DNA Unwinding Is an MCM Complex-dependent and ATP Hydrolysis-dependent Process*

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Minichromosome maintenance proteins (Mcm) are essential in all eukaryotes and are absolutely required for initiation of DNA replication. The eukaryotic and archaeal Mcm proteins have conserved helicase motifs and exhibit DNA helicase and ATP hydrolysis activities in vitro. Although the Mcm proteins have been proposed to be the replicative helicase, the enzyme that melts the DNA helix at the replication fork, their function during cellular DNA replication elongation is still unclear. Using nucleoplasmic extract (NPE) from Xenopus laevis eggs and six purified polyclonal antibodies generated against each of the Xenopus Mcm proteins, we have demonstrated that Mcm proteins are required during DNA replication and DNA unwinding after initiation of replication. Quantitative depletion of Mcms from the NPE results in normal replication and unwinding, confirming that Mcms are required before pre-replicative complex assembly and dispensable thereafter. Replication and unwinding are inhibited when pooled neutralizing antibodies against the six different Mcm2–7 proteins are added during NPE incubation. Furthermore, replication is blocked by the addition of the Mcm antibodies after an initial period of replication in the NPE, visualized by a pulse of radiolabeled nucleotide at the same time as antibody addition. Addition of the cyclin-dependent kinase 2 inhibitor p21cip1 specifically blocks origin firing but does not prevent helicase action. When p21cip1 is added, followed by the non-hydrolyzable analog ATPγS to block helicase function, unwinding is inhibited, demonstrating that plasmid unwinding is specifically attributable to an ATP hydrolysis-dependent function. These data support the hypothesis that the Mcm protein complex functions as the replicative helicase.

Eukaryotic cells duplicate their genome in the tightly controlled and cell cycle-dependent process of replication. Multiple origins of replication are used by eukaryotes to efficiently replicate the entire genome. Activation of individual origins requires an ordered series of protein assembly and enzymatic activities (reviewed by Bell and Dutta (1)). The eukaryotic SV40 viral replication initiator and the Escherichia coli replication initiation mechanism provide paradigms for normal eukaryotic genomic DNA replication. First, an origin is marked by SV40 virus T-antigen (2) or DnaA protein in E. coli (3), the paralog of the origin recognition complex in eukaryotes. Then, this protein platform promotes loading of a replicative helicase, the function of which is to melt the double-stranded DNA helix to allow template-directed, semiconservative DNA replication (4). SV40 viral replication relies on T-antigen as both the trans-acting origin determinant and the initiating helicase (2). The helicase locally melts the DNA, and an initiating polymerase is loaded onto the activated origin to start the duplication of the replicon.

Helicases are protein machines that melt a duplex nucleic acid and are essential for cellular processes that use nucleic acid templates (5). Helicases involved in DNA replication, such as SV40 T-antigen (6, 7), T7 bacteriophage DNA helicase (8), and E. coli DnaB (9), are generally oligomeric and have six-fold symmetry about the axis of the DNA substrate, probably to substantially increase their stability on the substrate and therefore their processivity (10, 11). A replicative helicase has not been identified in eukaryotes, but the highly conserved minichromosome maintenance protein (Mcm) complex is the best candidate (4). There are 134 putative helicase open reading frames in the Saccharomyces cerevisiae genome (12); therefore, it remains to be seen whether the Mcms, or another molecule, accomplishes the function of a replicative helicase in eukaryotes.

Mcm proteins form a family of conserved molecules that are essential for initiation of DNA replication and are activated following action of the S-phase kinases (including Cdk2 and Cdc7, which specifically activate substrates at the origin) (Mcm proteins reviewed in Refs. 13–15). The Mcm genes were initially identified in S. cerevisiae genetic screens that isolated mutants that failed to replicate a minichromosome containing a single origin of replication (16) and in a screen for cell cycle mutants that arrest at the G1/S transition (17). All eukaryotes contain six orthologous Mcm proteins, members of the “AAA+ ATPase” family of proteins (18), that function as heteromultimeric complexes (19–22). These proteins are all essential for initiation of DNA replication in eukaryotes. The sequencing of the complete genomes of several archaebacteria has shown that Mcm proteins are also present in the domain Archaea (15). We and others previously demonstrated that MtMcm, the unique Mcm protein of Methanobacterium thermoautotrophicum, contains helicase activity and DNA-dependent ATPase activity (23–25). MtMcm helicase unwinds DNA duplexes in a 3′→5′ direction and can unwind up to 500 bp in vitro. The kinetics, processivity, and

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1 The abbreviations used are: Mcm, minichromosome maintenance protein; Cdk, cyclin-dependent kinase; Cdc, Cdk complex; NPE, nucleoplasmic extract; RC, replicative complex; ELB, egg lysis buffer; RLF, replication licensing factor; Ara-dCTP, cytosine β-d-arabinofuranoside 5′-triphosphate.

