A DNA Helicase Activity Is Associated with an MCM4, -6, and -7 Protein Complex*

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All six minichromosome maintenance (MCM) proteins have DNA-dependent ATPase motifs in the central domain which is conserved from yeast to mammals. Our group purified MCM protein complexes consisting of MCM2, -4 (Cdc21), -6 (Mis5), and -7 (CDC47) proteins from HeLa cells by using histone-Sepharose column chromatography (Ishimi, Y., Ichinose, S., Omori, A., Sato K., and Kimura, H. (1996) J. Biol. Chem. 271, 24115–24122). The present study revealed that both ATPase activity and DNA helicase activity that displaces oligonucleotides annealed to single-stranded circular DNA are associated with an MCM protein complex. Both ATPase and DNA helicase activities were co-purified with a 600-kDa protein complex that is consisted of equal amounts of MCM4, -6, and -7 proteins. An immunodepletion of the MCM protein complex from the purified fraction using anti-MCM4 antibody resulted in the severe reduction of the DNA helicase activity. Displacement of DNA fragments by the DNA helicase suggested that it migrated along single-stranded DNA in the 3' to 5' direction, and the DNA helicase activity was detected only in the presence of hydrolyzable ATP or dATP. These results suggest that this helicase may be involved in the initiation of DNA replication as a DNA unwinding enzyme.

There are at least six MCM1 proteins that play an essential role in eukaryotic DNA replication as follows: MCM2, -3, -4 (Cdc21), -5, -6 (Mis5), and -7 (CDC47) proteins (see reviews in Refs. 1–3). Genetic evidence suggests that these proteins are required for the initiation of DNA replication in yeast (4, 5). The inactivation of human MCM2 protein with antibody consistently inhibits the initiation of DNA replication (6). Interaction between the MCM proteins has been reported in yeast (4, 7), and the protein complexes containing MCM proteins are detected in Drosophila (8), Xenopus (9), mouse (10, 11), and human cells (12–14). In human cells, MCM4, -6, and -7 proteins form a stable complex, and MCM2 is loosely associated with this complex (12, 15, 16). MCM3 and -5 proteins also form a stable complex (10, 13). All six of the MCM proteins contain DNA-dependent ATPase motifs in a central domain that is conserved from yeast to mammals, and these motifs are found in several enzymes that unwind the DNA duplex (17). These findings suggest that MCM protein complexes may be involved in the initiation of DNA replication as an enzyme that unwinds DNA in the origin region. However, DNA helicase activity of MCM proteins has not been reported.

MCM proteins bind with chromatin before the initiation of DNA replication and are then released from the chromatin as DNA replication proceeds (18–20). Our group found that MCM protein complexes containing MCM2, -4, -6, and -7 proteins bound tightly with histone H3 in vitro and were purified to near homogeneity by histone-Sepharose column chromatography (21). In the present study, it is shown that both ATPase and DNA helicase activities are associated with an MCM4, -6, and -7 protein complex.

EXPERIMENTAL PROCEDURES

Purification of MCM Proteins—MCM protein complexes containing MCM2, -4, -6, and -7 were purified from 0.2 M NaCl-soluble extracts (560 mg of protein) of HeLa cells (1 × 10⁸ cells) by ammonium sulfate fractionation (0–65% saturation instead of 35–65% saturation) and DE52 (Whatman, United Kingdom) column and histone H3/H4-Sepharose column chromatographies as described previously (21). However, the pooled DE52 fraction in 0.5 M NaCl was first loaded onto Sepharose column (8 ml) cross-linked with bovine serum albumin (1 mg/ml) and then unbound fractions were loaded to the histone H3/H4-Sepharose column. The purified MCM proteins were diluted to 0.15 M NaCl with buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (buffer A) plus 0.01% Triton X-100 and concentrated to 1–2 mg/ml with Centricon 30 (Amicon, Beverly, MA).

Glyceral Gradient Centrifugation—The MCM proteins purified by the histone-Sepharose column chromatography were centrifuged at 36,000 rpm for 16 h (TLS55, Beckman, Fullerton, CA) in a 15–30% linear glycerol gradient in 200 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, and 0.01% Triton X-100. Five drops were collected from the bottom of the centrifugation tube. Fractions 1–3 in the first glyceral gradient centrifugation were diluted with the above buffer and then concentrated with Centricon 30. They were re-centrifuged at 36,000 rpm for 14 h in a 15–30% linear glycerol gradient in 10 mM potassium-phosphate, pH 7.5, 0.5 mM EDTA, 0.15 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% Triton X-100. To cross-link proteins in the 2nd gradient fractions, 1 µl of 0.2 M dithiobis-succinimidyl propionate (DSP) that had been dissolved with dimethylformamide was added to 10 µl of each fraction, and the mixture was incubated for 1 h at 23 °C. The reaction was quenched by adding 1 µl of 1.4 M ethanolamine HCl, pH 8.0 (22). After 10 min, proteins in the mixture were analyzed by a 4% polyacrylamide gel containing SDS (23) in the absence of any reducing reagents and then stained with silver (Wako, Osaka, Japan). Thymoglobin (669 kDa), ferritin (440 kDa), and catalase (232 kDa) were used as protein molecular markers (Pharmacia, Uppsala, Sweden).

