The ATPase activity of MCM2–7 is dispensable for pre-RC assembly but is required for DNA unwinding

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Eukaryotes have six minichromosome maintenance (MCM) proteins that are essential for DNA replication. The contribution of ATPase activity of MCM complexes to their function in replication is poorly understood. We have established a cell-free system competent for replication in which all MCM proteins are supplied by purified recombinant *Xenopus* MCM complexes. Recombinant MCM2–7 complex was able to assemble onto chromatin, load Cdc45 onto chromatin, and restore DNA replication in MCM-depleted extracts. Using mutational analysis in the Walker A motif of MCM6 and MCM7 of MCM2–7, we show that ATP binding and/or hydrolysis by MCM proteins is dispensable for chromatin loading and pre-replicative complex (pre-RC) assembly, but is required for origin unwinding during DNA replication. Moreover, this ATPase-deficient mutant complex did not support DNA replication in MCM-depleted extracts. Altogether, these results both demonstrate the ability of recombinant MCM proteins to perform all replication roles of MCM complexes, and further support the model that MCM2–7 is the replicative helicase. These data establish that mutations affecting the ATPase activity of the MCM complex uncouple its role in pre-RC assembly from DNA replication.

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Introduction

Eukaryotic DNA replication initiates at multiple sites within the genome, occurring asynchronously throughout S phase (Gilbert, 2001). In *Xenopus laevis* early embryo, DNA replication initiates every 5–15 kb along chromosomes with no specificity in origin sequence (Hyrien and Mechali, 1993; Walter and Newport, 1997; Blow *et al*, 2001). Eukaryotes

share highly conserved mechanisms that regulate *in trans* the initiation of DNA replication from chromosomal origins (Bell and Dutta, 2002). During the G1 phase of the cell cycle, the stepwise assembly of the origin recognition complex (ORC), Cdc6, Cdt1, and the minichromosome maintenance (MCM) complex leads to the formation of the pre-replicative complex (pre-RC) marking replication origins (Diffley *et al*, 1994). At the G1/S transition, the activities of Cdc7/Dbf4 and Cdk2/cyclin E convert the pre-RC into the pre-initiation complex (pre-IC) (Jares and Blow, 2000; Walter, 2000). Cdc45 loads onto the chromatin, possibly through a direct interaction with the MCM proteins, and the activation of a chromatin-bound helicase results in the unwinding of DNA at the origins (Mimura and Takisawa, 1998; Zou and Stillman, 1998). Subsequently, the components of the elongation machinery, including RPA and DNA polymerases, are assembled at unwound origins, allowing for DNA synthesis to proceed (Mimura *et al*, 2000; Walter and Newport, 2000).

The MCM proteins were initially identified from screens for yeast mutants defective in minichromosome maintenance and cell cycle control (Moir *et al*, 1982; Maine *et al*, 1984; Takahashi *et al*, 1994). Six of the MCM proteins (MCM2–7) form a complex that is essential for the initiation and elongation of eukaryotic DNA replication in yeast, and are highly conserved from yeast to mammals (Tye, 1999; Forsburg, 2004). In addition, MCM8 and MCM10 are also important at later steps of DNA replication (Forsburg, 2004; Maiorano *et al*, 2005). MCM2–7 proteins are similar to each other, especially within a central 200-amino-acid core domain. As members of the AAA + superfamily of proteins, all MCM proteins contain an ATPase motif within this core, including Walker A and B motifs, which are highly conserved in DNA helicases (Koonin, 1993). MCM proteins exist predominantly as heterohexameric complexes of approximately 600 kDa (Fujita *et al*, 1997; Kubota *et al*, 1997). However, following cellular fractionation and biochemical purification, additional subcomplexes, including MCM2,4,6,7, MCM3,5, and MCM4,6,7, have also been identified (Prokhorova and Blow, 2000). Based on physical interaction studies *in vivo*, all six MCM subunits associate in a complex with equal stoichiometry (Forsburg, 2004). Most evidences suggest that MCM proteins productively bind to chromatin almost exclusively as a heterohexameric complex (Fujita *et al*, 1997; Prokhorova and Blow, 2000). The regulation of MCM binding to chromatin is a key, yet poorly understood, event in controlling origin activity and usage.

Human MCM4,6,7 subcomplex purified from HeLa cells, and recombinant MCM4,6,7 complexes from mouse, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* exhibit DNA helicase activity *in vitro* (Ishimi, 1997; Lee and Hurwitz, 2000; You *et al*, 2002; Kaplan *et al*, 2003). Moreover, the single MCM protein from the archaeon *Methanobacterium thermoautotrophicum* (MtMCM) contains processive helicase

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activity *in vitro*, suggesting that an MCM complex may evolutionarily function as the replicative DNA helicase in archaea and eukaryotes (Kelman *et al.*, 1999; Chong *et al.*, 2000; Shechter *et al.*, 2000). Although one study showed a seven-fold arrangement around a central axis of the MtMCM protein (Yu *et al.*, 2002), subsequent electron micrograph reconstruction of MtMCM unambiguously reveals a hexameric ring-shaped formation, with each monomer organized symmetrically around a six-fold axis (Pape *et al.*, 2003), consistent with the crystal structure of an MtMCM protein fragment (Fletcher *et al.*, 2003).