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directionality of MtMcm support its role as a replicative helicase in *M. thermotolerant. Furthermore, the structure of the MtMcm protein, determined by x-ray crystallography and via electron microscopy, is a hexameric ring, consistent with known helicases (26, 27).

To date, no helicase activity has been reported in vitro for the eukaryotic hexameric Mcm2–7 complex. However, the Mcm4,6,7 subcomplex purified from human cells in culture (28) or recombinant Mcm subcomplexes from mouse (29), *S. pombe* (30), and *S. cerevisiae* (31) exhibit in vitro helicase activity.

In *Xenopus* egg extracts, yeast, and human cells, the number of origin recognition complex molecules bound to the chromatin is approximately the same as the number of active replication origins (32), whereas there is a large excess of chromatin-bound Mcm proteins (33). However, the mechanism of the eukaryotic Mcm complex action and localization is still poorly understood, and conflicting data have led to diverging views. Immunofluorescence observations of the Mcm proteins suggest that they do not colocalize with replication foci after initiation of replication (34–36) but bind to sites close to the point of synthesis (35). However, there is an absolute requirement for Mcm proteins to replicate the eukaryotic genome, because induction of the rapid degradation of yeast Mcm proteins quickly halted the progression of those cells through S-phase (37). Therefore, the question of the dynamics and function of the Mcm complex after initiation is still unresolved.

*Xenopus laevis* egg extracts support the assembly and replication of varied DNA templates (38). Fractionation of egg extract and isolation of a concentrated nuclear extract yield two components, egg cytosol and nucleoplasmic extract (NPE). The ordered events of the cell cycle and progression through S-phase can be experimentally divided into two steps: assembly in the egg cytosol and activation and replication in the NPE (39). In *Xenopus* egg extracts, DNA replication is regulated by activation of individual replication origins. A competent replication origin requires stepwise assembly of the pre-replicative complex (pre-RC), consisting of origin recognition complexes 1–6, Cdc6, Cdt1, and Mcm2–7 proteins (1, 40). The S-phase-promoting kinases, Cdk2 and Cdc7, phosphorylate their substrates, including the Mcm proteins (41), to promote DNA replication (39). Immediately after S-phase-promoting kinase action, Cdc45 is loaded onto the chromatin and likely contacts the Mcm proteins (42); the putative helicase melts the DNA, and replication proceeds (1). Only a small subset of the large number of Mcm complexes on chromatin in *Xenopus* egg extracts is used for replication (33, 43). Initiation of replication in *Xenopus* egg extracts occurs asynchronously and with irregular spacing (44, 45), whereas the timing of origin firing is temporally controlled by the damage-checkpoint kinases ATR and ATM in the absence of damage (46).

Here, we have studied the dynamics and function of the *Xenopus* Mcm protein complex. Using the biphaseic NPE system, in which pre-RC formation and template assembly is experimentally separated from initiation, template melting, and DNA replication, we confirm the absolute requirement for the Mcm proteins to be assembled on the DNA template before origin firing. We further demonstrate that DNA template unwinding is dependent on ATP hydrolysis after initiation and is resistant to dilution, consistent with a tightly bound helicase, likely the Mcms, performing the unwinding. We show that a pool of neutralizing antibodies directed against each of the six Mcm proteins block incorporation and template unwinding. These observations all support the idea that the Mcm complex in *Xenopus* egg extracts serves as the replicative helicase.

### EXPERIMENTAL PROCEDURES

**Materials**—[^20]-α-dATP was purchased from Amersham Biosciences, SYBR Gold was purchased from Molecular Probes, cytosine β-arabinofuranoside 5-triphosphate (Ara-dCTP) was purchased from Sigma (catalog number C-3639). All incubations in extracts were performed at 22°C. All radionucleotide-containing gels were dried and exposed to a PhosphorImager plate, read in a PhosphorImager (Molecular Dynamics). SYBR-Gold stained gels were scanned using a FluorImager (Molecular Dynamics). All image levels were adjusted, and incorporation was quantitated in ImageQuant software (Molecular Dynamics).

**Buffer**—Egg lysis buffer (ELB) contained 200 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES, pH 7.8, 1 mM dithiothreitol, and 50 mg/ml cycloheximide.

**Extracts**—Interphase *Xenopus laevis* egg extracts were prepared as described in the presence of cycloheximide (47). Before experimental use, each batch of extract was assayed for competent and timely nuclear assembly, and only extracts that formed rounded nuclei with a nuclear envelope within 30 min were used. Membrane-free clarified egg cytosol and NPE were prepared as described (39).

**Plasmid Replication and Unwinding Assays in NPE**—For replication and unwinding assays, Qiagen-purified plasmid DNA (pBlueScriptII) was used, and assays were performed as described (48). Unwinding reactions were run on a 22.5% chloroquine-agarose gel in Tris-phosphate EDTA, as described (46).

