

The *Methanobacterium thermoautotrophicum* MCM protein can form heptameric rings

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Mini-chromosome maintenance (MCM) proteins form a conserved family found in all eukaryotes and are essential for DNA replication. They exist as heteromultimeric complexes containing as many as six different proteins. These complexes are believed to be the replicative helicases, functioning as hexameric rings at replication forks. In most archaea a single MCM protein exists. The protein from *Methanobacterium thermoautotrophicum* (mtMCM) has been reported to assemble into a large complex consistent with a dodecamer. We show that mtMCM can assemble into a heptameric ring. This ring contains a C-terminal helicase domain that can be fit with crystal structures of ring helicases and an N-terminal domain of unknown function. While the structure of the ring is very similar to that of hexameric replicative helicases such as bacteriophage T7 gp4, our results show that such ring structures may not be constrained to have only six subunits.

INTRODUCTION

DNA replication is fundamental to all organisms, and studies using prokaryotic and viral models have contributed greatly to our understanding of the mechanistic details. An important element involves processive replicative helicases that are initially assembled at replication origins (Dean *et al.*, 1992). The replicative helicase is typically a complex of six identical subunits assembled into a ring-shaped structure. Strong evidence exists for hexameric helicases, such as bacteriophage T7 gp4 (Yu *et al.*, 1996) and *Escherichia coli* DnaB (Kaplan, 2000), that unwinding involves the ring encircling and moving along one of the DNA strands. The prime candidate for the replicative helicase in eukaryotes is a complex of six non-identical but highly

conserved subunits known as MCM 2-7 (Tye, 1999). Hexamers containing stoichiometric amounts of each of the six mini-chromosome maintenance (MCM) proteins can be readily purified as a globular complex from a number of eukaryotes (Adachi *et al.*, 1997). Although genetic evidence strongly implicates the MCM hexamer as the replicative helicase that melts origin DNA and unwinds growing forks (Aparicio *et al.*, 1997; Labib *et al.*, 2000), the purified MCM hexamer failed to demonstrate such an activity *in vitro* (Ishimi, 1997). Interestingly, weak helicase activity can be reconstituted from a metastable hexamer containing only three of the six subunits (Ishimi, 1997; Lee and Hurwitz, 2001). To understand how the MCM helicase works, it is important to study the structure of the assembled helicase. However, in order to study the anatomy of a functional helicase, successful assembly of an active and stable helicase is necessary.

The best model for the eukaryotic MCM helicase may be the archaeal homolog, a 3'-5' ATP-dependent helicase presumed to contain six identical subunits (Kelman *et al.*, 1999; Chong *et al.*, 2000; Shechter *et al.*, 2000). The eukaryotic MCM helicase has no known bacterial homolog, but there is a single highly conserved progenitor homolog in all archaea studied (Tye, 2000). The *Methanobacterium thermoautotrophicum* MCM (mtMCM) protein is 34% identical and 51% similar in sequence to the MCM2-7 protein family with the strongest homology concentrated in three conserved motifs, two of which are the Walker A and B nucleotide binding sequences (Koonin, 1993). mtMCM, a single polypeptide of 75 kDa, self-assembles on purification into a large-molecular-weight complex that contains robust helicase activity.

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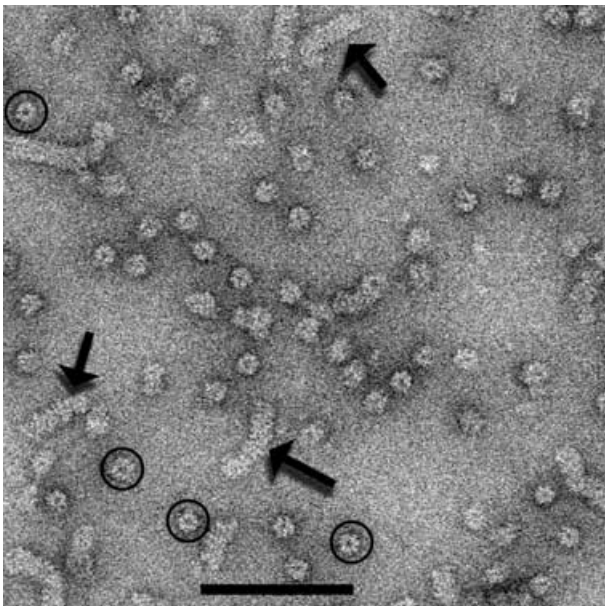


Fig. 1. An electron micrograph showing both single rings (circles) and larger aggregates (arrows) of the mtMCM protein. The scale bar is 1000 Å.

