Sepharose (Amersham Biosciences) column equilibrated in Buffer C containing 5 mM imidazole. The column was washed with 600 ml of Buffer C containing 5 mM imidazole and then 500 ml of Buffer C containing 60 mM imidazole. Bound proteins were eluted with a 500 ml, 60 mM to 1 M imidazole gradient in Buffer C. Peak fractions containing His-Mcm5p were pooled (FrII, 40 ml, 0.25 mg/ml) and then dialyzed first against Buffer A containing 0.5 M NaCl, but no DTT, and then against Buffer A containing 100 mM NaCl. After dialysis, the preparation was clarified by centrifugation for 10 min at 12,000 rpm in an SS34 rotor at 4 °C, then applied to a 1-ml Mono Q column equilibrated in Buffer A with 100 mM NaCl. The column was washed with the same buffer then eluted with a 20 ml, 100–500 mM NaCl gradient in Buffer A. Peak fractions were pooled (FrIII, 9 ml, 0.5 mg/ml) and stored at −80 °C.

Purification of His-Mcm6p—The Mcm6p-expressing cells were lysed as described above except that DTT was omitted, the pH was 7.9, and 5 mM imidazole was added to the cell slurry. After centrifugation, the supernatant was applied to a 25-ml Ni-NTA Sepharose column equilibrated in Buffer C containing 5 mM imidazole. After washing the column with 800 ml of Buffer C containing 5 mM imidazole, followed by 1 liter of Buffer C with 60 mM imidazole, bound protein was eluted with a 250 ml, 60–500 mM imidazole gradient in Buffer C. Peak fractions containing Mcm6p were pooled (FrII, 125 ml, 0.56 mg/ml) and dialyzed against Buffer A for 2 h. The preparation was then applied to a 4-ml EAH Sepharose (Amersham Biosciences) column equilibrated in Buffer A, and the column was washed with Buffer A containing 100 mM NaCl. The column was eluted with a 40-ml, 100–500 mM NaCl gradient in Buffer A (40 ml). Peak fractions were pooled (FrIII, 65 ml, 0.6 mg/ml), dialyzed against Buffer A for 2 h, and then applied to an 8-ml Mono Q column equilibrated in Buffer A. The column was eluted with an 80 ml, 0.1–0.5 M NaCl gradient. Peak fractions containing Mcm6p were pooled (FrIV, 30 ml, 0.44 mg/ml) and stored at −80 °C.

Purification of Mcm7p—Cell lysate of Mcm7p-expressing cells was treated with 0.3 g/ml ammonium sulfate. After centrifugation for 30 min at 12,000 rpm in an SLA1500 rotor at 4 °C, the pellet was resuspended in Buffer A containing 0.25 g/ml ammonium sulfate and then centrifuged as above. This step was repeated using Buffer A containing 0.2 g/ml ammonium sulfate. The resulting pellet was resuspended in 100 ml of Buffer A and dialyzed against Buffer A for 2 h (final conductivity equal to 110 mM NaCl). The protein (FrII, 100 ml, 3.6 mg/ml) was applied to a 120-ml Fast Flow Q Sepharose column equilibrated in Buffer A, and the column was washed with the same buffer. The column was eluted with a 1.2-liter, 0–500 mM NaCl gradient in Buffer A. Peak fractions containing Mcm7p were pooled (FrIII, 105 ml, 2.4 mg/ml) and treated with 0.3 g/ml ammonium sulfate. After centrifugation, the pellet was resuspended in 100 ml of Buffer B, then dialyzed overnight against Buffer B containing 100 mM NaCl. The protein was diluted to a conductivity equal to 80 mM NaCl with Buffer B and then applied to a 170 ml Heparin agarose column equilibrated in the same buffer. The column was washed with Buffer B and then eluted with a 1.5-liter, 0–500 mM NaCl gradient in Buffer B. Peak fractions containing Mcm7p were pooled (FrIV, 450 ml, 0.30 mg/ml) and 0.3 g/ml ammonium sulfate was added. After centrifugation the pellet was resuspended in 25 ml of Buffer A and dialyzed against Buffer A for 2.5 h. The protein was diluted with Buffer A before being applied to an 8-ml Mono Q column equilibrated in Buffer A. Bound proteins were eluted with an 80-ml, 0–500 mM NaCl gradient in Buffer A. Peak fractions containing Mcm7p were pooled and then stored at −80 °C.

Reconstitution of Mcm2-7p—An equimolar amount (44 nmol) of each MCM protein was mixed and concentrated using a Centricon-50 ultrafiltration device at 4 °C to a final volume of 2.5 ml and a conductivity equivalent to 50 mM NaCl in Buffer A. After incubation at 15 °C for 30 min, the sample was applied to a 1-ml Mono Q column equilibrated in Buffer A. The column was eluted with a 20-ml, 0–500 mM NaCl gradient in Buffer A. A portion (3 mg in 250 μl) of the peak fractions containing all six MCM proteins was dialyzed against Buffer A with 50 mM NaCl and then was applied to a Superose 6 gel filtration column as described below. A portion (2 μl) of the peak fractions from the gel filtration column was examined for ATPase activity as described below, except that the reactions were incubated for 60 min at 30 °C. The presence of Mcm2p and Mcm4p in the gel filtration fractions was determined by Western blotting using standard protocols.