**Production of Recombinant Proteins**—*Xenopus Geminin* was expressed in BL21(DE3) cells from a pET28A plasmid (gift of Dr. M. Michael) according to standard protocols. Hexahistidine-tagged human p21WAF1 protein was expressed in BL21(DE3) cells, solubilized in 8 M urea from inclusion bodies, purified on a nickel column, renatured in aqueous solution using Non-Detergent Sulfobetaine (NDSB201, Calbiochem), as described, and concentrated on a MonoQ column.

**Purification of Antibodies**—Sera from rabbits immunized against the six Mcm proteins were individually purified to collect total IgG. First, the serum was dialyzed overnight against 10% glycerol, 25 mM Tris-HCl, pH 8.0, and 25 mM NaCl and applied to a DEAE-Affigel blue (Bio-Rad) column. The flow-through was collected and applied to a 1-mL MonoQ column on fast protein liquid chromatography. The proteins were eluted with a 25–500 mM NaCl gradient, and the peak of IgG was collected after identification on a Coomassie Blue-stained gel of the collected fractions. The pool of IgG was then raised to 50% ammonium sulfate, and the precipitate was resuspended in 10% glycerol, 25 mM Tris-HCl, pH 8.0, and 25 mM NaCl. Finally, the pool of IgG was bound in batch to protein A-Sepharose for 1 h and washed with 500 mM NaCl containing buffer and then the application buffer, followed by elution with 100 mM glycine-HCl, pH 2.5, and neutralization with Tris-HCl, pH 8.8 (final concentration, 100 mM) and 10% glycerol. The final pH was neutral, and each pool of IgG was ~2 mg/ml.

### RESULTS

**Mcm Proteins Are Required on the Template for Unwinding and Replication**—Mcm proteins are required for DNA replication and unwinding in *Xenopus* egg extracts, including in NPE (39). To determine exactly when the Mcm proteins are required, we produced antibodies specifically directed against all six individual *Xenopus* Mcm proteins. Total IgG from the immunized rabbit sera were biochemically purified to high concentration and purity, as shown in Fig. 1A. Each purified antibody directed against the Mcm2–7 proteins specifically recognized a single polypeptide in NPE of the correct size on an immunoblot, as seen in Fig. 1A. *Xenopus* egg cytosol contains large amounts of Mcm proteins (33), which become quantitatively imported into the nucleus and are therefore enriched in NPE (Fig. 1C). Therefore, we depleted the Mcm complex from both the clarified egg cytosol and the NPE (as seen in Fig. 1D, Mcm3 and Mcm6 are removed upon immunodepletion with an Mcm6 antibody).

We confirmed the requirement for Mcm proteins by deleting egg cytosol of Mcms and assaying for replication of a plasmid template. Fractionation of the egg extract using polyethylene glycol precipitation had previously demonstrated that a fraction required for replication licensing and pre-RC assembly, called replication licensing factor (RLF) M, contained Mcm...
proteins (49). As seen in Fig. 2a, plasmid DNA replication is inhibited after Mcm depletion from the cytosol and is partly recovered after addition of the Mcm-containing RLF-M fraction. However, this requirement could be attributable to a function during origin assembly or any subsequent step: initiation, template unwinding, or replication.

To assess whether the Mcm proteins are required during the elongation phase, we attempted to use the Mcm antibodies in a neutralizing fashion. The addition of purified IgG from preimmune serum to plasmid assembled in egg cytosol, followed by NPE containing radionucleotides, had no effect on replication as measured by incorporation (Fig. 2b, compare lane 1 with lane 2). The addition of purified anti-Mcm2–7 antibodies modestly reduced DNA replication (Fig. 2b, compare lane 3 with lanes 1 and 2). We reasoned that the antibodies might be quenched by the excess of Mcm proteins and thus cannot completely neutralize Mcm function on the template. Depleted NPE was perfectly functional to promote DNA replication (Fig. 2b, compare lane 1 in the top and bottom panels) and was not affected by addition of the control IgG (Fig. 2b, lane 2, bottom panel). However, upon addition of the Mcm2–7 antibodies before addition of the NPE, incorporation into the assembled plasmid template was completely blocked (Fig. 2b, lane 3, bottom panel). These data establish that we successfully generated neutralizing antibodies against the Mcm proteins and strongly support the hypothesis that the Mcm proteins are required at some point or points during DNA replication initiation and/or progression. Because the depletion of Mcms from the NPE demonstrated that the remaining Mcms in solution are dispensable for DNA replication, only those complexes previously assembled on the template are required for replication.