RESULTS

Electron micrographs of mtMCM display fields of abundant ring-like structures (Figure 1, circles). While most of the objects are consistent with projections of randomly-oriented rings, many larger aggregates are also seen (Figure 1, arrows). These larger structures are consistent with side views of ‘stacks’ of rings. An extensive analysis (see Supplementary data available at *EMBO reports* Online) involving 81749 images of the single rings has shown that these single rings are heptameric. This analysis involved images of 55925 rings from one preparation (with an N-terminal His tag), and 25824 rings from a different construct (with a C-terminal His tag), prepared and purified in a different laboratory.

A reconstruction generated from 14785 images (Figure 2) showed that the mtMCM ring was ~100 Å high (along the symmetry axis) and ~150 Å wide (perpendicular to the symmetry axis). Different reconstructions were generated by forcing the number of images to be comparable in each of the projection directions, or by first averaging the projections and then using these averages for back projection. The size of the central channel was found to be variable among these different reconstructions, while all other features remained relatively constant. Thus, no reliable conclusions can be drawn about the diameter of this central channel.

Molecular modeling

Two crystal structures now exist for ring helicases: bacteriophage T7 gp4 (Singleton *et al.*, 2000) and the plasmid-encoded RepA helicase (Niedenzu *et al.*, 2001). The gp4 protein actually contains two domains, a helicase and a primase (Bernstein and Richardson, 1988), but the crystal structure is of the helicase domain alone. The gp4 helicase domain (Singleton *et al.*, 2000), the full-length gp4 (Egelman *et al.*, 1995) and RepA (Scherzinger

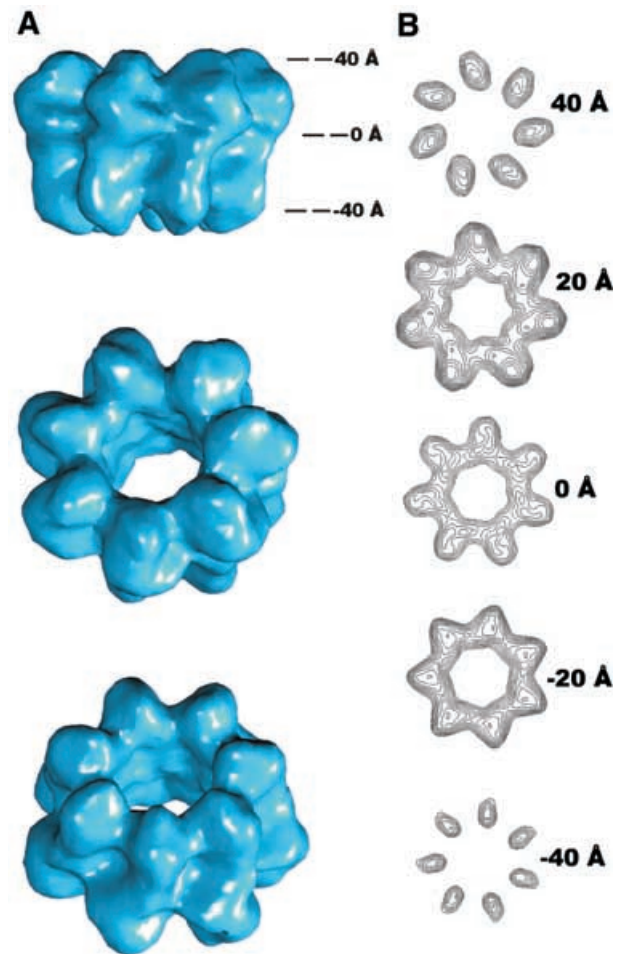


Fig. 2. Surfaces (A) and cross-sections (B) from the 3D reconstruction of mtMCM. The surface at the top is oriented so that the 7-fold axis is vertical, and the axial position of the sections (B) at -40, 0 and 40 Å are indicated. A very small detached density was present in the central channel, but was removed since simulations showed that it was consistent with noise. The heptamer is ~100 Å in length along the 7-fold axis. The surface in the center corresponds to a ‘top’ view (showing the helicase domain, Figure 4).