Gel Filtration Analysis—Gel filtration was performed at 4 °C in Buffer A containing 100 mM NaCl using a Superose 6 gel filtration column (Amersham Biosciences). To test for interactions between MCM proteins, the indicated amounts of proteins were mixed, diluted to a conductivity equivalent or below 100 mM NaCl and then, if necessary, concentrated to 2 mg/ml using a Centricon-50 ultrafiltration device (Amicon). The sample was incubated at 15 °C for 30 min. Any debris was removed by centrifugation for 10 min at 12,000 rpm in a table top centrifuge, and then the sample was applied to the gel filtration column and eluted in Buffer A containing 100 mM NaCl. After the first 5.9 ml, fractions of 175 μl were collected at 4 °C. A portion of each fraction, as indicated, was analyzed by SDS-PAGE (6%). Densitometric analysis was performed using a laser densitometer and ImageQuant software (Molecular Dynamics).

Nucleotide Hydrolysis—Nucleotide hydrolysis was measured by thin layer chromatography. For analysis of individual MCM proteins and the pairwise combinations, each reaction (15 μl) contained 1 mM [α-^{32}P]ATP (8–30 μCi/ml; PerkinElmer Life Sciences), 20 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, and 2 mM DTT as well as 0.5 μM of each MCM protein. Reactions were incubated for 30 min at 30 °C and then quenched by addition of 15 μl of 50 mM EDTA, pH 8.0. For the Mcm3/7p mutant study, 18 μM of Mcm3p and 0.5 μM of Mcm7p, mutant or wild type, was used, and the reactions were incubated for 20 min at 30 °C. A portion (1 μl) of each reaction was spotted onto a polyethyleneimine cellulose thin layer chromatography sheet (EM Science) and then developed in 0.6 M potassium phosphate pH 3.4 for 35 min. The amount of ADP produced was quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics). After determining the volume of the ADP spot and the ATP spot, the amount of ADP produced (in μM) was determined by: ((volume ADP)/(volume ADP + volume ATP)) × 1000 μM.

RESULTS

Assembly of Mcm2-7p from Individual Subunits—Mcm2-7p copurify as a heterohexamer from many different eukaryotes (9, 24–27). Assembly of Mcm2-7p may require a certain phosphorylation state, or may require a particular order of subunit addition (*i.e.* like the $\gamma/\tau\delta\delta'\chi\psi$ clamp loader, Ref. 28). To test this we constructed *E. coli* expression plasmids for each MCM protein and purified the individual MCM proteins as described under "Experimental Procedures." Mcm5p and Mcm6p had low yields and therefore ten histidine residues were placed on the N termini of these proteins for purification by nickel affinity chromatography. Yields were 10–200 mg of pure protein from 24 liters of cell culture. The final preparation of each protein is shown in Fig. 1*A*.

To determine whether these six recombinant MCM proteins could assemble into a heterohexamer, the six individual MCM proteins were combined and analyzed by ion exchange chromatography on a Mono Q column and then by gel filtration. Results of the Mono Q column showed that several MCM proteins resolved from one another, but toward the end of the gradient all six of the proteins co-eluted, indicating that the Mcm2-7p complex may have assembled (not shown). Some MCM proteins (Mcm2p and/or Mcm4p) appeared in stoichiometric excess, indicating that the putative hexamer formed but eluted along with excess single subunits. Fractions containing the putative Mcm2-7p complex were pooled and analyzed by gel filtration on a Superose 6 column to separate any free proteins from the heterohexamer (Fig. 1*B*). All six subunits co-eluted at the expected size of a Mcm2-7p hexamer comprised of one of each subunit (607 kDa) suggesting that heterohexamer had formed. The complex resolves from the excess Mcm2p/Mcm4p and elutes earlier than any of the MCM proteins analyzed individ-

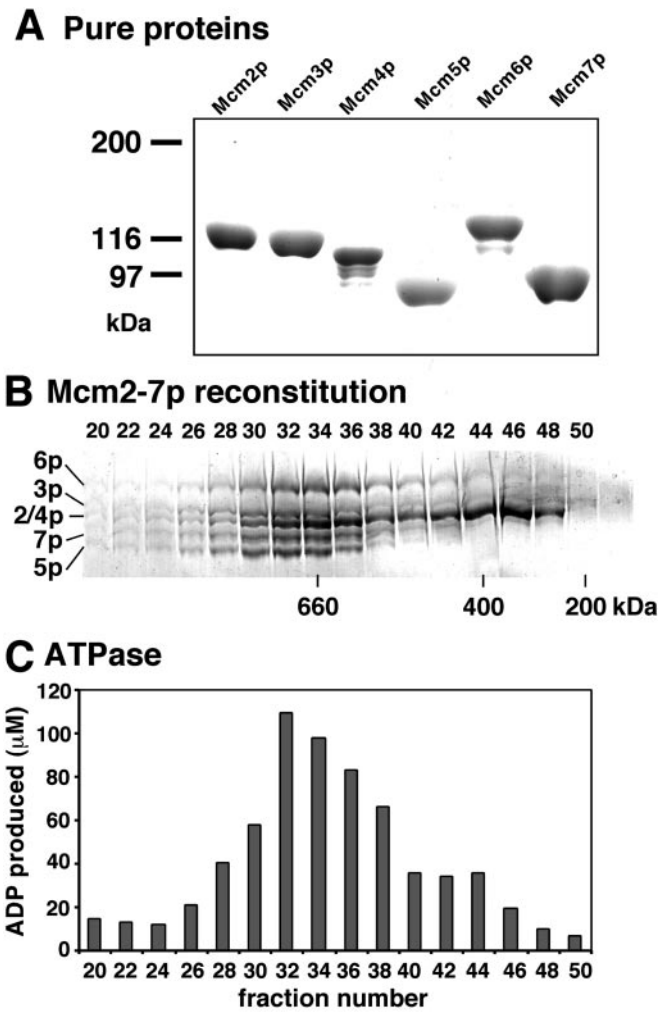

FIG. 1. **Purification and reconstitution of Mcm2-7p.** *A*, Coomassie Blue-stained SDS-polyacrylamide gel (6%) of 2.5 μg of each MCM protein. Mcm2p (99 kDa) and Mcm6p (113 kDa) both migrate slightly larger than their predicted sizes. *B*, Mcm2-7p-containing fractions from a Mono Q column were pooled and analyzed by gel filtration. The fraction numbers are *above* the gel, and the migration of size standards is indicated at the *bottom*. Each MCM protein is identified on the *left* of the gel. *C*, ATPase assays of the gel filtration column fractions. The assays were incubated for 60 min. Fraction numbers correspond to the fractions in *B*.