We then asked whether the Mcm proteins are required in the NPE for plasmid DNA unwinding. As demonstrated previously, Mcm proteins are required in the egg cytosol for the template assembly step and, as a consequence, for subsequent DNA unwinding (48). We show that Mcm-depleted NPE is capable of unwinding a plasmid template (Fig. 2c, lane 2). The addition of either purified mouse IgG or purified pre-immune rabbit IgG did not affect plasmid unwinding (Fig. 2a, lanes 3 and 4, compared with lane 2), whereas the addition of Mcm2–7 antibodies

**Fig. 1.** Purification and characterization of Mcm antibodies and the distribution of Mcm proteins in Xenopus egg extracts. a, 4 ml of each purified Mcm antibody was separated on SDS-PAGE and stained with Coomassie Blue to demonstrate the purity and concentration of the antibodies. The antibody purification scheme is indicated. b, 0.2 ml of NPE was immunoblotted with each purified Mcm antibody indicated to demonstrate the specificity of the purified antibodies. Mcm2 migrated as a doublet, as did recombinant Mcm2 isolated from baculovirus-infected insect cells (data not shown). c, 1.5 ml of egg cytosol, NPE, or RLF-B (without Mcm proteins) or RLF-M (normally containing Mcm proteins) (49) fractions produced from residual egg extract after the NPE preparation were immunoblotted for all six Mcm proteins (Mcm2–7), Mcm3, Mcm7, origin recognition complex 2 (Orc2), or proliferating cell nuclear antigen (PCNA). Because the residual egg extract was depleted of Mcm proteins, the RLF-M fraction did not contain Mcms. d, egg extract cytosol or NPE was subjected to successive rounds (Rd1, Rd2, Rd3) of Mcm protein depletion using anti-Mcm6 serum. 0.5 ml after each round of depleted extracts was immunoblotted for the presence of Mcm3 or Mcm6 protein, as indicated.
FIG. 2. Mcm proteins are required for replication progression and unwinding. a, pBluescriptII plasmid was incubated at 50 ng/ml in undepleted, Mcm2-7-depleted, or Mcm proteins in the RLP-M fraction added to Mcm2-7-depleted egg extract cytosol, followed by the addition of a 2-fold volume of NPE containing [32P]dATP. The reaction was stopped after 30 min, and plasmid DNA replication was observed after electrophoresis through a 0.8% agarose gel and autoradiography. b, pBS plasmid was incubated at 50 ng/ml in egg cytosol for 60 min, followed by the addition of a 2-fold volume of ELB, purified pre-immune IgG (Pre-Imm. abs), or purified IgG directed against all six Mcm proteins, as indicated, and incubated for 10 min. One volume of NPE (top gel) or Mcm-depleted NPE (bottom gel), containing [32P]dATP, was then added. The reaction was stopped after 30 min and analyzed as above. c, pBS plasmid was incubated at 50 ng/ml for 60 min in egg cytosol diluted 2-fold in ELB, followed by the addition of a 0.6-fold volume of ELB or purified antibodies, as indicated. Then Mcm-depleted NPE containing 50 mg/ml aphidicolin was added, and the reactions were stopped after 30 min. Plasmid DNA topoisomers were analyzed on a chloroquine-agarose gel. d, pBS plasmid was incubated at 50 ng/ml in 50%-diluted egg cytosol for 60 min, followed by the addition of a 2-fold volume of ELB, purified pre-immune IgG, or purified IgG directed against individual Mcm proteins and incubated for 10 min. One volume of Mcm-depleted NPE containing [32P]dATP was then added. The reaction was stopped after 30 min, and replication was observed after electrophoresis through a 0.8%-agarose gel. e, pBS plasmid was incubated at 50 ng/ml in egg cytosol for 60 min, followed by the addition of a 2-fold volume of ELB, control mouse IgG, purified pre-immune IgG (Pre-Imm. abs), or purified IgG directed against Mcm2 protein and incubated for 10 min. One volume of Mcm-depleted NPE containing [32P]dATP was then added. The reaction was stopped after 30 min, and replication was observed after electrophoresis through a 0.8%-agarose gel. The reactions were performed in the same experiment and extract, but the reaction product images were spliced together for clarity.

entirely blocked unwinding (Fig. 2c, lane 5). These data strongly suggest that, despite the high concentration of Mcm proteins in the NPE, there is little, if any, exchange of active Mcm proteins assembled on the DNA template.

As shown previously (48, 50), template unwinding is most likely attributable to replicative helicase function. Therefore, this observation further supports the hypothesis that the Mcm proteins are required at some point or points during DNA replication initiation and/or helicase progression to melt the DNA template.

