Novel DNA Binding Properties of the Mcm10 Protein from Saccharomyces cerevisiae

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The Mcm10 protein is essential for chromosomal DNA replication in eukaryotic cells. We purified the Saccharomyces cerevisiae Mcm10 (ScMcm10) and characterized its DNA binding properties. Electrophoretic mobility shift assays and surface plasmon resonance analysis showed that ScMcm10 binds stably to both double strand (ds) DNA and single strand (ss) DNA. On short DNA templates of 25 or 50 bp, surface plasmon resonance analysis showed a ~1:1 stoichiometry of ScMcm10 to dsDNA. On longer dsDNA templates, however, multiple copies of ScMcm10 cooperated in the rapid assembly of a large, stable nucleoprotein complex. The amount of protein bound was directly proportional to the length of the DNA, with an average occupancy spacing of 21–24 bp. This tight spacing is consistent with a nucleoprotein structure in which ScMcm10 is aligned along the helical axis of the dsDNA. In contrast, the stoichiometry of ScMcm10 bound to ssDNA of 20–50 nucleotides was ~3:1 suggesting that interaction with ssDNA induces the assembly of a multisubunit ScMcm10 complex composed of at least three subunits. The tight packing of ScMcm10 on dsDNA and the assembly of a multisubunit complex on ssDNA suggests that, in addition to protein–DNA, protein–protein interactions may be involved in forming the nucleoprotein complex. We propose that these DNA binding properties have an important role in (i) initiation of DNA replication and (ii) formation and maintenance of a stable replication fork during the elongation phase of chromosomal DNA replication.

MCM10 is a ubiquitous, conserved gene essential for DNA replication in eukaryotes. It was first discovered in yeast genetic screens designed to detect mutants defective in DNA synthesis and minichromosome maintenance (1, 2). In vivo, Mcm10 associates with chromatin and chromosomal replication origins in human cells (hMcm10), Xenopus laevis (XMcm10), Schizosaccharomyces pombe (SpMcm10), and Saccharomyces cerevisiae (ScMcm10) (3–6). In S. cerevisiae, initiation of chromosomal replication occurs at multiple specific sites known as autonomously replicating sequences (ARSs) (7). Mutations in MCM10 enhance the loss rate of plasmids bearing specific ARSs (8), suggesting a function for ScMcm10 in initiation.

In eukaryotic systems replication initiation is a cell cycle-regulated process characterized by a multistep sequential loading of ORC, Cdc6, Cdt1, and the Mcm2–7 complex onto the origin in G1, to form the pre-RC complex. Binding of ORC, Cdc6p, and Cdt1p to chromatin is a prerequisite for the recruitment of Mcm2–7 (9, 10). The next step in the assembly of the initiation replication apparatus in S. cerevisiae involves protein kinases (Cdc28 and Cdc7/Dbf4), and recruitment of Mcm10, Cdc45, and the GINS complex. The mechanism for targeting Mcm10 to replication origins is unknown. However, recent studies in S. cerevisiae have shown that Mcm10 and Mcm2–7 physically interact (6, 11). It is now believed that in late G1, chromatin-bound Mcm2–7 is responsible for the recruitment of Mcm10 presumably via protein-protein interactions (12). Prior studies in the Xenopus laevis system reached similar conclusions (4). Additional interactions of Mcm10 with other components of the pre-RC cannot be excluded (13).

A key step in the initiation of replication is the local melting of an origin DNA sequence, which occurs at the G1/S transition and throughout the S phase. The mechanism of this essential DNA-melting process is not understood. There is no evidence in S. cerevisiae that the assembled pre-RC complex leads to the melting of an origin DNA sequence. This unwinding may occur only following the recruitment of Mcm10, raising the possibility that Mcm10 is a key component of the initiation machinery responsible for this process. Results of a study in the Xenopus egg extract system (4), which showed that omission of Xcm10 blocks unwinding of plasmid DNA and initiation of DNA replication, are consistent with this notion. An additional function of Mcm10 in initiation is in the recruitment of Cdc45 to replication origins, presumably via Mcm10-Cdc45 physical interactions (5, 14). Cdc45 is believed to be important for the activation of replication origins and the assembly of the replication elongation complex (15). Upon initiation of DNA replication, ScMcm10 moves from the origin to origin-proximal sequences suggesting that ScMcm10 associates with moving replication forks (12) and is consistent with the observation that elevated

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2 The abbreviations used are: ARS, autonomously replicating sequence; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; GST, glutathione S-transferase; BSA, bovine serum albumin; RU, resonance unit; ORC, origin recognition complex; pre-RC, pre-replicative complex.
temperatures cause pausing of replication forks in a mcm10-1 ts mutant (8). Both ScMcm10 and SpMcm10 interact with DNA polymerase α supporting the notion that replication fork movement requires Mcm10. ScMcm10 and polymerase α form a complex on and off the DNA in vivo (12). In S. pombe, SpMcm10 stimulates the activity of polymerase α in vitro and associates with a primase activity (16, 17) that has not been reported in other eukaryotes (18).