et al., 1997) assemble into hexameric rings. A sequence alignment of MCM, gp4 and RepA (Figure 3) shows that the nucleotide binding site (involving the Walker A and B motifs) in both MCM and gp4 is in the C-terminal half of the protein.

We have identified the helicase domain in the MCM heptameric ring by positioning X-ray crystal structures of both RepA (Figure 4A and D) and the T7 gp4 helicase domain (Figure 4B and E) into the three-dimensional (3D) reconstruction of MCM. In the best fit of the gp4 helicase domain into the reconstruction, the crystal structure protruded significantly out of the electron microscopic (EM) surface, and steric clashes were present with neighboring subunits in the heptamer. Unlike T7 gp4, the RepA helicase crystallized as a hexameric ring with perfect 6-fold symmetry. We extracted one subunit from the RepA hexamer and docked this structure into the MCM reconstruction. The RepA structure provided a significantly better fit to the MCM surface (Figure 4A and D) than did the gp4 structure (Figure 4B and E). In a model of the MCM heptamer generated using the best fit orientation of the RepA structure, the subunit-subunit

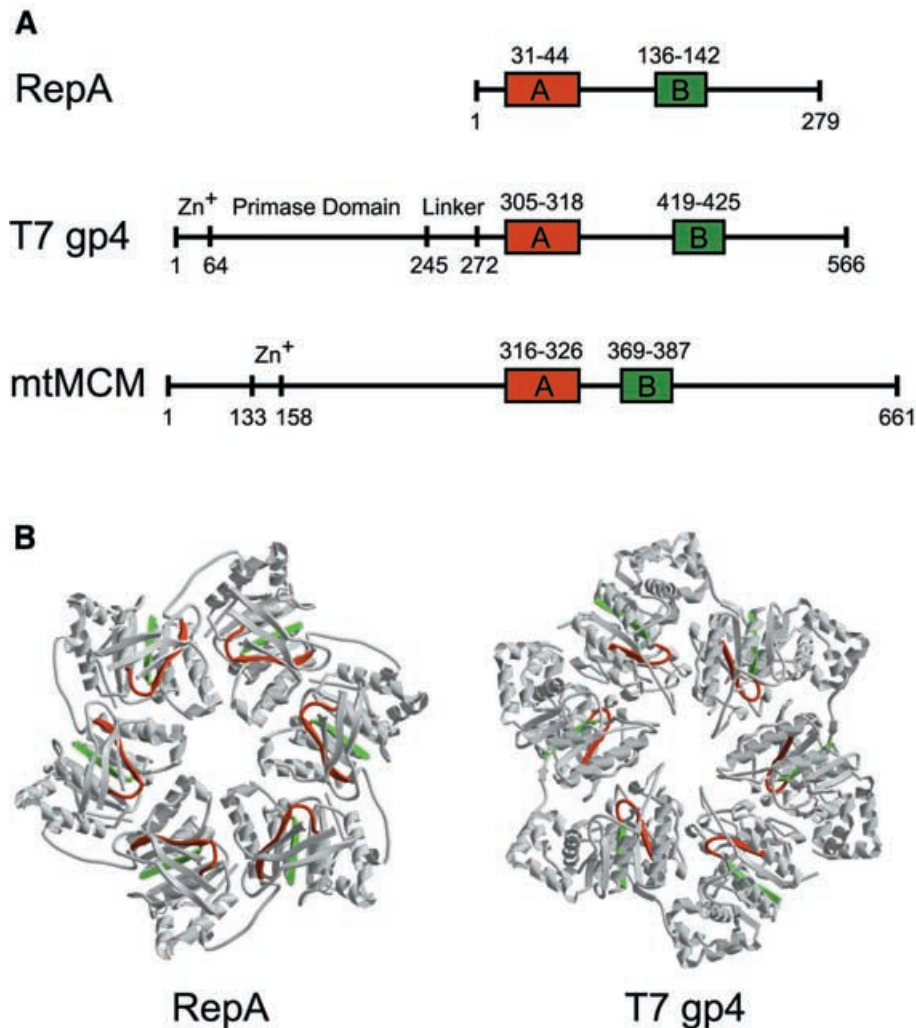


Fig. 3. A comparison between the location of the Walker A and B motifs in mtMCM and in two helicase proteins that form hexameric rings, RepA and T7 gp4 (A). The T7 gp4 protein has a primase domain located on the N-terminal end of the protein and a small linker region located between these two domains (Singleton *et al.*, 2000). The mtMCM protein has an N-terminal domain of unknown function, with no homology to any protein other than MCMs. (B) The location of the Walker A (red) and B (green) motifs are shown for the RepA (Niedenzu *et al.*, 2001) and T7 gp4 (Singleton *et al.*, 2000) crystal hexamers.