ually (compare Figs. 1 and 4*A*). Furthermore, the Mcm2-7p complex elutes earlier that any of the pairwise complexes described later (see Fig. 4*B*). However, it is still possible that other subcomplexes, comprised of fewer subunits, elute at the same position as Mcm2-7p heterohexamer. Indeed, there appears to be some slight variation in subunit ratios in different fractions (compare the Mcm2/4 band in fractions 30 and 32 in Fig. 1*B*). Mcm2p and Mcm4p did not resolve in the SDS gel, but the presence of both proteins in the Mcm2-7p heterohexamer was confirmed by Western blotting (not shown). The results indicate that assembly of the Mcm2-7p complex can proceed without a particular subunit order, or need for phosphorylation.

It has been reported that the Mcm2-7p heterohexamer in budding yeast is an ATPase (29), however in other systems such an activity has not been detected (7). Fig. 1*C* shows that the majority of ATPase activity co-elutes with Mcm2-7p (30 pmol/μg/min in fraction 32). Addition of single strand DNA did not affect this activity (not shown). A weaker, but significant ATPase activity is associated with later fractions (fractions 38–48) that do not appear to contain all six subunits. As we will show later in this study and has been shown in other studies (29), there are pairwise complexes of MCM proteins that contain ATPase activity (see Fig. 2*A*). Thus some of the activity in fractions 38–48 may be the result of those smaller complexes. As noted above, there are some slight variations in MCM protein ratios from fraction to fraction. We cannot completely rule out the possibility that the ATPase activity in these fractions is due to contaminating subcomplexes comprised of fewer than six subunits or that the hexamer dissociates into smaller subcomplexes when diluted into the ATPase assay. Next, we examined the individual MCM proteins for ATPase activity.

ATPase Activities of the MCM Subunits and Subcomplexes—Study of individual MCM proteins showed very little or no ATP hydrolysis activity (Fig. 2*A*). Longer incubation times or higher concentrations of Mcm2p, 3p, 4p, 5p, or 6p (for example, 100 ng/μl for 60 min) did not yield significant ATP hydrolysis (data not shown). However, when Mcm7p was assayed at a higher protein concentration, some hydrolysis of ATP was observed (Fig. 2*A, inset*; 7 pmol/μg/min). The ATPase activity is likely intrinsic to Mcm7p, rather than due to a contaminant, as mutation of the invariant lysine in the Mcm7p ATP site (described in more detail below) resulted in a mutant protein, $Mcm7p^{P-loop}$ that lacked significant ATPase activity (Fig. 2*A, inset*). We next tested pairwise combinations of MCM proteins for ATPase activity.

Each of the fifteen different combinations of two MCM proteins was tested for ATPase activity. The results in Fig. 2*A* show that three of the pairwise combinations result in ATPase activity. The Mcm3/7p pair produced the most active ATPase (Fig. 2*A*; ~220 pmol/μg/min). As this study was performed, a report appeared that supports this observation (29). In addition, ATPase activity is associated with the Mcm4/7p pair (~104 pmol/μg/min). A still weaker activity is associated with the Mcm2/6p pair (35 pmol/μg/min), consistent with a previous study (29). These assays were performed in the absence of DNA; however the presence of M13mp18 single strand DNA (330 ng) did not affect any of the ATPase activities documented herein (data not shown). It is also interesting to note that the pairwise complexes have significantly higher ATPase activity than a heterohexamer containing all six MCM proteins (compare Fig. 1*B* with Fig. 2*A*). This trend of the Mcm2-7p heterohexamer having less activity than complexes comprised of fewer subunits has been observed by others (7, 29).

The results of Fig. 2*A* show that significant ATPase activity requires at least two MCM proteins. This observation is consistent with the location of ATP sites at the interfaces of AAA+ protein complexes. Take for example the circular pentameric $\gamma_3\delta\delta'$ clamp loader. Only the γ subunits bind ATP and thus the pentamer contains three ATP sites (16, 30). The three ATP sites are located at the δ'/γ_1, γ_1/γ_2 and γ_2/γ_3 interfaces (Fig. 2*B*). Residues from both subunits are important for ATP hydrolysis: One subunit binds ATP and the neighboring subunit contains an arginine residue, which is thought to be important for catalysis. The putative catalytic arginine is embedded in an "SRC" motif in both γ and δ′, and this motif is conserved in clamp loader subunits from bacteria, T4 phage, eukaryotes, and Archaea (31–33).