To determine which of the individual Mcm proteins are required for replication progression and template unwinding, we added individual purified anti-Mcm antibodies to the NPE replication reaction. As shown in Fig. 2d, the addition of control IgG did not affect the level of incorporation into the plasmid DNA template as compared with the addition of buffer alone (compare lanes 1 and 2). The addition of anti-Mcm2 antibodies resulted in a modest, but reproducible, increase in incorporation (Fig. 2d, lane 3, and Fig. 2e), consistent with a negative role for Mcm2 in promoting DNA replication suggested previously by the inhibitory function of Mcm2 in vitro helicase assays (51). The addition of anti-Mcm3, anti-Mcm4, anti-Mcm5, anti-Mcm6, and anti-Mcm7 antibodies inhibited nucleotide incorporation with various effects (Fig. 2d, lanes 4–9).

To determine whether the Mcm proteins are required only during initiation of DNA replication, during replication progression, or both, we analyzed the kinetics of incorporation into a plasmid template in the NPE. A plasmid template assembled in egg cytosol was added to NPE, pulsed with radionucleotides at the time points indicated, and followed by a chase with excess unlabeled nucleotides 5 min later, as indicated (Fig. 3a). Incorporation occurred mainly within the first 15 min after the addition of NPE, peaking between 5 and 10 min. From these experiments, we chose to add the neutralizing Mcm antibodies at early time points during replication in the NPE, up to 15 min. As shown in Fig. 3b, the addition of anti-Mcm2–7 antibodies to a plasmid replicating in NPE at early time points inhibited incorporation dramatically compared with the control (Fig. 3b, compare lanes 6–8 with lanes 1–3). In contrast, the addition of the neutralizing antibodies at later time points, after the bulk of incorporation was completed (Fig. 3a, lane 4), did not inhibit incorporation as efficiently (Fig. 3b, compare lane 10 with lane 5). This is consistent with a requirement for the Mcm proteins during elongation. However, the asynchrony of initiation of replication in NPE that we have documented (46), and the fact that unwinding of a 3-kb plasmid molecule is completed in 3 min (48), suggests that this experiment might not fully differentiate between initiation and elongation.

Therefore, to better distinguish events occurring during replication initiation and helicase progression, we used the reversible polymerase inhibitor Ara-dCTP to separate helicase function from DNA incorporation. The addition of Ara-dCTP to NPE entirely inhibits incorporation into a plasmid template, whereas concomitant addition of excess dCTP with the inhibitor rescues incorporation (Fig. 3c). Ara-dCTP can also be used in place of aphidicolin to trap the “U-form” replication intermediate, indicative of helicase action, and can subsequently be chased out by the addition of dCTP, allowing other topoisomerases to accumulate (Fig. 3d and Ref. 48). Although the polymerases are inhibited by Ara-dCTP, the helicase will continue to function and, consequently, will be disposable for DNA incorporation once it has completed its function and unwound the template. We therefore probed the role of the Mcm proteins by measuring nucleotide incorporation into a plasmid template incubated in NPE contain-
Mcm proteins are required only for replication during template unwinding. a, pBS plasmid was incubated at 50 ng/ml in egg cytosol for 30 min, followed by the addition of a 2-fold volume of NPE. The reaction was pulsed with [32P]dATP and chased with 1 mM unlabeled dATP at the time points indicated. The reaction was stopped at 30 min and analyzed on a 0.8%-agarose gel. b, pBS plasmid was incubated at 50 ng/ml in egg cytosol for 30 min, followed by the addition of a 2-fold volume of Mcm-depleted NPE containing [32P]dATP. At 0, 2.5, 5, 10, or 15 min after the addition of NPE, as indicated, an aliquot was added to a tube containing one-half volume of ELB (lanes 1–5) or purified IgG directed against all six Mcm proteins (lanes 6–10). The reaction was stopped after 30 min and analyzed on a 0.8%-agarose gel. c, pBS plasmid was incubated at 50 ng/ml in egg cytosol, followed by the addition of a 2-fold volume of NPE containing [32P]dATP and 200 mM Ara-dCTP in the absence or presence of 1 mM dCTP. The reaction was stopped after 30 min and analyzed on a 0.8%-agarose gel. d, pBS plasmid was incubated at 50 ng/ml in egg cytosol, followed by the addition of a 2-fold volume of NPE containing 200 mM Ara-dCTP. At 0, 5, 10, 15, 20, or 25 min, an aliquot of the reaction was either stopped or added to a tube containing a dCTP chase to 1 mM final concentration. The latter reactions were all stopped at 26 min after NPE addition. The reaction products were analyzed on a chloroquine-agarose gel. e, pBS plasmid was incubated at 50 ng/ml in egg cytosol, followed by the addition of a 2-fold volume of NPE containing [32P]dATP and 200 mM Ara-dCTP. At 0, 5, 10, 15, or 20 min, an aliquot was added to a tube containing one-half volume of ELB (top panel) or purified IgG directed against all six Mcm proteins (bottom panel). One min later, 1 mM dCTP was added to promote nucleotide incorporation. The reactions were stopped after 60 min and analyzed on a 0.8%-agarose gel.