interface was very similar to that in the RepA hexamer structure. This suggests that, as seen in RepA (Figure 3B), the nucleotide binding site in MCM lies near the subunit–subunit interface. Interestingly, neither RepA (Scherzinger *et al.*, 1997) nor MCM requires a nucleotide cofactor for ring assembly, in contrast to ring helicases such as SV40 large T antigen (Bullock, 1997), where the nucleotide cofactor is essential for ring assembly, or helicases such as T7 gp4 (Patel and Hingorani, 1993) and *E. coli* DnaB (Jezewska and Bujalowski, 1996), where the nucleotide cofactor is important for the stabilization of the ring.

DISCUSSION

Contrary to the predictions of previous studies and the expectations from the hexameric eukaryotic enzyme, electron microscopy and image analysis indicates that the archaeal MCM helicase can exist as a heptameric ring. An obvious question arises as to whether the heptameric rings could be due to a contaminant, such as GroEL, which does form heptameric rings (Chen *et al.*,

1998). We can exclude this possibility for four reasons. (i) The purified mtMCM protein runs as a single band on Coomassie Blue-stained SDS gels at the same time that EM fields (Figure 1) show large numbers of rings. If the heptameric rings were due to a contaminant, this contaminant could only be present as a very small fraction of the total protein and could not account for the highly abundant rings. We can eliminate the possibility that a GroEL contaminant is comigrating with MCM and therefore not resolved on SDS gels, since the molecular weight of GroEL is 57 kDa, while the molecular weight of the mtMCM is 76.5 kDa. (ii) GroEL forms double heptameric rings, while we observe single heptameric rings. Due to the dihedral symmetry of the double GroEL rings (head-to-head packing), end views of the GroEL rings show no chirality (handedness) (Falke *et al.*, 2001). In contrast, we observe a chirality in such end views (see Supplementary figure 1D) that likely corresponds to projections of single rings. (iii) The use of an antibody raised against the mtMCM protein shows binding to these rings in electron micrographs