Alignment of the γ and δ′ sequences with MCM proteins of budding yeast, shows that each MCM subunit contains an "SRF" motif in the same position as the SRC motif of the clamp loader subunits (Fig. 2*C*), suggesting that this arginine may be catalytic. This motif is also conserved in MCM proteins from other species (not shown). The fact that individual MCM proteins have very little or no ATPase activity, and that ATPase activity is detected

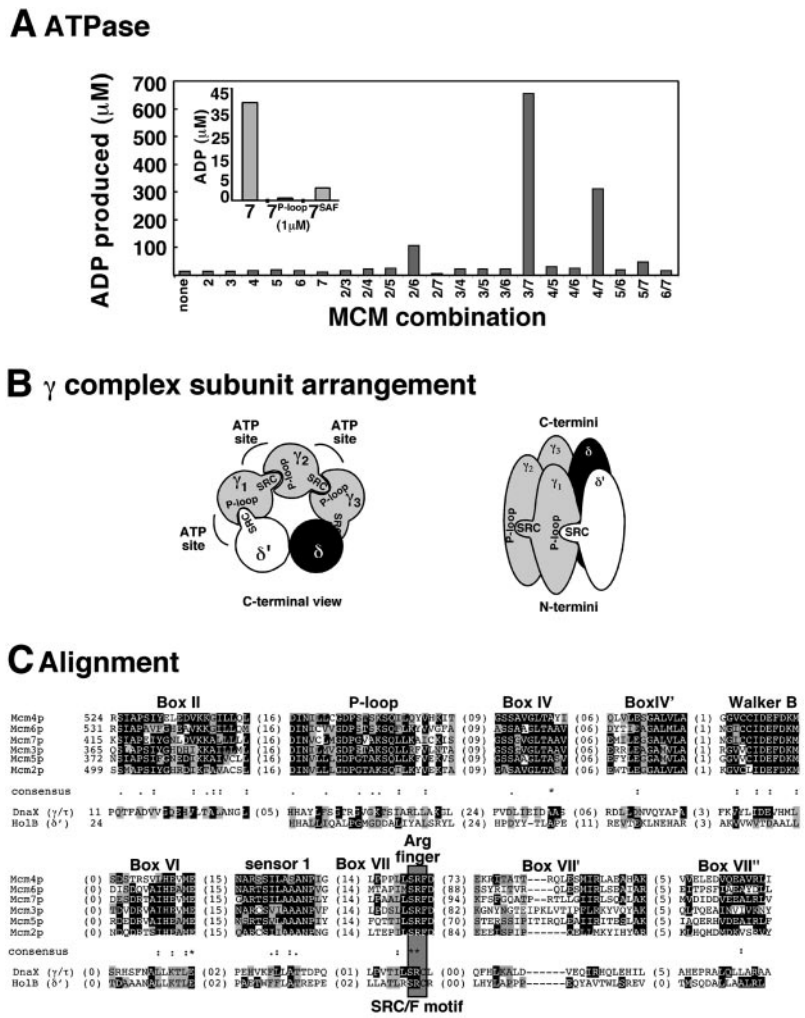

FIG. 2. **ATPase analysis of MCM proteins.** *A*, each MCM protein, individually or in combinations of two, was analyzed for ATPase activity. The *inset* shows ATP hydrolysis by Mcm7p, wild type or mutant, under more sensitive conditions; other single subunits gave no signal. *B*, schematic of the *E. coli* γ complex clamp loader, based on the structure looking down the C-terminal face (*left diagram*) or from the side (*right diagram*) (16). The arginine fingers are indicated by *SRC*, and the ATP binding sites by *P-loop*. *C*, alignment of Mcm2-7p and the γ (DnaX) and δ′ (HolB) subunits of γ complex. The Mcm2-7p proteins were aligned using ClustalX (48). Only the sequences within the *boxes* defined in Ref. 15 are shown. In addition, the corresponding sequences in the γ and δ′ subunits are shown *below* the *consensus line* and were positioned according to the alignment in Ref. 15. In the consensus line, positions that are fully identical are marked by a *star*; the *colon* marks positions that are highly conserved residues, and the *dot* marks those that are less conserved. Identical amino acids at a given position are *boxed* in *black*, similar amino acids at a given position are *gray*.

when two subunits are mixed, is consistent with ATP sites that require a catalytic arginine from a second subunit.

Analysis of the Mcm3/7p ATP Site—The hypothesis that there is only one complete ATP site in Mcm3/7p, and that ATP hydrolysis requires the Arg residue of the SRF motif of one subunit and the ATP site of the other, was tested in the experiments of Fig. 3. The Arg residue of the SRF motif in Mcm3p (R542) and in Mcm7p (Arg-593) were each changed to Ala and the resulting mutant proteins, Mcm3p^{SAF} and Mcm7p^{SAF}, were purified. The prediction is that only one combination of wild type and mutant protein will result in loss of ATPase activity, namely the one that participates in the ATP site at the interface of the Mcm3/7p complex (see *diagrams* in Fig. 3*A*).

In Fig. 3*A*, the bipartite construction of the ATP site is illustrated such that the subunit to the left contributes the presumptive catalytic arginine, and the subunit to the right binds the ATP to be hydrolyzed. This left-to-right or clockwise orientation is that observed from the C-terminal face of the subunits in multimeric AAA+ machines (see γ complex in Fig. 2*B*). Two subunit arrangements for the Mcm3/7p complex are possible. The arrangement to the *left* in Fig. 3*A* predicts that only mutation of the arginine in Mcm7p will decrease ATPase activity; the corresponding mutation in Mcm3p should have no effect. Conversely, the arrangement to the right predicts that only mutation of the arginine in Mcm3p will have an adverse effect on the Mcm3/7p ATPase.

We first tested whether the Mcm3p^{SAF} mutant retained ATPase activity when mixed with wild type Mcm7p. The results in Fig. 3*A* show that the Mcm3p^{SAF} mutant failed to produce ATPase activity. Thus, one may predict that the Arg of Mcm7p may not be needed for hydrolysis of ATP bound to Mcm3p. As the results in Fig. 3*A* show, this prediction is upheld: the combination of Mcm7p^{SAF} and Mcm3p formed as active an ATPase as wild type Mcm3/7p.