Mcm proteins are tightly bound to the DNA template (52, 53), and we have demonstrated that the archaeal Mcm homolog in M. thermoautotrophicum forms a stable double hexamer, resistant to high salt treatment, chelating agents, and dilution (23). Because we hypothesized that the Mcm complex is responsible for DNA unwinding, we tested whether the unwinding reaction was resistant to dilution. An unwinding reaction sensitive to dilution would support a model in which the helicase is not tightly bound to the template. An unwinding reaction that is not sensitive to dilution would be consistent with the tightly bound Mcm complex serving to unwind the DNA. The unwinding reaction requires concentrated S-phase kinases in the NPE to activate the origins of replication (46, 48). Therefore, we diluted the unwinding reactions at the same time as NPE addition and also 2 min after NPE addition to rule out an effect on unwinding attributable to dilution of the requisite kinase activity. In this preparation of NPE, unwinding was complete by 8 min after the addition of NPE in the absence of dilution (Fig. 5a). As seen in Fig. 4a, unwinding activity was substantially reduced when dilution was performed at the same time as NPE addition. However, the unwinding reaction was entirely resistant to dilution 2 min after NPE addition (Fig. 4b), strongly supporting the hypothesis that unwinding is attributable to a tightly template-bound helicase. This observation is consistent with Mcms acting to unwind the DNA.
ment for kinase activity in the unwinding reaction. Purified, recombinant p21cip1 protein was added to the unwinding reaction at various time points, and the reaction was allowed to proceed for 16 min. p21cip1 addition at 0, 1, and 2 min resulted in an inhibition of unwinding, but p21cip1 addition had no effect between 4 and 8 min (Fig. 5b). In a parallel experiment, the addition of ATPγS, a non-hydrolyzable analog of ATP, resulted in a more potent inhibition of template unwinding and inhibition at later time points than the p21cip1 addition (Fig. 5c, d).

Finally, a control addition to the NPE of Geminin, a protein inhibitor of Cdt1 and pre-RC formation, did not have any effect on template unwinding, consistent with its role as an inhibitor upstream of origin firing and therefore not required in the NPE (Fig. 5d).

p21cip1 protein addition at 1 or 2 min after NPE addition blocks unfired origins and therefore traps uninitiated plasmid molecules before helicase action, as observed by less negatively supercoiled species than the U-form on the chloroquine-agarose gel. Therefore, we reasoned that we could experimentally separate helicase action from initiation by inhibiting origin firing with p21cip1 protein. The following experiments were performed in an NPE preparation that exhibited complete template unwinding to have a consistent end point for the reaction. p21cip1 protein was added to an unwinding reaction at 90 s after the addition of NPE, and an aliquot of the reaction was stopped at time points afterward. As seen in Fig. 5e, lane 6, by the presence of remaining ground-state topoisomers, p21cip1 prevented complete unwinding after 10 min, as compared with the complete unwinding with the same NPE preparation seen in the absence of p21cip1 (Fig. 5a, lanes 6 and 7, and Fig. 5f, lane 2). Therefore, we conclude that the increasing extent of unwinding after p21cip1 addition in the first 3 min (Fig. 5e, lanes 2–5) is solely attributable to helicase action after initiation of replication. This 3-min time frame for unwinding after initiation is consistent with the observation by Walter et al. (48) that there was a 3-min window between the first appearance of the U-form and the first appearance of fully replicated product.

Because helicase action is ATP hydrolysis-dependent and
because we demonstrated (Fig. 5c) that ATP/"S can block U-
form accumulation, we probed the requirement for ATP hydrol-
ysis after S-phase kinase action by treatment of an unwinding
reaction with p21cip1. First, as shown in Fig. 5f, lane 2, the
plasmid template was completely unwound in 10 min in the
absence of p21cip1. p21cip1 protein was added to the unwinding
reaction 90 s after the addition of NPE, and ATP/"S was added
to aliquots of the reaction at time points afterward, as indicated
(Fig. 5f, lanes 3–7). We observed a very similar extent of un-
winding over time upon ATP/"S addition (Fig. 5f, lanes 3–7)
compared with stopping the reaction entirely (5, lanes 2–6).
Therefore, we conclude that unwinding after Cdk2 action is
entirely attributable to an ATP hydrolysis-dependent step. Be-
cause the Mcm complex is an ATP hydrolysis-dependent heli-
case, this observation is consistent with the Mcms serving to
unwind the DNA template.