Immunodepletion Analysis—Five µl of the MCM proteins (1.5 µg) (fractions 1–3 in the 1st glyceral gradient centrifugation) was incubated for 1 h at 4 °C on a rocking platform with 15 µl of anti-MCM4 antibody beads (24) or with control rabbit IgG beads that had been washed with buffer A containing 0.1 M NaCl, 1 mg/ml bovine serum albumin, and 0.05% Nonidet P-40. After centrifugation, the supernatant was saved (unbound). The beads were washed three times with phosphate-bufffered saline containing 0.05% Nonidet P-40, and proteins bound to the beads were eluted twice with 20 µl of 0.1 M glycine, pH 2.5, and 0.15 M...
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NaCl. The eluted solution was neutralized by adding 1 μl of 1 M Tris. The proteins unbound and unbound to the beads were electrophoresed in a 10% acrylamide gel containing SDS and then stained with silver.

**DNA Helicase Activity**—A 17-mer oligonucleotide (5′-GTTTTC-CCACTGCAAGC-3′, 40 primer for M13 dideoxy sequencing, U.S. Biochemical Corp.), a 24-mer oligonucleotide (5′-CGACGTTGCAA-AACGACGGCCAGT-3′), and a 37-mer oligonucleotide (5′-AATTCG-AGCTGCTAACCAGAAAAGTTGTAA-3′) were labeled at the 5′-end with polynucleotide kinase in the presence of [γ-32P]ATP, and the resultant 17-mer and 24-mer oligonucleotides were annealed to single-stranded M13mp18 DNA; the 37-mer oligonucleotide was annealed to single-stranded M13mp19 DNA (25). The annealed oligomers were purified by centrifuging at 28,000 rpm for 15 h (TLS-55, Beckman) in a linear sucrose gradient from 5 to 20% in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM NaCl. To prepare the 3′-labeled helicase substrate, unbalanced 37-mer (3 pmol) annealed to M13mp19 DNA was labeled at the 3′-OH in the reaction medium (180 μl) consisting of 25 units of DNA polymerase I (Toyobo, Japan) and 5 mM MgCl2 in a 50 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, and 0.1 mM NaCl. The mixture was incubated for 20 min at 30 °C and then for an additional 15 min with a further addition of unlabeled dCTP to the final concentration of 50 μM. The 3′-labeled helicase substrate was purified as described above. DNA helicase substrates to examine the directionality of the translocation were prepared as follows. First, 0.5 pmol of the 3′-labeled helicase substrate (37-mer oligonucleotide) or the 3′-labeled helicase substrate (40-mer oligonucleotide) was digested with SmaI. The digestion was terminated by the addition of EDTA and NaCl at final concentrations of 8 mM and 0.1 M, respectively. The aliquots of the digested DNA were used for the DNA helicase assay as described below (26). Approximately 5 fmol of the annealed oligomer was incubated at 37 °C for 30 min with the MCM proteins in 50 mM Tris-HCl, pH 7.5, 20 mM β-mercaptoethanol, 5 mM MgCl2, 5 mM ATP for other nucleotides in Fig. 6, and 0.5 mg/ml bovine serum albumin. The reaction was terminated by adding SDS to a final concentration of 0.2%, and an aliquot was electrophoresed in a 12% acrylamide gel in Tris-borate/EDTA (TBE). The labeled oligomer in the gel was detected by autoradiography or by using a Bio-Image Analyzer (BAS2000, Fuji, Japan).

**DNA Helicase Activity**—MCM protein complexes were incubated at 37 °C for 30 min with 2 μCi of [γ-32P]ATP (3000 Ci/mmol) and 2 μg of M13mp18 DNA containing 5 fmol of the DNA, 26% of the oligonucleotide was annealed under the conditions described under “Materials and Methods.” The reaction mixture including [γ-32P]ATP was incubated with the gradient fractions (17-mer) as indicated at the top. The positions of the annealed oligomer (annealed) and released oligomer (17-mer) are indicated. ATPase activity in these fractions was measured under the conditions described under “Materials and Methods.” The reaction mixture including [γ-32P]ATP was incubated with the gradient fractions (4 μl) in the presence of single-stranded DNA, and the released 32P was detected by thin layer chromatography. The position where released Pi migrated is indicated.