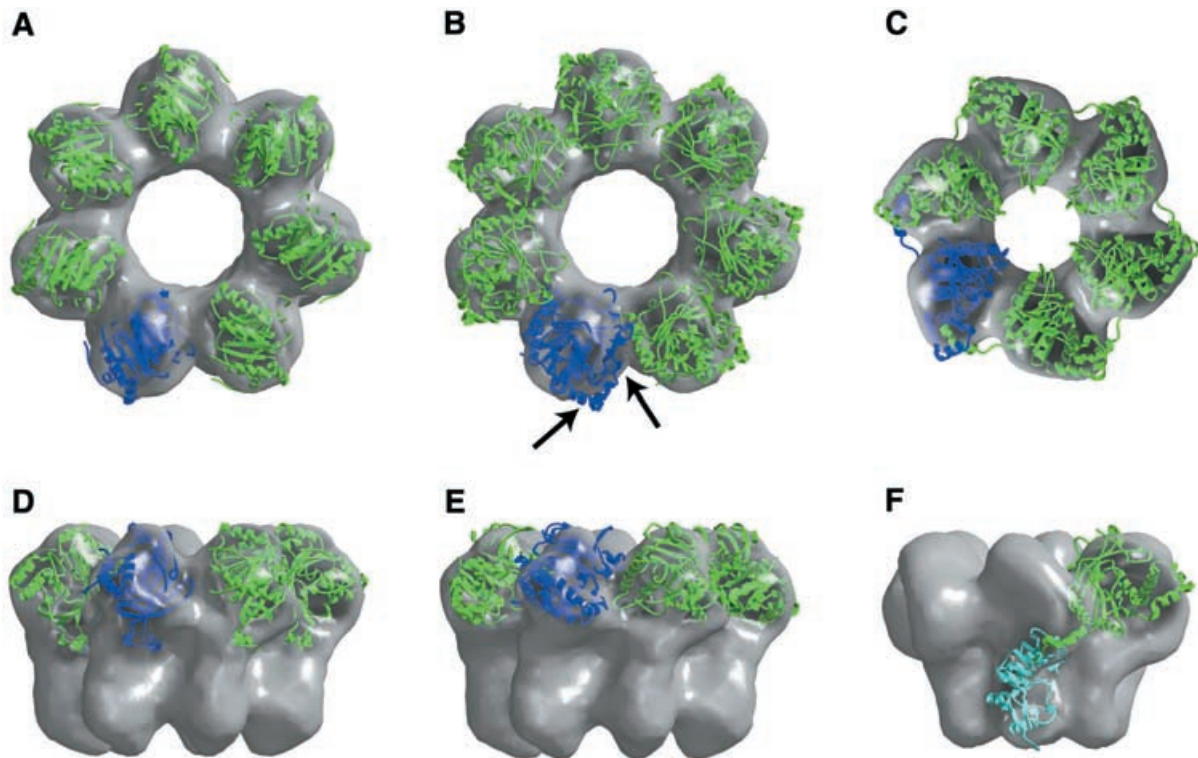


Fig. 4. Locating the helicase domain in the mtMCM heptamer. Crystal structures of both the RepA (A and D) and T7 gp4 (B and E) helicase domains have been docked into the mtMCM reconstruction (gray surface). To help in visualization, one subunit is shown in blue and the remainder are in green. For comparison, a model for the location of the helicase (green) and primase (cyan) domains in the EM reconstruction of the T7 gp4 hexamer (VanLoock *et al.*, 2001) is shown in (C) and (F). The arrows in (B) indicate those regions of the gp4 helicase structure that penetrate the MCM EM reconstruction surface.

(data now shown). Since the binding was largely disordered, these complexes were not amenable to further image analysis. (iv) The same heptameric structure is seen after purification of the protein in two different laboratories (Cornell and CARB) using three different clones (with an N-terminal His tag, a C-terminal His tag and a Zn²⁺-domain mutation). However, preliminary observations of mtMCM protein from yet another clone show that both heptameric and hexameric rings can form (X. Yu and E.H. Egelman, unpublished data), suggesting that there are unknown factors that might affect this polymorphism.

The finding that the archaeal MCM rings can be heptameric is unexpected, given that the eukaryotic MCM proteins have been observed to assemble into hexameric rings. In *Xenopus* egg extracts, three different heterohexamers have been observed, containing either MCM(2 + 3 + 4 + 5 + 6 + 7), MCM(2 + 3 + 4 + 6 + 7 + 7) or MCM(4 + 6 + 7)₂ subunits (Prokhorova and Blow, 2000). A hexameric complex of the human MCM(4 + 6 + 7)₂ subunits has been described (Sato *et al.*, 2000). The observation that different MCM complexes formed heterohexamers was interpreted as being consistent with the role of MCM proteins in being replicative helicases. However, other proteins containing the RecA-like nucleotide binding core present in all helicases have been observed to form rings with different symmetries. The meiotic eukaryotic RecA homolog, Dmc1, forms rings with eight subunits, and it has been suggested that this is the active form of the protein (Passy *et al.*, 1999). While the *Thermus aquaticus* RecA protein forms rings with six subunits (in addition to helical filaments) (Yu *et al.*, 1995), the *E. coli* RecA protein and the

human Rad51 protein form rings with eight subunits (X. Yu and E.H. Egelman, unpublished data). Both the *E. coli* and *Thermus thermophilus* RuvB protein form hexameric rings when bound to DNA (Stasiak *et al.*, 1994; Miyata *et al.*, 2000). Remarkably, heptameric rings of the *T. thermophilus* RuvB protein have been observed when the rings are not bound to DNA (Miyata *et al.*, 2000). This raises the possibility that the mtMCM protein might form a hexameric ring when bound to DNA. Since we have been unable to obtain conditions for electron microscopy where the MCM rings are unambiguously bound to DNA, this will need to be tested in future work.