**DISCUSSION**

Previous studies of the human (28), mouse (29), *S. pombe* (30,
54), and *S. cerevisiae* (19) Mcm protein complexes, the single
*M. thermoautotrophicum* Mcm protein (23–25), and the *Sulfolo-
bus solfataricus* Mcm protein (55) have demonstrated *in vitro*
helicase activity for the Mcm complex. The MtMcm protein ex-
phibited potent helicase activity, consistent with that required for
a replicative helicase. These observations confirmed that the
Mcm proteins are a DNA helicase and strengthened the hypo-
thesis that they serve as the replicative helicase during DNA
replication. Furthermore, yeast expressing rapidly and inducibly
degradable (degron) mcn genes require all Mcm proteins to com-
pletely progress through S-phase (37). However, because Mcm
proteins play a role in genome stability (56, 57), it is formally
possible that degradation of Mcms trigger a checkpoint response
leading to inhibition of DNA replication. Here, we wanted to
show directly that the Mcm proteins are specifically required to
melt the DNA on a properly assembled and biologically active
DNA replication template. Therefore, we used NPE, which allows
dNA replication to be broken into two steps: 1) assembly of a
chromatinized template and pre-RC formation in the clarified
egg cytosol; and 2) initiation by S-phase kinases at the previously
assembled origin of replication and replication fork progression
and incorporation in the NPE. We also developed antibodies
against all six Mcm proteins, purified and concentrated them,
and used them in a neutralizing fashion to inhibit the function of
the Mcm proteins in the egg extracts. Considering that these
antibodies have effectively a 6-fold lower individual specific ac-
tivity than the pool of all of them, their individual neutralizing
effects might be accordingly lower.

**Mcm Proteins Are Required for Template Unwinding and
Replication**—A replicative helicase activity is absolutely re-
quired for DNA replication to occur, because the template must
be melted for polymerization of new daughter strands from the
template in a semi-conservative fashion. As discussed above,
previous work has strongly demonstrated the intrinsic DNA
helicase activity of many different Mcm proteins and com-
plexes. Our results are in agreement with the hypothesis that
the Mcm complex is the replicative helicase. First, we demon-
strate that Mcm proteins are absolutely required for DNA
replication, because depletion of the Mcms from egg cytosol
prevented replication, whereas re-addition of an Mcm-contain-
ing extract fraction recovered the activity. Interestingly, al-
though the soluble and non-template-bound Mcm proteins are
imported into the nucleus during NPE preparation, we show that
they are entirely dispensable for DNA replication and plasmid
unwinding, consistent with functional Mcms only being
present as assembled on the template. Second, we demon-
strate that neutralizing antibodies directed against all six Mcm
proteins block DNA replication and template unwinding when
added to the NPE step of the reaction, at which time origin
firing occurs, and the helicase melts the template.

We also added the individual neutralizing Mcm antibodies to
replication and unwinding experiments, in addition to adding
all six together, to probe whether the individual Mcm poly-
peptides have discrete functions or whether they are collectively
required. Our experiments did show a more pronounced inhibi-
tory effect on replication and unwinding (data not shown) by
the Mcm3 and Mcm7 antibodies and a more modest inhibition
by the Mcm4, Mcm5, and Mcm6 antibodies. However, these
antibodies were not affinity purified, and therefore the pool of
all six antibodies has an effective 6-fold higher specific activity.
Therefore, the lack of inhibition by any of the antibodies should
not be viewed as if the corresponding Mcm protein is not
required. We therefore only considered the effect on the entire
complex. Interestingly, the Mcm2 antibody alone caused an
increase in incorporation relative to the control (Fig. 2 and data
not shown) consistent with the previously reported data that
Mcm2 is inhibitory for helicase activity (51).

**DNA Helicase Requirement during Elongation**—These ob-
servations support the hypothesis that the Mcms are required
factors for DNA replication initiation and must be present on
properly assembled templates. To more completely demon-
strate that the Mcms are functioning specifically to unwind the
template, we added the neutralizing antibodies at various time
points after replication proceeded. We also added the neutralizing
antibodies while replication, but not unwinding, was inhibited
in the presence of a reversible polymerase inhibitor, Ara-dCTP.
Using these approaches, we demonstrated that the antibodies
impact replication only during the earlier time points, during
which time the putative helicase is functioning. Additionally,
we also demonstrated that the unwinding reaction is resistant
to extreme dilution, after a lag period for initiating kinase
activity, consistent with unwinding being performed by a
tightly bound factor, likely the Mcm proteins.

**Functional Separation of Initiation from Elongation**—We
have demonstrated previously that origin firing activity is
asynchronous on plasmid templates in the NPE (46). We also
show in Fig. 3 that incorporation occurs in a window of ~15
min in the NPE on a 3-kb plasmid, compared with an observed
window of 3 min for a subset of molecules to completely repli-
cate (48). Therefore, to isolate the role of the Mcm proteins
after initiation, we used the Cdk2 inhibitor p21cip1 to block
further initiation and to separate the two phases of initiation
and elongation. Using this approach, we demonstrated that
plasmid unwinding after kinase action and initiation is an ATP
hydrolysis-dependent action. This observation is consistent
with the known requirement for ATP-binding and hydrolysis
of the eukaryotic (29, 58) and archaeal Mcm proteins (23–25).
Indeed, a functional synergy between the Mcm subunits is
required for ATPase activity in the full complex (19, 58, 59).
This cooperative effect requires an arginine finger and P-loop
motifs in the Mcm proteins (19).