**RESULTS**

ATPase and DNA Helicase Activities in the MCM Protein Fraction—MCM protein complexes containing MCM2, -4, -6, and -7 were purified to near homogeneity from 0.2 M NaCl-soluble HeLa whole cell extracts by ammonium sulfate precipitation, DEAE cellulose (DE52), and histone H3/H4-Sepharose column chromatographies (21). The fractions eluted at near 1 M NaCl in the histone-Sepharose column chromatography contained almost equal amounts of the four MCM proteins. The immunoprecipitation with anti-MCM4 antibody indicated that these four proteins form complexes. The purified MCM protein fraction was examined for the DNA helicase activity that displaces oligonucleotides (17-mer) annealed to single-stranded circular DNA. During the incubation of 800 ng of MCM proteins with 5 fmol of the DNA, 26% of the oligonucleotide was released from the DNA (data not shown). The histone-Sepharose fraction was fractionated by glyceral gradient centrifugation (Fig. 1). Almost equal amounts of the four MCM proteins cosedimented at a position of approximately 230 kDa, and MCM4, -6, and -7 proteins were also detected in the fractions sedimenting faster than catalase (Fig. 1A). When both the DNA helicase (Fig. 1B) and ATPase (Fig. 1C) activities in the glyceral gradient fractions were measured, the activities were observed to have cosedimented. The main peak of these activities was detected at fraction 2, where almost equal amounts of MCM4, -6, and -7 were present, but the peak did not coincide with the position where four MCM proteins sedimented (fraction 4). The active fractions (fractions 1–3) were pooled and were titrated...
for DNA helicase activity (Fig. 2A). The 17-mer was displaced from the annealed DNA in a dose-dependent manner, and a large portion of 17-mer was displaced in the presence of 300 ng of MCM proteins. Purification of DNA helicase proceeded by the glycerol gradient centrifugation, since the specific activity of DNA helicase increased compared with that in the fraction before the centrifugation. The pooled fraction was subjected to immunodepletion analysis using an anti-MCM4 antibody (Fig. 2, B and C). The supernatant obtained after the incubation of the pooled MCM proteins with anti-MCM4 antibody beads contained only a small amount of DNA helicase activity (Fig. 2B, lanes 4 and 5), and it contained less than 25% of total MCM proteins (Fig. 2C, lanes 4–6). The MCM4 antibody precipitated MCM4, -6, and -7 proteins in addition to a small amount of MCM2 protein. In contrast, the supernatant obtained after the incubation with control antibody beads retained DNA helicase activity (Fig. 2B, lanes 2 and 3) and contained almost all MCM proteins (Fig. 2C, lanes 1–3). These results indicated that the MCM proteins that form complexes were required for the DNA helicase activity. However, it remains to be determined why the supernatant after incubation with anti-MCM4 antibody beads did not contain any DNA helicase activity.

**MCM Protein Composition of Active DNA Helicase**—To verify the relationship between the DNA helicase activity and the composition of MCM proteins, the pooled fractions in the 1st glycerol gradient centrifugation were re-fractionated by glycerol gradient centrifugation (Fig. 3). The MCM proteins sedimented at a position between 225 kDa (catalase, fraction 5) and 440 kDa (ferritin, fraction 2) (Fig. 3A). The peak of the DNA helicase activity was detected in fraction 3 (approximately 350 kDa) (Fig. 3B), where almost equal amounts of MCM4, -6, and -7 were present (Fig. 3A). These results and the data in Fig. 2 suggest that a trimeric complex consisting of MCM4, -6, and -7 has the DNA helicase activity, since the sum of the molecular mass of MCM4, -6, and -7 proteins is 293 kDa. Next, proteins in these fractions were cross-linked with DSP and then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3C). Two major complexes of MCM proteins were found as follows: one migrated slightly faster than thyroglobulin (669 kDa) and the other migrated as a roughly 400-kDa protein. Uncross-linked MCM proteins were not detected (data not shown). These complexes were also detected without using DSP by native agarose gel electrophoresis (data not shown). The amount of the larger complex was almost proportional to the DNA helicase activity shown in Fig. 3B, and the most active fraction 3 contained only the larger complex. These results suggested that a larger complex of approximately 600 kDa was formed by the interaction of two of the trimeric complexes of MCM4, -6, and -7 proteins and that this complex functions as a DNA helicase.