The loss of ATPase activity with Mcm3p^{SAF}/7p may be due to inability of the mutant to form a complex with Mcm7p. In Fig. 3*C*, a mixture of Mcm3p^{SAF} and Mcm7p was analyzed by gel filtration to determine if they form a complex. The results show that Mcm3p^{SAF} and Mcm7p co-elute earlier than either subunit alone, indicating that they form a complex. A similar analysis using wild type Mcm3p and Mcm7p yields the same result.

The above results suggest that the Mcm3/7p ATPase binds ATP in Mcm7p, not Mcm3p. If this is the case, then mutation of the Mcm3p ATP site should not affect the Mcm3/7p ATPase (see *diagram* in Fig. 3*B*). To test this, the P-loop motifs of Mcm3p and Mcm7p were mutated. The P-loop or Walker A motif is conserved in many nucleotide-binding proteins and binds the phosphate moiety of the nucleotide (34, 35). The invariant lysine of the P-loop motif, Lys-415 of Mcm3p and Lys-466 of Mcm7p, were changed to Ala and the mutant proteins, Mcm3p^{P-loop} and Mcm7p^{P-loop}, were purified and examined for ATPase activity in combination with the appropriate wild type protein. The results, in Fig. 3*B*, confirm the prediction that mutation of the Mcm7p P-loop produces an inactive ATPase with wild type Mcm3p. Also, as predicted, mutation of the Mcm3p P-loop has no effect on the Mcm3/7p ATPase. This result is consistent with a coincident study on P-loop mutants

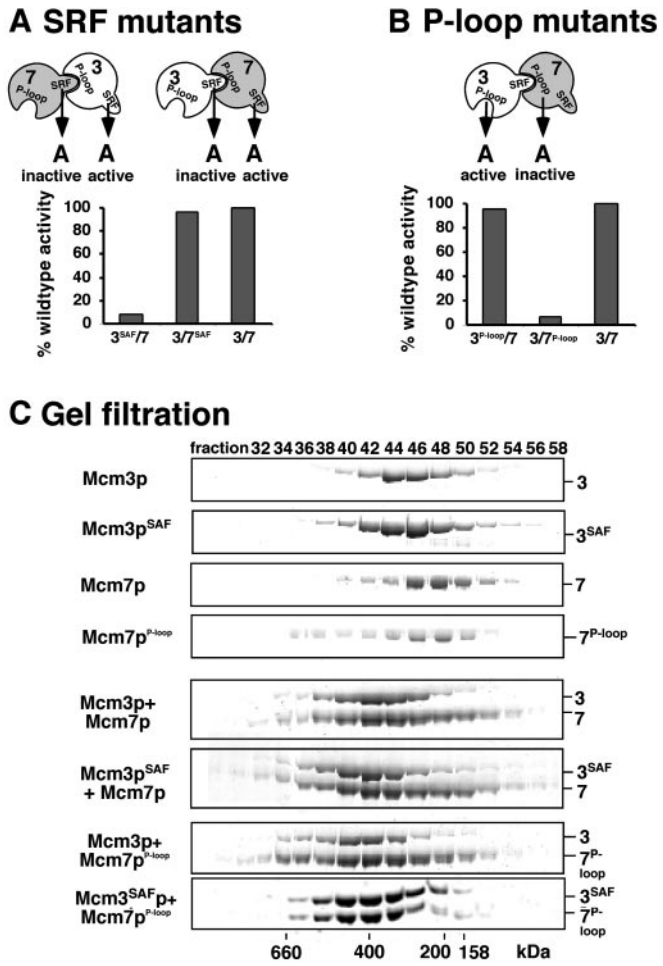

FIG. 3. **Mutation of the Mcm3p SRF motif eliminates Mcm3/7p ATPase activity.** The effect of changing the conserved Arg in the SRF motif and the conserved Lys of the P-loop motif of either Mcm3p or Mcm7p on the Mcm3/7p ATPase was determined. *A*, mutation of the SRF motifs in Mcm3p and Mcm7p is expected to correspond to one of the two predictions shown. The results, shown as a percentage of wild type Mcm3/7p activity, indicate that the prediction on the right is the correct model. *B*, the results of mutating the P-loop motifs in Mcm3p and Mcm7p lead to the prediction shown here. The prediction is fulfilled by the results, expressed as a percentage of wild type Mcm3/7p activity. *C*, mutant and wild type Mcm3p and Mcm7p, together or separately, were analyzed by gel filtration. Fraction numbers for each analysis are shown *above* the top gel, and the elution positions of molecular size standards are shown *below* the bottom gel. Positions of the Mcm3p and 7p are shown to the *right* of each gel.