Previous studies with Mcm10 in other systems showed that Mcm10 binds DNA. Using a filter binding assay Fien and Hurwitz (16) reported that SpMcm10 from S. pombe binds well to ssDNA but barely interacts (20-fold lower affinity) with dsDNA. It has been suggested that binding of SpMcm10 to ssDNA is important for the recruitment of polymerase α (16). Recently, it has been reported that a DNA binding activity is also associated with XMcm10 protein from X. laevis. Measurements of fluorescence anisotropy were used to show binding of XMcm10 to short, 25-nucleotide-long oligonucleotides (18). These studies have shown that XMcm10 has similar affinities for ssDNA and dsDNA. Unlike SpMcm10, which harbors a single DNA-binding domain in the N-terminal half of the protein, XMcm10 seems to contain two distinct domains for binding DNA. The biological implication of having two DNA-binding domains is not clear.

It appears that there are differences in the quaternary structure of Mcm10 from different organisms. Although SpMcm10 and XMcm10 may be a homodimer in solution (17, 18), a recent electron microscopy study suggested that human hMcm10 has a hexameric ring structure (19). The same study reported that hMcm10 interacts with ssDNA but failed to bind dsDNA. The differences in structure and DNA binding properties may reflect differences in the function of Mcm10 in various organisms as well as in the protein preparations.

Here we report, for the first time, the characterization of the DNA binding properties of purified Mcm10 from S. cerevisiae. We show that ScMcm10 forms a stable complex with dsDNA and ssDNA. In addition, we demonstrate that dsDNA longer than 50 bp sustains oligomerization of ScMcm10. The number of ScMcm10 molecules bound is directly proportional to the size of the dsDNA, suggesting that ScMcm10 is tightly packed on the dsDNA, perhaps in a head to tail oligomeric structure. In contrast to a 25-bp-long dsDNA, which supports the binding of a single copy of ScMcm10, ssDNA containing only 20 nucleotides may sustain binding of as many as three copies of ScMcm10, suggesting that a ScMcm10 complex composed of at least 3 subunits assembles on ssDNA. We believe that these distinct binding properties to dsDNA and ssDNA are important for the ScMcm10 functions in initiation, formation of replication forks, and the maintenance of replication fork progression during chromosomal DNA replication.

**EXPERIMENTAL PROCEDURES**

**Expression of ScMCM10—Escherichia coli strain BL21-Codon Plus (DE3) pLysS/pDEST15-ScMCM10** cells were grown in the LB medium (2 liters) by shaking in a New Brunswick incubator-shaker at 37 °C until they reached a density of A600 = 0.8. Then isopropyl 1-thio-β-D-galactopyranoside was added (1 mM final concentration) to induce the expression of the ScMcm10 protein. Induction was performed for 2 h at 37 °C. Then, cells were harvested by centrifugation and resuspended (16 ml) in Buffer P plus 10% sucrose. The cell suspension was frozen in liquid nitrogen and stored in a freezer at −80 °C.

**Purification of the ScMcm10 Protein**—All operations were carried out at 0–4 °C. Frozen cells were thawed on ice. Extracts were obtained by first incubating the cells with lysozyme to generate spheroplasts essentially as previously described (21). After a 45-min period of incubation on ice, the cell suspension was supplemented with Nonidet P-40 and NaCl to 0.1% and 1 M final concentration, respectively. This was followed by further incubation on ice for additional 30 min. Then, the extracts were centrifuged for 50 min in a Sorvall centrifuge, with an SS34 rotor, to separate the soluble and insoluble fractions. The soluble fraction was diluted 5× with Buffer P and loaded onto a glutathione-Sepharose (Amersham Biosciences) column (4 ml) pre-equilibrated with Buffer P plus 0.2 M NaCl. After collecting the flow through, the column was washed with Buffer P plus 0.2 M NaCl (80 ml). Following the wash, the ScMcm10 protein was eluted with Buffer P plus 0.2 M NaCl plus 10 mM reduced glutathione. Fractions (4 ml each) containing the GST-ScMcm10 protein were identified by a Western blot using anti GST polyclonal antibody (Sigma). All fractions containing ScMcm10 were pooled and loaded on an S-Sepharose (Amersham Biosciences) column (1 ml) pre-equilibrated with Buffer P plus 0.2 M NaCl. Then the column was washed with Buffer P plus 0.2 M NaCl (20 ml), followed by a wash with Buffer P plus 1 M NaCl. The ScMcm10 protein was eluted in the high salt buffer. It was identified by a Western blot using anti GST antibodies, Coomassie Blue staining, and the ability to bind DNA as determined by the EMSA assay.