Because MCM4,6,7 has associated DNA helicase activity whereas the heterohexameric MCM2–7 does not, it has been proposed that MCM4,6,7 subcomplex may function as the catalytic core and possibly as the exclusive replicative helicase during DNA replication. However, there is no evidence for the formation of a functional MCM4,6,7 complex *in vivo*. In addition, *S. cerevisiae* degron mutants of MCM proteins demonstrated that all six proteins are necessary not only for initiation but also for elongation of DNA replication, suggesting that the entire heterohexameric complex is maintained at replication forks until termination of DNA replication (Labib *et al.*, 2000). However, one cannot rule out that in these experimental conditions, reducing MCM levels in cells may trigger a checkpoint as observed in mammalian cells (Cortez *et al.*, 2004). MCM proteins are essential and are required at multiple steps during DNA replication, including pre-RC and pre-IC assembly, origin unwinding, and elongation (Pacek and Walter, 2004; Shechter *et al.*, 2004). However, genetic analysis of MCM mutants has not identified mutations that separate the assembly role of MCMs from their role during unwinding, as the phenotype analyzed in the screens was inhibition of DNA replication.

Xenopus cell-free egg extracts support one round of cell-cycle-regulated chromosomal replication (Blow and Laskey, 1986). Previous studies in *Xenopus* using immunodepletions have demonstrated that a complex containing all six MCM proteins (MCM2–7) was required for one round of semiconservative DNA replication (Chong *et al.*, 1995; Madine *et al.*, 1995; Kubota *et al.*, 1997).

We have expressed and purified various recombinant *Xenopus* MCM complexes from baculovirus-infected cells. We demonstrate that the recombinant MCM2–7 complex is biologically active by its ability to (1) bind to chromatin assembled in cell-free extracts depleted of endogenous MCM proteins, (2) participate in pre-RC assembly, and (3) support DNA replication in MCM-depleted egg extracts. A mutation of the conserved lysine residue in the Walker A motif in both MCM6 and MCM7 subunits of the MCM2–7 complex (MCM2–7_{67KA}) results in the inhibition of ATPase activity of the complex. This ATPase-deficient MCM complex was able to support pre-RC assembly but was not able to support DNA replication. Our data suggest that ATP binding and/or hydrolysis by MCMs is dispensable for the early initiation steps but essential for DNA unwinding during DNA replication.

Results

Expression and purification of recombinant MCM complexes

To study the functions of MCM proteins, we reconstituted heteromeric MCM complexes using the baculovirus expression system. Each of the six *Xenopus* MCM cDNAs was

inserted into the pFastBac vector, with a C-terminal FLAG tag fused to MCM7 to facilitate purification of protein complexes. In addition, we introduced a lysine to alanine point mutation within the conserved Walker A motif of MCM6 (MCM6_{6KA}) and MCM7 (MCM7_{7KA}). This conserved lysine residue is essential for nucleotide binding of ATPases and DNA unwinding activities of helicases (You *et al.*, 2002). Mutations at corresponding sites in *S. pombe* and *S. cerevisiae* MCM7 confer the most severe phenotype as compared to the corresponding mutation in other MCMs (Schwacha and Bell, 2001; Gomez *et al.*, 2002). Using the baculovirus expression system, we expressed the following different recombinant complexes: wild-type complexes (MCM2–7, MCM4,6,7); single-mutant complexes (MCM2–7_{6KA}, MCM2–7_{7KA}, MCM4,6,7_{7KA}); and a double-mutant complex (MCM2–7_{67KA}). All complexes were purified to near homogeneity (Figure 1B–D, F, and G) through three purification steps: anti-FLAG immunoprecipitation, glycerol gradient centrifugation, and Mono Q ion exchange chromatography (Figure 1A and E). The Coomassie stain of the SDS–PAGE following the last purification step is shown for all complexes (Figures 1B–D, F, G, and 2B). The identity of the subunits was confirmed by Western blotting using specific antibodies directed against each *Xenopus* MCM protein (Figure 2A).

The intermediate glycerol gradient centrifugation purification step showed that wild-type and mutant MCM4,6,7 containing fractions cosedimented and peaked at approximately 600 kDa, suggesting that MCM4,6,7 exists predominantly as a hexameric complex (data not shown). These fractions were pooled and further purified using Mono Q ion exchange chromatography (Figure 1E–G). Glycerol gradient sedimentation of MCM2–7, MCM2–7_{7KA}, and MCM2–7_{67KA} also yielded a high-molecular-weight complex of approximately 600 kDa (Figure 1B–D), consistent with the predicted heterohexameric size. Furthermore, electrophoresis of the wild-type and mutant MCM complexes on a nondenaturing gradient acrylamide gel confirmed that only 600 kDa complexes were purified and complex formation was not affected by the mutation in the MCM subunit (data not shown).

Next, we performed co-immunoprecipitation studies on the recombinant MCM complexes to confirm the interactions between subunits. We used an antibody against *Xenopus* MCM6 to immunoprecipitate the recombinant complexes. We detected the presence of all three proteins (MCM4, MCM6, and MCM7) in the MCM6 immunoprecipitate of MCM4,6,7 and MCM4,6,7_{7KA} protein complexes (Figure 2C). To verify that recombinant MCM2–7 complex was not dissociated into MCM2,4,6,7 and MCM3,5 subcomplexes, we immunoprecipitated with an MCM6 antibody and performed Western blotting with an antibody against a representative MCM subunit of each known subcomplex (Figure 2D). Co-immunoprecipitation assays were also performed with MCM2–7_{67KA} to confirm complex stability (data not shown). Taken together, our analyses establish that recombinant MCM proteins assemble into stable heterohexameric complexes and that the formation of these complexes is not affected by the mutation in MCM6 and/or MCM7.

Mutation in the MCM Walker A motif results in loss of ATPase and DNA helicase activities of the complexes
MCM proteins are the best candidates for the replicative helicase, the enzyme that melts DNA at the replication fork.