Although we cannot exclude the presence of double heptamers in our preparations, the majority of the rings appear to be single heptamers. Previous studies of mtMCM protein by gel filtration or glycerol gradient centrifugation (Kelman *et al.*, 1999; Chong *et al.*, 2000; Shechter *et al.*, 2000), as well as by scanning transmission electron microscopy (Chong *et al.*, 2000), have reported a single high-molecular-weight species, interpreted as being a double hexamer. The resolution of these techniques may not be great enough to distinguish between a double hexamer and a double heptamer. For example, a single mutation in the zinc-finger domain of the mtMCM protein causes a shift of the putative double hexamer to a significantly lower apparent molecular mass, as judged by gel filtration (Poplawski *et al.*, 2001). In contrast to what has been observed for the mtMCM, a study of the *Sulfolobus solfataricus* MCM protein using gel filtration and glycerol gradient centrifugation found two species,

X. Yu *et al.*

and these were interpreted as a monomer and a hexamer (Carpentieri *et al.*, 2002).

At this time, we are unable to verify the physiological relevance of the heptameric structure of the archaeal MCM helicase or the subunit composition of the active eukaryotic MCM helicase. Assuming that the heptamer is the physiological form of the archaeal MCM helicase, an important lesson to be learned from this study may be that the essence of a processive helicase lies not in the 3- or 6-fold symmetry of the ring but in the topological constraints imposed by the ring itself when it encircles DNA. If the MCM rings can exist in two states, with either six or seven subunits (consistent with our unpublished observations), an interesting possibility arises for the loading of these rings on DNA. A topological problem exists for binding any preformed ring to other than the ends of DNA, and the inability of preformed SV40 large T rings to bind origin DNA was used as an argument that these rings encircle the DNA (Dean *et al.*, 1992). The possibility that preformed MCM rings with seven subunits lose one subunit, encircle DNA and then close to form a ring with six subunits is highly speculative, but interesting. It has previously been shown that the hexameric *E. coli* rho transcription-termination rings can actually be found with a missing subunit, as well as with an opened cleft induced by binding RNA to an external high-affinity RNA binding site (Yu *et al.*, 2000). A ring-opening model for the hexameric T7 helicase primase has also been proposed, based upon kinetic data (Ahnert *et al.*, 2000). Taken together, this provides a testable hypothesis that needs to be considered in future studies of MCM rings.

METHODS

Sample preparation. The mtMCM protein was overexpressed and purified from *E. coli* as described previously (Poplawski *et al.*, 2001). Samples were applied to carbon-coated grids and negatively stained with 2% uranyl acetate. Specimens were examined in an FEI Tecnai 12 electron microscope at an accelerating voltage of 80 keV and a nominal magnification of 30 000 \times .

Image analysis and modeling. Electron micrographs were scanned on a Leaf 45 densitometer at a raster corresponding to 3.9 Å/pixel. The SPIDER software package (Frank *et al.*, 1996) was used for most of the image processing and O (Jones *et al.*, 1991) was used for the molecular modeling. Models were built by first generating 3D densities from atomic coordinates of individual subunits, and filtering these volumes to a resolution comparable to that of the MCM reconstruction. We then docked the low-resolution surfaces into the MCM reconstruction using shape as our primary guide. Transformations used in docking the surfaces were applied to the structure coordinates. The enantiomorphic ambiguity present in the 3D reconstruction was resolved by choosing the structure that gave the best fit with the crystal models. BOBSCRIPT (Esnouf, 1999) was used for displaying molecular models within EM surfaces.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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