**Sedimentation Analysis on Glycerol Gradients**—Sedimentation analysis was performed in a linear 20 to 40% glycerol gradient (5 ml) that contained, in addition to glycerol, Buffer P plus enzyme. Expression of the GST-ScMcm10 fusion protein is under the control of the inducible T7 RNA polymerase promoter.

**Radioactively Labeled DNA Substrates**—DNA substrates were labeled in vitro with [γ-³²P]ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed using the bio-spin P-30 columns from Bio-Rad.

**Buffers**—Buffer P contained 20 mM sodium phosphate, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonfluryl fluoride, and a protease inhibitor mixture described before (20). Buffer H contained 50 mM Hepes, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol. Buffer T contained 50 mM Tris-HCl, pH 8.1, 10 μM ZnCl₂, 1 mM EDTA, 1 mg/ml BSA, and 20 mM β-mercaptoethanol. Buffer HBS contained 10 mM Hepes, pH 7.4, 3 mM EDTA, 0.15 M NaCl, and 0.05% Surfactant P20.

**DNA and Synthetic Oligonucleotides**—All DNA substrates used are described in the supplemental data.

**Growth of E. coli Cells**—E. coli BL21-Codon Plus (DE3) pLysS/pDEST15-ScMCM10 cells were grown in the LB medium (2 liters) by shaking in a New Brunswick incubator-shaker at 37 °C until they reached a density of A600 = 0.8. Then isopropyl 1-thio-β-D-galactopyranoside was added (1 mM final concentration) to induce the expression of the ScMcm10 protein. Induction was performed for 2 h at 37 °C. Then, cells were harvested by centrifugation and resuspended (16 ml) in Buffer P plus 10% sucrose. The cell suspension was frozen in liquid nitrogen and stored in a freezer at −80 °C.
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0.2 M NaCl. Sedimentation was carried out in a Beckman ultracentrifuge (SW 50.1 rotor) at 50,000 rpm, 4 °C, for 36 h. After centrifugation, we collected 0.2-ml fractions from the bottom of the tube. We then tested aliquots of each fraction for DNA binding. The remaining material of each fraction was precipitated by trichloroacetic acid, and the proteins were analyzed by SDS-PAGE, essentially as described before (20). In parallel, we performed an identical sedimentation analysis of the following molecular weight markers: BSA (67 kDa), aldolase (154 kDa), and catalase (232 kDa). We identified these proteins by their migration on an SDS-PAGE gel.

**DNA Binding Reactions**—We used DNA mobility shift assays in agarose gels to test the binding of purified ScMcm10 protein to dsDNA. A standard DNA binding assay for EMSA analysis (20 μl) contained Buffer H supplemented with BSA (1 mg/ml), 0.1 M NaCl, 0.05% surfactant P20), used interchangeably in the binding assay, had no effect on the binding of ScMcm10 to DNA.

**DNA Binding Analysis by Surface Plasmon Resonance**—SPR analysis was performed using the BIAcore T100. Biotinylated DNA probes were immobilized to the surface of a streptavidin-coated sensor chip (BIAcore type SA). The amounts of ligands (ScMcm10, Abf1p) were diluted at increasing concentrations (2–4 ng), and purified ScMcm10 protein (0.1–0.4 μg of the S-Sepharose fraction, unless indicated otherwise). The assays were performed in an ionic environment that does not exceed 0.15 M NaCl and analyzed by electrophoresis on agarose gels, essentially as described before (20). Buffer H, Buffer T, or Buffer HSB (10 mM Hepes, pH 7.4, 3 mM EDTA, 0.15 M NaCl, 0.05% surfactant P20), used interchangeably in the binding assay, had no effect on the binding of ScMcm10 to DNA.