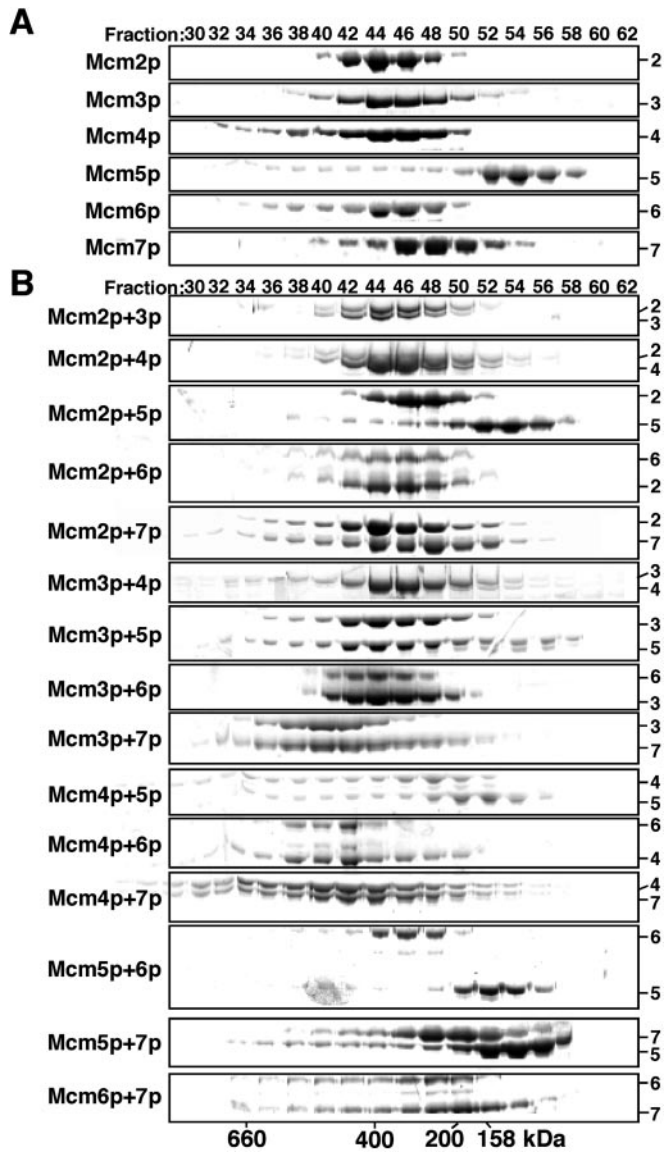

FIG. 4. **Physical interactions between MCM proteins.** Interactions among the MCM proteins were examined by gel filtration analysis as described under "Experimental Procedures." Analysis of the individual MCM proteins is shown in the first group of six. Column fraction numbers are shown *above* the top gel. The second group comprises the analysis of each of the fifteen pairs of MCM proteins. The elution positions of size standards are shown *below* the last gel. Each MCM protein is identified to the *right* of each SDS gel.

of Mcm3p and Mcm7p (29). Gel filtration analysis of Mcm3p and Mcm7p^{P-loop} show that the two proteins interact, and thus the inability to form a complex is not the cause for the loss of ATPase with Mcm3p/7p^{P-loop} (Fig. 3*C*).

Overall, these results demonstrate that the Mcm3/7p ATPase binds ATP in Mcm7p, and hydrolysis requires a catalytic Arg within the SRF motif of Mcm3p. Interestingly, the ATPase activity intrinsic to Mcm7p may also utilize an interfacial catalytic arginine since Mcm7p oligomerizes on its own (discussed later, in Fig. 4). In further support of this conclusion, the Mcm7^{SAF} mutant has very little ATPase activity compared with wild type Mcm7p (Fig. 2*A*, *inset*).

Arrangement of MCM Proteins—Use of a catalytic arginine in Mcm3p for hydrolysis of ATP bound to Mcm7p predicts how the subunits are arranged when viewed from the C termini (as in Fig. 3). By analogy to $\gamma_3\delta\delta'$, Mcm7p is immediately clockwise of Mcm3p when viewed from the C-terminal face of these proteins, just as γ_1 is clockwise of δ' (Fig. 2*B*). Next we examined the fifteen different pairwise combinations of MCM proteins for interaction by gel filtration to fill in the other positions around the MCM ring.

A stabile interaction between proteins is indicated when the elution volume of one or both proteins is altered from its elution volume alone. As a control, we first determined the elution volume of each of the individual MCM proteins in the gel filtration column (Fig. 4*A*). The oligomeric state of each MCM protein can be estimated from the elution volume. However the results are only approximate since the migration of a protein through the gel filtration resin is affected by both shape and size. The monomeric protein masses, predicted from gene sequences, are: Mcm2p, 99 kDa; Mcm3p, 107 kDa; Mcm4p, 105 kDa; Mcm5p, 86 kDa; Mcm6p, 113 kDa; and Mcm7p, 95 kDa. The results demonstrate that Mcm5p elutes at a position consistent with a monomer, whereas Mcm2p, Mcm3p, Mcm4p, Mcm6p and Mcm7p elute at volumes consistent with a size range of 200–300 kDa, and thus may form oligomers.

Stabile interaction between MCM proteins was tested by

mixing pairs of MCM proteins and analyzing their elution behavior. As illustrated in the experiments of Fig. 3, an interaction between Mcm3p and Mcm7p is observed using this technique (Fig. 4*B*, *panel 9*). In addition, a complex between Mcm4p and Mcm7p is also detected (Fig. 4*B*, *panel 12*); Mcm4p and Mcm7p co-elute at a position earlier than either protein alone, indicating that they form a Mcm4/7p complex. A Mcm4/7p complex is consistent with the production of ATPase activity by this pair of proteins. Densitometric analysis reveals that the ratio of Mcm3p to Mcm7p (Fig. 4*B*, *panel 9*), and of Mcm4p to Mcm7p (Fig. 4*B*, *panel 12*), in the peak fractions is 1:1 and 0.9:1, respectively.

In addition to the Mcm3/7p and Mcm4/7p complexes, interactions are also detected between Mcm3p and Mcm5p, and between Mcm4p and Mcm6p (Fig. 4*B*, *panels 7* and *11*, respectively). Interactions between Mcm3p and Mcm5p have been reported previously in many different systems (7, 24, 27, 36–39). Consistent with the previous isolation of this complex (7), the Mcm3/5p complex elutes at a volume consistent with a heterodimer containing one each of Mcm3p and Mcm5p (predicted size, 188 kDa). Densitometric analysis indicates a ratio of 1:1 Mcm3p to Mcm5p in the peak fraction. The Mcm4/6p complex peaks at an elution volume consistent with a hetero-oligomer and the ratio of Mcm4p to Mcm6p is ~1:1 in the peak fraction.