To know whether ATP-binding activity resides in the MCM protein complex, MCM proteins pooled after glycerol gradient centrifugation were incubated with \([\alpha-32P]ATP\) under ultraviolet light and then the proteins were separated by SDS-polyacrylamide gel electrophoresis (Fig. 4). When DNA polymerase I (molecular mass, 103 kDa) from *E. coli* was incubated with \([\alpha-32P]ATP\) in the polyacrylamide gel (Fig. 4B) suggested that the labeled 100-kDa band was MCM6 or MCM4 protein. These results are consistent with the notion that the MCM protein complex retains ATPase and DNA helicase activities.

**Characterization of ATPase and DNA Helicase Activities**—ATPase activity in the pooled MCM proteins (fractions 1–3 in Fig. 1) was examined. Only a small amount of ATPase activity (7 pmol) was detected in the presence of 0.6 μg of the MCM
proteins, and this activity was greatly enhanced by the addition of single-stranded DNA, poly(dT), or tRNA to the reactions and slightly stimulated by the addition of double-stranded DNA (Fig. 5). At maximum, 37 pmol of ATP was hydrolyzed with 0.6 μg of the MCM proteins in the presence of poly(dT). Consistent with these data, the DNA helicase activity was detected only in the presence of ATP or dATP (Fig. 6), but it was not detected in the presence of AMP-PNP or ATP-γS, which are non-hydrolyzable analogues of ATP, in place of ATP, and the activity was not detected in the presence of other rNTP. These results indicate that the DNA helicase activity in purified MCM proteins is dependent on the presence of hydrolyzable ATP. With the decrease of the ATP concentration from 5 to 1 mM in the reaction, the DNA helicase activity was greatly reduced, suggesting that MCM proteins require a relatively high concentration of ATP for DNA helicase activity.

Direction of Translocation of MCM Proteins—The direction of the movement of the MCM proteins on the DNA strand was examined by the strategy illustrated in Fig. 7 (25). A pair of linearized helicase substrates (substrates A′ and B′) were prepared by digesting substrates A and B with Smal. If MCM proteins move from 3′ to 5′ along the single-stranded DNA segment, the translocation would displace the radioative 18-mer from substrate A′. In contrast, the radioactive 22-mer would be liberated from substrate B′, if the enzyme migrates in a 5′ to 3′ direction. Pooled MCM proteins were incubated with substrate A′, B′, or a mixture of A′ and B′. As shown in Fig. 8A, MCM proteins displaced the radioactive 18-mer from substrate A′, but did not displace the 22-mer from substrate B′. SV40 T antigen, which migrates from 3′ to 5′ (29), displaced the 18-mer, and a mouse DNA helicase B, which migrates from 5′ to 3′ (25), displaced the 22-mer under these conditions (Fig. 8A).

When a mixture A′ and B′ of DNA helicase substrates was incubated with the fractions obtained by the 1st glycerol gradient centrifugation (Fig. 1), activity that displaced only 18-mer from the substrate A′ was detected at the same position as the activity that displaced 17-mer annealed to single-stranded circular DNA (Fig. 8B). These results suggest that MCM proteins move unidirectionally in a 3′ to 5′ direction along the DNA strand to which it binds. Purified MCM proteins were incubated with several DNA helicase substrates where different sizes of oligonucleotides were annealed (Fig. 9). The proportion of displaced oligonucleotide was decreased with the increase in the size of the oligonucleotides. Only a small percentage of 37-mer was displaced in the presence of 300 ng of MCM proteins, suggesting that the processibility of DNA unwinding is not high.

DISCUSSION

I showed that both ATPase and DNA helicase activities were present in the purified MCM protein fraction. The results of the immunodepletion experiment using anti-MCM4 antibody suggested that the DNA helicase activity required the MCM protein complexes (Fig. 2). The peak of DNA helicase activity was detected at a position of about 350 kDa in the glycerol gradient centrifugation where almost equal amounts of MCM4, -6, and -7 proteins were present (Fig. 3). Consistently, it was suggested that MCM6 or MCM4 protein was capable of binding ATP (Fig. 4). The protein cross-linking of the glycerol gradient fractions indicated that these three MCM proteins formed a hexameric complex. The hexameric complex may be separated into two trimeric complexes during glycerol gradient centrifugation. A main peak of MCM proteins in the glycerol gradient centrifugation (Figs. 1 and 3) was detected at a position near 230 kDa, where four MCM proteins were present. In the same position, mainly a roughy 400-kDa complex was detected (Fig. 3). These results suggest that stable dimers of MCM proteins are present in purified MCM proteins and that they interact to form tetramers of 400 kDa, but they probably
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Do not function as a DNA helicase. Since DNA helicase activity was detected only in the fractions lacking MCM2 protein, it is possible that the presence of MCM2 protein disturbs the assembly of MCM4, -6, and -7 proteins to inhibit the DNA helicase activity.