Figure 1 Purification of recombinant *Xenopus* MCM complexes. Recombinant protein complexes expressed in baculovirus-infected cells (see Materials and methods) were purified following schemes (A) for MCM2-7, MCM2-7_{7KA}, and MCM2-7_{67KA}, and (E) for MCM4,6,7 and MCM4,6,7_{7KA}. Protein fractions eluted from (B) MCM2-7 glycerol gradient, (C) MCM2-7_{7KA} glycerol gradient, (D) MCM2-7_{67KA} glycerol gradient, (F) MCM4,6,7 Mono Q column, and (G) MCM4,6,7_{7KA} Mono Q column were analyzed by 10% SDS-PAGE and stained with Coomassie blue. Molecular weight standards were simultaneously analyzed and are indicated in (B-D).

DNA helicases are molecular motors that use the energy of ATP hydrolysis to catalyze the unwinding of duplex DNA into single strands (Patel and Picha, 2000). Thus, we examined the enzymatic activities associated with the purified recombinant complexes by determining if these complexes displayed ATPase and DNA helicase activities. First, we measured the ATPase activity of the different recombinant complexes. ATPase activity cosedimented with the *Xenopus* MCM2-7 and MCM2-7_{7KA} complexes across each respective glycerol gradient elution profile (Figure 3A and B). No ATPase activity was observed with the double-mutant complex, MCM2-7_{67KA} (Figure 3C-E), while the ATPase activity of the single-mutant complexes containing a point mutation in either MCM6 or MCM7 was significantly lower than wild-type MCM2-7 (Figure 3D and E; at 180 ng of protein complex, MCM2-7_{6KA} displayed ~50% of MCM2-7 activity). ATP hydrolysis by these hexameric complexes was not stimulated

by DNA (data not shown), as previously reported in *S. cerevisiae* (Schwacha and Bell, 2001; Davey *et al.*, 2003).

We also tested the ATPase activities of wild-type and mutant MCM4,6,7 across the Mono Q elution profile (Figure 3F and G). The peak of enzymatic activity coincided with fractions 42-44 of the MCM4,6,7 elution profile. Mutation in the Walker A motif of MCM7 resulted in almost a complete reduction of ATPase activity of the MCM4,6,7 complex (Figure 3H and I; at 120 ng of protein complex, MCM4,6,7_{7KA} displayed 16% of MCM4,6,7 activity).

Next, we determined whether these complexes had associated DNA helicase activity by testing the ability of these proteins to displace a ³²P-labeled 17-mer oligonucleotide annealed to M13-ssDNA. MCM4,6,7 displayed DNA helicase activity (Figure 4A and B), which coincided with the MCM4,6,7 Mono Q elution profile, similar to the observed ATPase activity (data not shown). No DNA helicase was

Figure 2 Characterization of purified recombinant MCM complexes. (A) Purified MCM2-7, MCM2-7^{7KA}, MCM4,6,7, and MCM4,6,7^{7KA} protein complexes from each final purification step were processed by PAGE followed by Western blot using the indicated MCM antibodies to confirm expression of MCM proteins within each recombinant complex. (B) Purified MCM2-7 complex was loaded onto a 3–8% NuPAGE Tris-acetate gel (Invitrogen) and Coomassie stained. Individual MCM subunits were also loaded onto the gel and processed by Western blot (data not shown) to identify the migration of each subunit within the complex. Based on these data, and the predicted molecular weight of each MCM protein, the position of each MCM subunit within the purified complex is indicated. (C, D) Co-immunoprecipitation analysis of purified MCM complexes. (C) MCM4,6,7 and MCM4,6,7^{7KA} complexes were immunoprecipitated with anti-MCM6 or preimmune antibody, and the immunoprecipitates were immunoblotted with anti-MCM4, anti-MCM6, and anti-MCM7 antibodies. (D) Purified MCM2-7 and MCM2-7^{7KA} were immunoprecipitated with anti-MCM6 or preimmune antibody, and the immunoprecipitates were immunoblotted with anti-MCM2, anti-MCM3, and anti-MCM7 antibodies.

observed with MCM4,6,7^{7KA} (Figure 4A and B). The heterohexameric MCM2-7 complex did not display DNA helicase activity *in vitro* (Figure 4A and B), although it displayed ATPase activity (Figure 3A, D, and E). Cdc45 is required for DNA unwinding and might function as a cofactor for MCM proteins (Mimura *et al*, 2000; Walter and Newport, 2000; Masuda *et al*, 2003; Pacek and Walter, 2004). We did not detect *in vitro* DNA helicase activity when we added Cdc45 protein to MCM2-7 complex (data not shown).

Pre-RC assembly and Cdc45 loading are independent of ATPase activity of the MCM complex

Initiation of DNA replication requires the sequential assembly of protein components of the pre-RC onto the chromatin. At the onset of S phase, the loading of Cdc45 and its association with the MCM complex is a critical step for origin firing and unwinding (Mimura and Takisawa, 1998; Mimura *et al*, 2000; Walter and Newport, 2000; Pacek and Walter, 2004). We tested the ability of recombinant MCM complexes to assemble onto chromatin. Recombinant MCM complexes were added to MCM-depleted extract in the presence of demembranated sperm nuclei. Following incubation, chromosomal DNA was purified and chromatin assembly of MCMs was determined by Western blotting with specific antibodies against different MCM proteins. The recombinant MCM2-7, MCM2-7^{7KA}, and MCM2-7^{67KA} protein complexes were loaded with similar efficiency onto the chromatin (Figure 5A). In contrast, we observed very low levels

of binding for the MCM4,6,7 and MCM4,6,7^{7KA} protein complexes onto the chromatin (Figure 5B).