Analysis of Mcm2p and Mcm6p by gel filtration gave no conclusive evidence of stabile complex formation (Fig. 4*B*, *panel 4*). This combination provides ATPase activity and thus is expected to form a complex. The mixture of Mcm2p and Mcm6p show that the two proteins co-elute from the gel filtration column, suggesting that they form a complex. Individually, Mcm2p and Mcm6p each elute as oligomers and migrate at the same position as the Mcm2/6p mixture. Thus, it is possible that a Mcm2/6p hetero-oligomer forms, but elutes at the same position as the homo-oligomers. Alternatively, the interaction between Mcm2p and Mcm6p, detected in the ATPase assays, may not be stabile to gel filtration. Similarly ambiguous results were also obtained with the combinations of Mcm2/3p, Mcm2/4p, Mcm3/4p, Mcm3/6p, and Mcm6/7p (Fig. 4*B*). In the absence of any convincing evidence of interaction between these pairs of proteins, we conclude that they do not interact. The protein-protein interactions that are observed, assuming the Mcm2/6p ATPase pair also interact, along with the documentation of an Mcm2-7p heterohexamer in many different systems (7, 9, 24–27, 29) predicts a unique arrangement of MCM proteins in a heterohexameric ring (Fig. 5).

DISCUSSION

Reconstitution of Mcm2-7p and Arrangement of Its Subunits—The individual MCM proteins have been expressed in *E. coli* and purified. These subunit preparations can be simply mixed together to reconstitute the Mcm2-7p heterohexamer, indicating that there is no obligatory subunit order of addition, or need for a particular phosphorylation state. No individual MCM protein displays significant ATPase activity even though they each have an ATP site. However, certain pairs of MCM proteins have ATPase activity. Examination of one of these ATPase pairs, Mcm3/7p, has revealed only one competent ATP site in which Mcm7p binds ATP and Mcm3p contributes a catalytic Arg residue. We also document which combinations of two proteins form hetero-oligomer pairs. The protein-protein contacts, along with information from the ATPase analysis, lead to a proposed arrangement of MCM proteins in a simple ring (Fig. 5). This model is not predicated in the Mcm2-7p heterohexamer having ATPase activity.

The proposed MCM ring includes the pairs of ATPases observed in Fig. 2 of this study: Mcm2/6p, Mcm3/7p, and Mcm4/7p, as well as the complexes observed in the gel filtration analysis of Fig. 4: Mcm3/5p, Mcm3/7p, Mcm4/6p, and Mcm4/7p. Protein-protein interaction studies herein indicate how the other subunits connect with Mcm3/7p and these side-by-side interactions are consistent with a ring as illustrated in Fig. 5. The ring in Fig. 5 is viewed from the C-terminal face of the complex. The arrangement predicts that Mcm4p of the Mcm4/7p ATPase binds the ATP, and that Mcm7p contributes the catalytic Arg residue. Likewise, the model predicts that the Mcm2/6p ATPase binds ATP in Mcm2p, and that Mcm6p contributes the catalytic Arg residue. For each of the six MCM proteins, mutation of the P-loop in the ATP binding site results in loss of cell viability in *S. cerevisiae* (29). Thus, each subunit is thought to interact with ATP. Whether all six subunits also hydrolyze ATP is unknown. Indeed, we cannot fully determine whether any of the ATP sites are active in the Mcm2-7p heterohexamer. Our ability to detect only three ATPase pairs should not be taken to imply that the other three subunits are not catalytic (explained in more detail later).

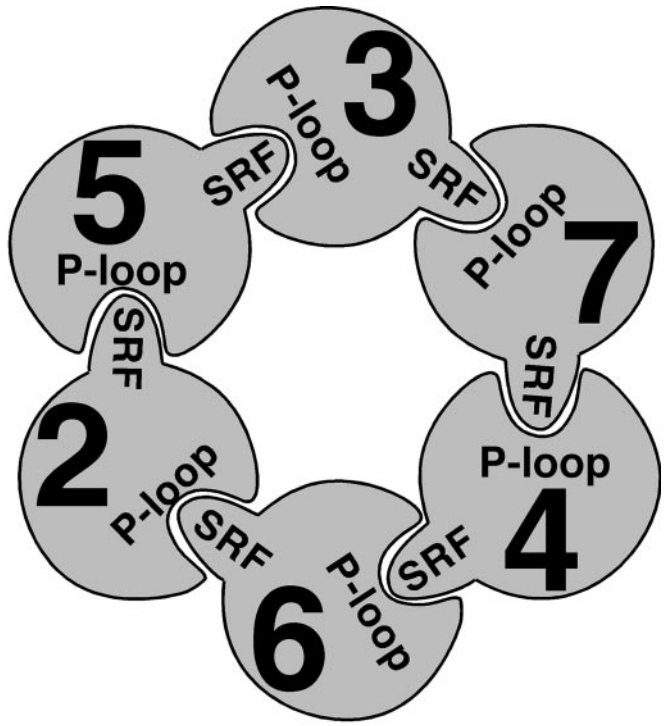

FIG. 5. **Model of the Mcm2-7p heterohexamer.** A model based on the interactions observed in the gel filtration analysis and ATPases analyses in this study as well as previous studies is shown. The orientation of the subunits around the ring is based on the view from the C-terminal face of multimeric AAA+ proteins, such as γ complex, in which the catalytic arginine in one subunit is clockwise of the ATP site in the other. The orientation of all subunits is anchored in the orientation of the Mcm3/7p ATPase pair established in Fig. 3. *SRF* represents the catalytic arginine motif and *P-loop* represents the ATP binding site.