The four MCM proteins of MCM2, -4, -6, and -7 were tightly bound to a histone-Sepharose column (21). Our group found that among these proteins, only MCM2 protein has an affinity for histone. Therefore, I speculate that a complex consisting of MCM2, -4, -6, and -7 is bound to the histone-Sepharose column through the interaction between MCM2 and histone (histone H3), and a trimeric complex of MCM4, -6, and -7 was dissociated from MCM2 protein during the elution of the MCM proteins with sodium chloride. Knipper’s group (12, 15, 16) has reported that MCM4, -6, and -7 proteins form a stable complex and that MCM2 protein is more loosely associated with the trimeric form of MCM proteins. My conclusion that a hexamer of MCM4, -6, and -7 has DNA helicase activity is consistent with the findings that several helicases including SV40 T antigen (30) function as a hexamer (31–33). However, a possibility that protein(s) bound to MCM proteins has intrinsically the DNA helicase activity cannot be ruled out.

The DNA helicase activity of MCM proteins can be expected from the finding that all of these proteins have motifs that are commonly present in the proteins possessing DNA-dependent ATPase activity (17). The DNA helicase activity may be crucial for the initiation as well as the elongation of DNA replication. MCM proteins are not located in the replication forks, since they are not co-localized with either replication proteins of proliferating cell nuclear antigen and replication protein A (human multisubunit single-stranded DNA-binding protein) or newly replicated DNA (18, 20, 24, 34). In addition, MCM proteins accumulate in chromatin at the G1 phase and detach from it as the DNA replication proceeds (18, 20, 24, 35). These findings, together with our results, suggest that MCM proteins are required at an early step of the bidirectional DNA replication as an enzyme that unwinds duplex DNA in the origin region. A number of DNA helicases have been identified in eukaryotes by the activity that displaces annealed oligonucleotides (36, 37). Among them, a mouse DNA helicase B (38) and DNA2 gene product in Saccharomyces cerevisiae (39) are good candidates for the enzyme involved in the progression of the replication forks. The complex of MCM4, -6, and -7 proteins is different from other DNA helicases in that it cannot displace longer oligonucleotides efficiently (Fig. 9). In addition, the specific activity of DNA helicase in the glycerol gradient fraction was low compared with other DNA helicases. In vivo, phosphorylation of the MCM4, -6, and -7 protein complex may stimulate the DNA helicase activity, or other replication proteins such as replication protein A may be involved in increasing the processivity of DNA unwinding by the MCM protein complex. Another possibility is that the DNA helicase activity of MCM proteins is required only at the initial step of DNA unwinding of the origin region, and other DNA helicases take over the role of MCM proteins in the DNA unwinding.

The origin recognition complex (ORC) consisting of six subunits was first identified in budding yeast as a complex that recognizes the 11-base pair consensus origin sequence (40), and it has been shown that the ORC is required for DNA replication (41–44). Homologues of ORC1 (45) and ORC2 (46) were recently identified in Xenopus, and it was shown that they are...
required for DNA replication in an in vitro system. A genetic interaction between ORC components and MCM proteins has been reported in S. cerevisiae (44, 47). Therefore, MCM protein complexes may be involved in the initiation of DNA replication as a DNA unwinding enzyme by interacting with ORC. Another complexes may be involved in the initiation of DNA replication been reported in interaction between ORC components and MCM proteins has

in vitro replication in the Xenopus system (48). In this system, both RLF (replication licensing factor)-B and RLF-M are required for DNA replication. RLF-M has been purified to homogeneity and consists of a complex of MCM proteins (48–50). It has been suggested that RLF-B is required for loading MCM proteins to chromatin (48). Coleman et al. (51) have reported that the binding of ORC2 and Cdc6 proteins to chromatin is required for the assembly of MCM 3 protein with chromatin, which leads to the initiation of DNA replication in the Xenopus system.

In the present study, it was demonstrated that both ATPase and DNA helicase activities were associated with purified MCM proteins. Although the issue of whether these activities are intrinsic features of MCM4, -6, and -7 protein complex needs further study, this paper presents the first biochemical evidence for the view that MCM protein complexes act as a DNA unwinding enzyme in the initiation of DNA replication.

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