These results were further confirmed by assessing the ability of these complexes to bind a biotinylated 1 kb dsDNA fragment following incubation in MCM-depleted extracts (Edwards *et al*, 2002). Both wild-type and mutant MCM2-7 complexes bound to the DNA with comparable efficiency (Figure 5C, lanes 1–5). Importantly, this binding was dependent on the pre-RC assembly, as it was inhibited in the presence of geminin and in Cdc6-depleted extracts (Figure 5C, lanes 6–9).

Next, we tested if recombinant MCM complexes were able to support loading of endogenous Cdc45 onto the chromatin, a subsequent step in pre-RC activation. Cdc45 loading is dependent on the presence of the MCM proteins on the chromatin and is required for the subsequent loading of DNA polymerases (Kubota *et al*, 1997; Mimura *et al*, 2000). Recombinant MCM2-7, MCM2-7^{7KA}, and MCM2-7^{67KA} complexes were able to support Cdc45 loading onto the chromatin to a similar extent (Figure 5A, lower panel). In contrast, neither MCM4,6,7 nor MCM4,6,7^{7KA} protein complexes supported Cdc45 loading onto chromatin (data not shown). The ability of the recombinant MCM2-7 complex to support endogenous Cdc45 loading onto chromatin establishes that the reconstituted protein complexes support this critical function of MCM proteins. Importantly, our results demonstrate that pre-RC assembly and subsequent Cdc45 assembly do not require ATPase activity of the MCM complex, but do require a heterohexameric MCM complex.

Figure 3 Mutations in Walker A motif of MCM6 and MCM7 subunits result in loss of ATPase activity of MCM2-7 complex. ATPase activity was measured by thin-layer chromatography (see Materials and methods) across each fraction (1 μ l) eluted from the glycerol gradient of (A) MCM2-7 complex, (B) MCM2-7_{7KA} complex, and (C) MCM2-7_{67KA} complex. (D) ATPase activity of MCM2-7, MCM2-7_{7KA}, MCM2-7_{6KA}, and MCM2-7_{67KA} was measured at the indicated amounts of protein complexes. (E) The corresponding pmol of phosphate (Pi) released was quantified using the PhosphorImager and plotted. (F, G) ATPase activity was measured across the indicated fractions (1 μ l) eluted from the Mono Q column of (F) MCM4,6,7 and (G) MCM4,6,7_{7KA}. (H) ATPase activity of MCM4,6,7 and MCM4,6,7_{7KA} was measured after Mono Q purification. (I) Quantification of phosphate released was plotted.

Origin unwinding is dependent on the ATPase activity of the MCM complex

Origin firing is associated with DNA unwinding, which can be measured by the transient association of the single-stranded DNA binding protein, RPA, to the chromatin. We tested if the wild-type and ATPase-deficient MCM complexes were able to support loading of RPA onto the chromatin. Recombinant MCM2-7 was able to load endogenous RPA onto the chromatin with similar efficiency as the control (Figure 6). In contrast, the loading of RPA was significantly reduced in the presence of MCM2-7_{67KA}, whereas the loading of endogenous Cdc45 was not affected (Figure 6, lane 3). These results suggest that the ATPase activity of the MCM2-7 complex is required for DNA unwinding, further supporting the hypothesis that the MCM complex is the replicative DNA helicase.

Intact ATPase activity of recombinant MCM2-7 is required to support pre-RC activation and DNA replication

To determine if recombinant MCM complexes can fulfill all MCM functions in replication, we tested whether recombi-

nant MCMs could support DNA replication in MCM-depleted extracts. We confirmed that, following depletion, all MCM proteins were quantitatively removed from the extract by Western blot (Figure 7A). DNA replication was assessed by incorporation of [α -³²P]dATP into chromosomal DNA followed by agarose gel electrophoresis. MCM-depleted egg extracts do not support DNA replication of demembrated sperm chromatin (Figure 7B, compare lane 1 to 8; Figure 7C, compare lane 1 to 4; and Figure 7D, compare lane 1 to 7). Addition of recombinant MCM2-7 complex to MCM-depleted extracts rescued DNA replication in a dose-dependent manner (Figure 7B). When replication reactions were run to completion (180 min), recombinant MCM2-7 complex was able to rescue DNA replication in MCM-depleted extracts at 170 nM, a concentration that is eight-fold lower than that of endogenous MCMs (data not shown). Therefore, the recombinant protein complex supports all replication functions of the endogenous MCM proteins in cell-free extracts. This establishes that recombinant MCM complex is fully active and that depletion of MCMs from the extracts did not remove any essential proteins apart from MCM2-7.

Figure 4 ATPase activity of MCM complex is required for DNA helicase activity *in vitro*. DNA helicase assays (5 fmol of template) were performed as described in Materials and methods. (A) Melting of a ³²P-labeled 17-mer oligonucleotide annealed to M13mp18 single-stranded DNA was measured at different concentrations of purified MCM4,6,7_{7KA}, MCM4,6,7, MCM2-7_{7KA}, and MCM2-7 complexes, as indicated. Lane 1 contained 5 fmol of template heated at 100°C for 2 min. Lane 2 contained 5 fmol of template with no added protein, incubated at 23°C for 1 h. (B) Quantification of displaced ³²P-labeled 17-mer oligonucleotides (femtomoles) for each titrated amount of protein was plotted.

Consistent with its inability to support DNA unwinding, the ATPase-deficient mutant MCM2-7 complex was not able to rescue DNA replication (Figure 7C and E), although it was able to efficiently support Cdc45 assembly onto chromatin. The single-mutant complex, MCM2-7_{7KA}, fully supported Cdc45 loading (Figure 5A) but only partially rescued DNA replication, as it shows less than 20% of the replication activity of wild-type MCM2-7 (Figure 7E, lane 3). Neither MCM4,6,7 nor MCM4,6,7_{7KA} protein complexes were able to rescue DNA replication in MCM-depleted extracts (Figure 7D and E), confirming that all six MCs are required for the initiation and, possibly, elongation of DNA replication. Taken together, our data demonstrate that ATP binding and/or hydrolysis by MCM protein complexes is dispensable for pre-RC assembly but is critical for later step(s) of DNA replication. In addition, this is the first biological analysis of *Xenopus* MCs demonstrating that the reconstituted MCM2-7 complex is functionally active in cell-free extracts as demonstrated by its ability to load onto chromatin, in addition to loading Cdc45 and RPA, and restore DNA replication in MCM-depleted egg extracts.