Furthermore, the effect of the addition of the Cdk2 inhibitor
p21cip1 on the unwinding reaction is consistent with a require-
ment for Cdk2 function between 0 and 2 min of the unwinding
reaction, after which time it is dispensable. The addition of
ATP/"S demonstrates a more extensive requirement over time
for ATP hydrolysis, consistent with the requirement of ATP for
Cdk2 activity and a subsequent requirement of ATP for heli-
case activity. Cdc7 kinase activity is required before Cdk2 in
*Xenopus* egg extracts (60). Therefore, we conclude from these
observations that the difference in the timing of U-form ap-
parence between the p21cip1 addition and the ATP/"S addition
is the time required for helicase action to unwind the DNA (Fig.
5, b and c, compare lanes 2–4).
Mcm-dependent DNA Unwinding—Verification of the role of the Mcm proteins as the replicative helicase will require complete reconstitution of the entire eukaryotic pre-RC and replication fork apparatus and mutations of the conserved ATPase and arginine finger domains. Some of the required factors for these reactions are probably still unknown and might not be easily expressed and purified. However, we have provided strong evidence that the Mcm proteins are specifically required for DNA replication, not just in template and pre-RC assembly, but in fork progression, and specifically, in template unwinding. These observations support the hypothesis that the Mcms are indeed the replicative helicase.

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REFERENCES
1. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
2. Kelly, T. J. (1988) J. Biol. Chem. 263, 17689–17692
3. Messer, W. (2002) FEMS Microbiol. Rev. 26, 355–374
4. Labib, K., and Diffley, J. F. (2001) Curr. Opin. Genet. Dev. 11, 64–70
5. von Hippel, P. H., and Delagoutte, E. (2003) BioEssays 25, 1168–1177
6. Li, D., Zhao, R., Lylestrom, W., Gai, D., Zhang, R., DeCaprio, J. A., Fanning, E., Jochimiak, A., Szakonyi, G., and Chen, X. S. (2003) Nature 423, 512–518
7. Dean, F. B., Borowiec, J. A., Eki, T., and Hurwitz, J. (1992) J. Biol. Chem. 267, 14129–14137
8. Toth, E. A., Li, Y., Sawaya, M. R., Cheng, Y., and Ellenberger, T. (2003) Mol. Cell 12, 1113–1123
9. San Martin, C., Rodermacher, M., Wolpersinger, B., Engel, A., Miles, C. S., Dixon, N. E., and Carazo, J. M. (1998) Structure 6, 501–509
10. Patel, S. S., and Picha, K. M. (2000) Annu. Rev. Biochem. 69, 651–697
11. Kelman, Z., Finkelstein, J., and O’Donnell, M. (1995) Curr. Biol. 5, 1239–1242
12. Shiratori, A., Shibata, T., Arisawa, M., Hanaoka, F., and Eki, T. (1999) Yeast 15, 219–253
13. Tye, B. K., and Sawyer, S. (2000) J. Biol. Chem. 275, 34833–34836
14. Forsburg, S. L. (2004) Mol. Cell 17, 383–405
15. Maine, G. T., Sinha, P., and Tye, B. K. (1984) Genetics 108, 365–385
16. Meir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (1982) Genetics 100, 547–563
17. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) Genome Res. 9, 27–43
18. Davey, M. J., Indiani, C., and O’Donnell, M. (2003) J. Biol. Chem. 278, 4491–4499
19. Crouse, M., Amargirola, F., Gicquet, S., and Mechali, M. (1998) Exp. Cell Res. 245, 282–289
20. Kubota, Y., Mimura, S., Ishino, S., Masuda, T., Nojima, H., and Takisawa, H. (1997) EMBO J. 16, 3320–3331
21. Thomas, P., Kubota, Y., Takisawa, H., and Blow, J. J. (1997) EMBO J. 16, 3312–3319
22. Shechter, D. P., Ying, C. Y., and Gautier, J. (2000) J. Biol. Chem. 275, 15049–15059
23. Chong, J. P., Hayashi, M. K., Simon, M. N., Xu, R. M., and Stillman, B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1530–1535
24. Kelman, Z., Lee, J. K., and Hurwitz, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14783–14788
25. Forsburg, S. L., Sherman, D. A., Ohtsue, S., Yasuda, J. R., and Hudson, J. A. (1997) Genetics 147, 1025–1041
26. Walter, J. (2003) J. Biol. Chem. 278, 39773–39778