The interactions observed in this study are also consistent with studies of MCMs from diverse organisms which have revealed not only the Mcm3/5p and Mcm3/7p complexes, but other complexes such as Mcm4/6/7p and Mcm2/4/6/7p (6, 7, 27, 29, 36–41), all of which can be assembled *in vitro* from these individual subunits.[2] An interaction between Mcm2p and Mcm4p has been reported for mammalian MCMs (42). However, this interaction was in the presence of Mcm6p and thus is also consistent with the model in Fig. 5. There may be additional interactions between MCM proteins that are not easily

[2] M. J. Davey and M. O'Donnell, unpublished data.

observed by gel filtration analysis or implied by ATPase analyses. Interestingly, we did not observe an interaction between Mcm2p and Mcm5p. Perhaps, the Mcm2-7p ring has a gap between these subunits. Alternatively, Mcm2p and Mcm5p may interact, but an interaction was not detected under the conditions used here.

In the arrangement proposed in Fig. 5, the Mcm4/6/7p subunits form one half of the hexameric ring and the Mcm2/3/5p subunits form the other half. Previous studies in other systems have shown that the Mcm4/6/7p complex unwinds DNA, and Mcm2p and Mcm3/5p complex interferes with this unwinding activity (6, 7). In those studies, the Mcm4/6/7p complex was shown to be a heterohexamer, probably a dimer of the Mcm4/6/7p heterotrimer. Electron microscope studies have revealed that the human Mcm4/6/7p complex is ring-shaped (10). Perhaps Mcm2p, 3p and 5p replace one of the Mcm4/6/7p trimers when Mcm2-7p is formed.

Why Bind ATP at an Interface?—The arrangement of ATP sites at the interface of subunits has implications for protein function. The placement of residues important for ATP binding and hydrolysis at one site in two different subunits provides a basis for communication between the subunits. For example, the ability of one subunit to perform a certain function or activity may be dependent on the nucleotide bound state of the neighboring subunit, which in turn could be sensed or communicated through positioning the catalytic arginine in or out of the ATP site. Specifically, ATP binding by one subunit may cause allosteric conformational changes in that subunit that may take the catalytic arginine located on its other side and place it in the proper orientation for catalysis of ATP bound to the neighboring subunit, triggering ATP hydrolysis.

Why Not Six ATPase Pairs?—ATPase analysis of pairwise combinations of MCM proteins revealed only three different ATPase pairs. One might expect six different ATP sites in the heterohexamer, one at each subunit interface, and therefore six ATPase pairs. Indeed, mutation of the P-loop of any one of the six MCM proteins inactivates Mcm2-7p (29). One possible explanation for detecting only three ATPase pairs may be that the three "inactive" pairs, Mcm2/5p, Mcm3/5p, and Mcm4/6p, require something extra to yield an active ATPase. For example, the arginine finger may not be positioned properly within the subunit pairs. Proper positioning may require interaction with other MCM proteins within Mcm2-7p. Alternatively, other factors may also be required. For example, DNA and/or other protein components of a replication fork may be required for all the sites to be active, and requiring their presence may ensure that Mcm2-7p is not active until it is properly arranged within a larger machinery.

It is also possible that not all of the ATP sites within Mcm2-7p are catalytic. A model of Mcm2-7p has been proposed in which three MCM proteins are non-catalytic, regulatory subunits (29). In this model there are two trimeric rings, one stacked upon the other. One ring contains the regulatory subunits and the other ring contains the catalytic subunits. The ATP sites are proposed to be at the interfaces between the regulatory and catalytic trimeric rings. This model nicely incorporates the observations that Mcm4/6/7p (the putative catalytic ring) is a helicase and Mcm2, 3, and 5p (the proposed regulatory ring) inhibits the helicase. However, the structures of AAA+ multi-protein machines show that ATP sites are formed at subunit interfaces that are "in plane" with one another, and thus the structural data are not consistent with a model in which ATP sites are formed at the stacking interface between two different protein rings.

The fact that only some ATP sites are utilized in a protein ring has been described for other helicases. For example, the T7 phage replicative helicase, gp4, is a ring shaped hexamer (43) that binds nucleotide with negative cooperativity (44). Presumably, some unoccupied sites are important to overall function of the hexamer. This negative cooperativity, along with other biochemical studies, led to the proposal of a mechanism similar to that of F_1 ATPase (45). This idea is largely upheld by structural studies of T7 gp4 (46). The structure suggests that the negative cooperativity is due to conformational changes induced in the ring by binding of nucleotide. A negative cooperative mechanism is certainly possible with Mcm2-7p, but since Mcm2-7p is a heterohexamer, it could simply contain some subunits that bind ATP, but do not hydrolyze it. Alternatively, all six MCM subunits may be catalytic, as indicated by the presence of an SRF motif in each subunit.

The exact role of the MCM heterohexamer in chromosomal replication is not understood. Mcm2-7p is the best candidate for the replicative helicase in eukaryotic cells. Consistent with a role at the replication fork, the MCM proteins associate with the origin before initiation of DNA replication (1, 2), and examination of Mcm4p and Mcm7p indicate that they travel with the replication fork (5). Only a subcomplex of Mcm4/6/7p has helicase activity *in vitro*; no helicase activity has been detected with Mcm2-7p (7). However, all six of the MCM proteins are required for ongoing replication in *S. cerevisiae* (3). One possible explanation for these seemingly conflicting observations is that Mcm2-7p heterohexamer requires the context of a replication fork for unwinding activity. For example, the unwinding activity of the *E. coli* replicative helicase is much greater when coupled to the replicative polymerase (47). A clear answer to the question of MCM function in DNA replication awaits the reconstitution of a eukaryotic replication fork *in vitro*.

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