Discussion

We have established a cell-free system in which MCM2-7 protein function is fully supported by purified recombinant

Figure 5 Full ATPase activity is dispensable for pre-RC assembly and loading of endogenous Cdc45 onto chromatin. Chromatin binding and DNA binding assays were performed as described in Materials and methods. (A) Recombinant MCM2-7 complexes (33 nM; lanes 2–4) were each incubated in MCM-depleted extracts supplemented with sperm nuclei. Lane 1: no protein was added to MCM-depleted extracts (–); lane 5: mock-depleted extract. Chromosomal DNA was purified from each reaction and the chromatin was loaded on 10% SDS-PAGE. The presence of MCs and Cdc45 on the chromatin was detected by Western blotting with indicated anti-MCM3, anti-FLAG, and anti-Cdc45 antibodies. (B) Chromosomal DNA was purified following incubation in MCM-depleted extracts in the presence or absence of recombinant MCM4,6,7 complexes (33 nM; lanes 2 and 3). Chromatin was subjected to SDS-PAGE and processed by Western blot using anti-MCM3 and anti-MCM6 antibodies. (C) Recombinant MCM2-7 and MCM2-7_{7KA} (33 nM) were incubated in MCM-depleted extracts supplemented with biotinylated 1 kb DNA fragment in the absence (lanes 3 and 4) or presence (lanes 6 and 7) of geminin. Lane 1: no DNA was added to extracts. No protein was added to MCM-depleted extracts (lane 2), to mock-depleted extracts (lane 5), to mock-depleted extract supplemented with geminin (lane 8), or to Cdc6-depleted extracts (lane 9). The presence of MCs on the DNA was detected by Western blotting with anti-MCM6 antibody.

MCM complexes. In this system, recombinant MCM proteins support all known enzymatic and biological activities of MCM proteins associated with DNA replication. Studies of complexes containing mutations in the Walker A motif of MCM6 and MCM7 subunits have allowed us to separate the role of MCs in pre-RC formation from their role in postinitiation of DNA replication.

Establishing a system to study the multiple roles of MCM proteins in replication

Genetic and biochemical studies have demonstrated the involvement of MCM proteins in the initiation and elongation steps of DNA replication (Labib *et al*, 2000; Pacek and Walter, 2004; Shechter *et al*, 2004). Different cell-free systems have been used to specifically study the biological activity of MCM

Figure 6 ATPase-deficient MCM2-7 complex cannot support efficient loading of endogenous RPA onto chromatin. Chromatin binding was performed as described in Materials and methods. Recombinant MCM2-7 and MCM2-7_{67KA} were incubated in MCM-depleted extracts supplemented with sperm nuclei (lanes 2 and 3). No protein was added to MCM-depleted extracts (lane 1) or to mock-depleted extracts (lane 4). The presence of MCMs, Cdc45, and RPA on the chromatin was detected by Western blotting with indicated anti-MCM6, anti-Cdc45, and anti-RPA antibodies.

proteins. For example, (1) an MCM multiprotein complex immunoprecipitated from *Xenopus* extracts using MCM3 antibodies restored replication activity in MCM-depleted extracts (Madine *et al*, 1995; Kubota *et al*, 1997), (2) biochemical fractionation using PEG precipitation of *Xenopus* egg extracts into two fractions, RLF-B and an MCM-containing RLF-M fraction, demonstrated that both fractions are required for licensing activity (Chong *et al*, 1995), and (3) chromatographic fractionation of *Xenopus* extracts demonstrated the ability of the heterohexamers MCM2-7 to bind to chromatin and provide licensing or RLF/M activity (Prokhorova and Blow, 2000).

In contrast to the above studies in which MCM complexes were partially purified from extracts, we reconstituted a fully active MCM2-7 complex from recombinant proteins. This recombinant heterohexamer, purified to near homogeneity, is able to bind to chromatin, load Cdc45 onto chromatin, and restore DNA replication in MCM-depleted egg extracts in a dose-dependent manner. These MCM activities are regulated and dependent upon pre-RC assembly (Figure 5C). Similar to what has been demonstrated *in vivo*, recombinant MCM-dependent DNA replication requires prior assembly of Cdc6 and is completely inhibited in Cdc6-depleted extracts (data not shown). These data support the model that all six members of the MCM complex are essential for pre-RC assembly as well as for postinitiation steps of DNA replication. Furthermore, a system dependent upon recombinant proteins allows us to assess the consequences of point mutations in single MCM subunits. An example of such

Figure 7 Recombinant MCM2-7 supports DNA replication. (A) MCM-depleted and mock-depleted extracts (1 μ l) were analyzed on SDS-PAGE, followed by Western blot using each of the six MCM antibodies. (B–D) DNA replication assays were performed as described in Materials and methods. Replication reactions were stopped at 90 min and genomic DNA replication was monitored by incorporation of [α -³²P]dATP followed by agarose gel electrophoresis. (B) Recombinant MCM2-7 complex was added to MCM-depleted extracts at the indicated amounts. Lane 2: 900 ng/ μ l (1500 nM); lane 3: 400 ng/ μ l (670 nM); lane 4: 100 ng/ μ l (170 nM); lane 5: 50 ng/ μ l (83 nM); lane 6: 10 ng/ μ l (17 nM); lane 7: 5 ng/ μ l (8 nM). No protein was added to MCM-depleted extracts (lane 1) or to mock-depleted extracts (lane 8). (C) Recombinant MCM2-7 (lane 2) and MCM2-7_{67KA} (lane 3) were added at 400 ng/ μ l (670 nM) to MCM-depleted extracts. No protein was added to MCM-depleted extracts in lane 1 or to mock-depleted extracts in lane 4. (D) Recombinant MCM complexes: MCM2-7 (lane 2), MCM2-7_{7KA} (lane 3), MCM4,6,7 (lane 4), MCM4,6,7_{7KA} (lane 5), and MCM7 (lane 6) were added at 400 ng/ μ l (670 nM) to MCM-depleted extracts. No protein was added to MCM-depleted extracts (lane 1) or to mock-depleted extracts (lane 7). (E) Genomic DNA replication was quantified as a percentage of mock-depleted activity.

a complex containing point mutations in MCM6 and MCM7 is discussed below.

Correlation between enzymatic and biological activities

As shown previously for recombinant MCM complexes from *S. cerevisiae*, *S. pombe*, and mouse, *Xenopus* MCM4,6,7 contains intrinsic ATPase and DNA helicase activities *in vitro* (Lee and Hurwitz, 2000; You *et al.*, 2002; Kaplan *et al.*, 2003). A mutation in the conserved lysine residue of the MCM7 Walker A motif results in a dramatic decrease in enzymatic activity associated with the MCM4,6,7 complex. This mutant complex MCM4,6,7_{KA} is stable (Figure 2C), and thus the loss of ATPase and DNA helicase activities cannot be attributed to complex instability. These results suggest that MCM7 may play a direct role in the enzymatic activities of the MCM complexes. When expressed and purified as a single subunit, MCM7 does not display ATPase or DNA helicase activity *in vitro* (Supplementary Figure S1B and C), consistent with previous observations (You *et al.*, 2002; Davey *et al.*, 2003). Thus, although MCM7 contains an ATP binding site, it requires other subunits to hydrolyze ATP, presumably MCM3 (Davey *et al.*, 2003).

MCM4,6,7 displays ATPase and DNA helicase activities *in vitro*, but it is not able to restore DNA replication in MCM-depleted egg extracts. Both MCM4,6,7 and MCM4,6,7_{KA} assemble onto chromatin very poorly as compared to endogenous MCMs or to the recombinant MCM2–7 complex. In turn, MCM4,6,7 and MCM4,6,7_{KA} fail to support the assembly of endogenous Cdc45 onto the chromatin. This suggests that MCM2, MCM3, and/or MCM5 are required for the efficient loading of MCM4,6,7 on the chromatin and are essential for the further assembly of Cdc45 onto the pre-RC. Therefore, even if MCM4,6,7 functions as the replicative helicase during elongation, MCM4,6,7 is not sufficient for the initiation step of DNA replication, as its loading requires all six MCM subunits.

S. cerevisiae MCM2–7 heterohexameric complex is an ATPase (Schwacha and Bell, 2001; Davey *et al.*, 2003). However, MCM2–7 purified from *S. pombe* does not have associated ATPase activity (Lee and Hurwitz, 2000). We found ATPase activity associated with the *Xenopus* MCM2–7 complex. A mutation in the Walker A motif of MCM6 or MCM7 causes a partial reduction in ATPase activity of the hexamer (Figure 3E). In *S. cerevisiae*, the same mutation in MCM6 or MCM7 results in a more dramatic decrease in ATPase activity of the heterohexamer; specifically, MCM complexes containing a point mutation in a single subunit displayed reduced ATPase activity up to 20-fold, suggesting that ATP hydrolysis occurs through the coordinate interactions among all six subunits (Schwacha and Bell, 2001). The reason for this difference is not known. Nonetheless, we show that the loading of MCM2–7_{KA} and MCM2–7_{KA} onto chromatin is not affected by the reduced ATPase activity of the complexes. More significantly, mutations in the Walker A motif of both MCM6 and MCM7 subunits resulted in the loss of ATPase activity of the MCM2–7 complex (Figure 3C–E). This ATPase-deficient MCM complex was sufficient to assemble onto chromatin with similar efficiency as the wild-type MCM2–7 complex (Figures 5A, C, and 6). This suggests that nucleotide binding and/or hydrolysis by MCM proteins is not a required step in pre-RC assembly.

Analysis of MCM2–7_{KA} showed that point mutations could separate the functions of MCM proteins. MCM2–7_{KA} is able to bind to chromatin and fully supports Cdc45 loading. In contrast, this complex was not able to support DNA replication in MCM-depleted egg extracts. We speculate that the failure to support DNA replication is due to the loss of ATPase activity of this mutant complex. Moreover, the loading of RPA was significantly reduced in the presence of MCM2–7_{KA} in comparison to MCM2–7, demonstrating the requirement of ATPase activity of the MCM complex during origin unwinding. This is in agreement with our recent observation that DNA unwinding specifically requires ATP hydrolysis (Shechter *et al.*, 2004) and strengthens the idea that MCM proteins are the replicative helicase.

Although the single-mutant MCM complex, MCM2–7_{KA}, is able to bind to chromatin and supports Cdc45 loading, it supports less than 20% of DNA replication in MCM-depleted extracts in comparison to wild-type MCM2–7. This result further shows that ATPase activity is a rate-limiting step following pre-RC assembly. The corresponding Walker A mutation in MCM7 in *S. pombe* or *S. cerevisiae* results in a nonfunctional MCM complex (Schwacha and Bell, 2001; Gomez *et al.*, 2002). In *S. cerevisiae*, the MCM2–7_{KA} complex has no detectable ATPase activity and consequently no *in vivo* activity (Schwacha and Bell, 2001).

Potential role of MCMs as the replicative helicase

In vitro DNA helicase activity has not been found associated with MCM2–7 in any system, including *Xenopus*. It can be speculated that the association of MCMs with other replication proteins, such as Cdc45, is required for the complex to have associated DNA helicase activity *in vivo*. In support of this idea, Cdc45 travels with the replication fork, as seen by chromatin immunoprecipitation studies (Aparicio *et al.*, 1997). In addition, targeted degradation of Cdc45 indicates that it is required for the progression of replication forks (Tercero *et al.*, 2000). Addition of Cdc45 to cell-free extracts prior to MCM immunoprecipitation stimulates the DNA helicase activity associated with MCM complexes (Masuda *et al.*, 2003). Furthermore, addition of neutralizing antibodies against Cdc45 in *Xenopus* egg extracts blocks DNA unwinding (Pacek and Walter, 2004). We did not detect *in vitro* helicase activity associated with MCM2–7 in the presence of Cdc45 (data not shown). This strongly suggests that the helicase activity requires additional factors or activation step(s) provided by structural modifications of MCM2–7 *in vivo*.

Abundance of MCM proteins

A possible role for MCMs in maintaining genome stability could explain why MCMs are so abundant and in vast excess as compared to the number of replication origins (Mahbubani *et al.*, 1997; Walter and Newport, 1997; Edwards *et al.*, 2002; Hyrien *et al.*, 2003). In *Xenopus* extracts, the number of ORC complexes correlates with the number of active origins, whereas the number of MCM complexes is about 10- to 40-fold higher (Mahbubani *et al.*, 1997; Walter and Newport, 1997). Consistent with observations that MCM proteins are present in excess, we show that the amount of recombinant MCM proteins that is able to support DNA replication is only a fraction of the amount of endogenous MCMs present *in vivo*. Under these conditions, complete genomic replication takes longer, suggesting that the rate of DNA replication is

slower, possibly due to less efficient origin firing. MCMs may have other biological activities, such as transcription and checkpoint responses (Bailis and Forsburg, 2004; Cortez *et al.*, 2004), in addition to their well-established role in DNA replication. One study demonstrated that reduction by siRNA of MCM7 to a level at which cells can divide and replicate normally, leads to a checkpoint defect as observed by radio-resistant DNA synthesis (RDS) following irradiation (Cortez *et al.*, 2004; Shechter and Gautier, 2004). As this RDS phenotype was not observed in cells with a similar reduction in MCM2 or MCM3 levels, the data support the idea that each MCM subunit may provide a unique and essential function to the MCM complex. Further studies will be required to define all biological role(s) of MCMs.

In conclusion, we have established a cell-free system from *Xenopus*, which is solely dependent on recombinant MCM proteins. Furthermore, using this system, we have established that MCM-dependent ATPase activity is required for DNA unwinding, but is dispensable for chromatin assembly.

Materials and methods

Cloning of wild-type and mutant forms of MCM cDNAs into baculovirus vectors

cDNAs of *Xenopus* MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 were cloned into pFastBac1 (GIBCO BRL) at the following restriction sites: *EcoRI* and *XhoI* (MCM2); *EcoRI* and *NotI* (MCM3); *BssHII* and *EcoRI* (MCM4); *EcoRI* and *XhoI* (MCM5); *BamHI* and *NotI* (MCM6); and *EcoRI* and *NotI* (MCM7). Mutations substituting lysine 404 to alanine of MCM6 and lysine 386 to alanine of MCM7 were generated by PCR-mediated mutagenesis. A sequence encoding a FLAG tag was added to the C-terminus of MCM7 gene by PCR. All MCM recombinant baculoviruses were generated based on the BAC-TO-BAC Baculovirus Expression System (GIBCO BRL). Introduction of MCM6_{6KA} and MCM7_{7KA} mutations and addition of the FLAG tag sequence were confirmed by DNA sequencing.

Expression of wild-type and mutant MCM proteins in insect cells

Recombinant MCM baculoviruses were amplified in Sf9 insect cells. For the expression of various MCM complexes, 3.6×10^8 High Five cells were simultaneously coinfecting with various combinations of the MCM baculoviruses, each at an MOI 5, and harvested at 72 h postinfection. Six different MCM complexes were expressed: MCM2,3,4,5,6,7 (MCM2-7); MCM2,3,4,5,6_{6KA},7 (MCM2-7_{6KA}); MCM2,3,4,5,6,7_{7KA} (MCM2-7_{7KA}); MCM2,3,4,5,6_{6KA},7_{7KA} (MCM2-7_{6KA},7_{7KA}); MCM4,6,7; and MCM4,6,7_{7KA}. MCM7 was also expressed as an individual subunit.

Purification of wild-type and mutant MCM proteins

Cells were lysed in Buffer MB (20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM PMSF, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride.HCl (AEBSF) 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml chymostatin). Lysates were sonicated with a microtip five times for 30 s. After incubation for 30 min on ice, the supernatant was collected by centrifugation at 35 000 g at 4°C for 30 min and incubated with anti-FLAG M2 agarose beads (Sigma) at 4°C overnight. After five washes with 20 volumes of Buffer MB, bound proteins were eluted with 1 mg/ml FLAG peptide in Buffer MB at 4°C for 2 h.

A portion of the proteins (200 µl) purified from anti-FLAG agarose beads was applied to a 5 ml 15–35% glycerol gradient in Buffer MB2 (20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM AEBSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml chymostatin). After centrifugation at 45 000 r.p.m. for 16 h (Beckman SW50.1 rotor) at 4°C, fractions (~200 µl) were collected from the bottom of the tube. Analysis of the MCM protein profile across the glycerol gradient was performed following SDS-PAGE by Coomassie blue staining.

Further purification of MCM complexes was carried out by fast protein liquid chromatography using a Mono Q column. Bound proteins were eluted with a 10 ml, 50–500 mM NaCl gradient in Buffer Q (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol). Peak fractions containing MCM complex proteins were detected following SDS-PAGE by Coomassie blue staining. Protein concentrations of the purified complex were determined using Bradford assay (Bio-Rad).

Immunodepletion of MCMs

Cytostatic factor (CSF)-arrested extracts were freshly prepared according to Murray (1991). Immunodepletions of *Xenopus* extracts were performed using α-MCM6 antibodies coupled to Protein A-Sepharose CL-4B (Amersham Biosciences). CSF extracts were released into interphase with 0.4 mM CaCl₂ and incubated at 22°C for 15 min. Three rounds of depletion were performed by rotating at 4°C for 20 min each time. Mock depletion was performed using preimmune serum coupled to protein A beads. MCM depletion was monitored by Western blot analysis using different MCM antibodies.

Replication assay

Chromosomal templates for DNA replication were prepared from demembranated *Xenopus* sperm nuclei as described by Murray (1991). Replication reactions were assembled by mixing either MCM-depleted or mock-depleted extracts with recombinant MCM proteins or Buffer MB. Demembranated sperm nuclei were added at 1000 nuclei/µl along with 1 µCi [α -³²P]dATP and the replication reaction was incubated at 22°C for 90 min. The reaction was stopped with 200 µl of replication stop buffer (80 mM Tris-HCl pH 8.8, 8 mM EDTA, 0.5% SDS), followed by incubation with proteinase K at 1 mg/ml for 1 h at 42°C, phenol-chloroform extraction, and ethanol precipitation. DNA synthesis was monitored by the incorporation of [α -³²P]dATP following agarose gel electrophoresis. Gels were exposed for autoradiography and quantified using the PhosphorImager.

Chromatin binding assay

For chromatin binding assay, purified recombinant MCM proteins were incubated in MCM-depleted extracts supplemented with 10 000 sperm nuclei/µl at 22°C. The reactions were incubated for 60 min for detection of MCM loading or for 110 min for detection of Cdc45 loading. Following incubation, each reaction was diluted with 800 µl of chromatin isolation (CI) buffer (100 mM KCl, 2.5 mM MgCl₂, 50 mM Hepes pH 7.8) supplemented with 0.125% Triton X-100. Chromatin was isolated through a 30% sucrose solution in CI buffer at 6000 g for 30 min at 4°C. The samples were run on 10% SDS-PAGE and analyzed by Western blotting.

For DNA binding assay, recombinant MCM proteins were incubated at 22°C for 20 min in MCM-depleted extracts supplemented with a biotinylated 1 kb DNA fragment that was prebound to streptavidin beads. The biotinylated DNA was generated by PCR using M13-ssDNA as a DNA template. Following incubation, the DNA-bound streptavidin beads (Dynabeads M-280 Streptavidin from DYNAL Biotech) were washed in XB buffer + 0.2% Triton X-100. The samples were run on 10% SDS-PAGE and analyzed by Western blotting.

ATPase assay

The ATPase reaction mixture (10 µl) contained 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 5 mM MgCl₂, 100 µM ATP, 0.8 nM [γ -³²P]ATP, and the enzyme fraction. Incubations were performed at 23°C for 1 h and reactions were stopped by addition of 1 µl of 0.5 M EDTA. A 0.7 µl portion of the reaction was spotted on a polyethyleneimine-cellulose thin-layer plate (Selecto Scientific) and the reaction products were separated by chromatography in 0.8 M acetic acid and 0.8 M LiCl. The thin layer chromatography plate was air dried and exposed to a PhosphorImager for imaging and quantification.

Helicase assay

A 17-mer oligonucleotide (5'-GTTTCCAGTCACGAC-3') was labeled at the 5'-end for 50 min at 37°C in a 20 µl reaction containing 18 pmol of 17-mer, 1 × polynucleotide kinase buffer, 20 µCi of [γ -³²P]ATP, and 15 U of T4 polynucleotide kinase. Formation and purification of DNA helicase template was described previously (Shechter *et al.*, 2000). In the helicase assay reaction,

5 fmol of labeled substrate was incubated with the indicated amounts of MCM proteins at 23°C for 1 h in a 20 µl reaction containing 25 mM Tris-HCl, 2.5 mM ATP, 5 mM MgCl₂, 1 mM DTT, and 200 µg/ml BSA. The reaction was stopped with 5 µl of 5 × stop solution (100 mM EDTA, 0.5% SDS, 0.1% bromophenol blue, 25% glycerol) and then run on a 10% polyacrylamide TBE gel. The gel was fixed in 20% trichloroacetic acid, dried, and exposed on a PhosphorImager for imaging and quantification.

MCM antibodies preparation

A 6 × HIS tag was added on the N-terminus of MCM2, MCM5, and MCM6 cDNA, and subcloned in pFastBac1 plasmid (GIBCO BRL). Sf9 insect cells were individually infected with each recombinant MCM baculovirus at MOI 10 and harvested 72 h postinfection. Recombinant MCM2, MCM5, and MCM6 proteins were each purified on a Ni²⁺ column (Qiagen) under denaturing conditions as described in Xpress System Protein Purification (Invitrogen). After 10% SDS-PAGE, protein bands were excised from the gel and used for immunization of rabbits. Production of MCM4 polyclonal antibodies was described previously (Hendrickson *et al*, 1996).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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