**RESULTS**

**Purified ScMcm10 Binds dsDNA**—We cloned ScMCM10 from *S. cerevisiae* as a fusion with GST and expressed the protein in *E. coli*. We then purified the fusion protein using affinity and conventional chromatography techniques as described under “Experimental Procedures.” Purified GST-ScMcm10 fusion was subjected to sedimentation in glycerol gradients. Following the centrifugation, aliquots of the glycerol gradient
fractions were analyzed by SDS-PAGE electrophoresis. As shown in Fig. 1A, a tight peak of a major band identified by Coomassie Blue staining (indicated by the arrow) contains a protein of a predicted size (∼90 kDa) corresponding to the GST-ScMcm10 fusion. Additionally, minor, faster migrating bands are also visible. These bands reacted with anti GST polyclonal antibodies (data not shown), suggesting they represent proteolytic cleavage products of the GST-ScMcm10 fusion, whose identity was confirmed by mass spectroscopy (matrix-assisted laser desorption ionization time).

The sedimentation rate of the GST-ScMcm10 fusion relative to molecular weight protein standards suggests that the protein is a monomer in solution (Fig. 1B). Its peak separated from BSA (67 kDa) by a single fraction and migrated significantly slower than aldolase (154 kDa). Similar sedimentation profile was obtained when the glycerol gradient was performed in a buffer containing 1 M NaCl (data not shown). This result differs from previous reported finding, which suggested that purified ScMcm10-His6 is a homocomplex of ∼800 kDa sensitive to high salt (0.5 M NaCl) (24). The reason for this discrepancy is not clear, and it may stem from the different approaches used to purify the ScMcm10 protein. It is also possible that contaminating DNA influenced the apparent size of the ScMcm10-His6 fusion protein.

To test whether the purified fusion protein binds DNA, we incubated aliquots of the glycerol fractions with a 32P-labeled dsDNA-300 fragment corresponding to the ARS1501 DNA sequence (25). The reaction mixtures were then loaded on an agarose gel and analyzed as described under “Experimental Procedures.” It is readily apparent that DNA binding activity co-migrated with the peak of the ScMcm10 protein (Fig. 1C). The extent of the observed DNA mobility retardation was unexpected for a protein of ∼90 kDa that is a monomer in solution, suggesting that several fusion molecules may have bound to the DNA, forming a large protein-DNA complex.

ScMcm10 Molecules Cooperate in the Assembly of a Multimeric Complex on Duplex DNA—The purified ScMcm10 is fused to GST. It is important to show that the GST moiety does not bind DNA and is not responsible for the formation of the large GST-ScMcm10-DNA complex due to its oligomerization. Using a glutathione-Sepharose column, we showed that the binding of GST to glutathione and the binding of Mcm10 to DNA are separable and distinct (supplemental Fig. S1A). This protein-DNA interaction was disrupted by >0.2 M NaCl (supplemental Fig. S1B). Removal of GST from Mcm10 by tobacco etch virus protease cleavage did not interfere with Mcm10 binding to DNA nor the formation of the large protein-DNA complex by gel retardation assay (supplemental Fig. S1C).

Considering that the purified Sc-Mcm10 protein is a monomer in solution (Fig. 1, A and B), the substantial retardation of DNA mobility observed in Fig. 1C could be explained if a single DNA molecule is associated with several copies of the ScMcm10 protein. Furthermore, because ScMcm10 is basic (pl of −10.46), the protein bound to the DNA may also contribute to a substantial retardation of the electrophoretic mobility shift by neutralizing phosphate-negative charges of the DNA backbone.

To gain an insight into the process of assembly of the Scmcm10-DNA complex, we titrated the protein in the DNA binding reaction and analyzed the products of these reactions as described in supplemental Fig. S.2. If ScMcm10 binds the DNA randomly and independently, a direct and proportional relationship between the amounts of protein added and protein-DNA complex formed is expected. Therefore, the degree of the electrophoretic mobility shift of the ScMcm10-DNA nucleoprotein complex should depend on the amount of protein added to the DNA binding reaction.

This analysis was performed using 32P-labeled ARS1501-300 and ARS1–100 (supplemental Fig. S.2A, lanes 1–5 and lanes 6–10, respectively. It is evident that the relationship between the amount of ScMcm10-DNA complex formed and protein added is complex. In supplemental Fig. S.2A the results showed that the quantitative accumulation of the large protein–DNA complex occurred within a narrow range of ScMcm10 concentrations (lanes 3, 4, 9, and 10). We noted that a large ScMcm10-DNA complex was visible even when it comprised a small fraction of total DNA present (supplemental Fig. S.2A, lanes 3 and 9). Quantifying the amount of free and bound 32P-labeled DNA (large complex) showed that the disappearance of free DNA was concomitant with the appearance of a large protein–DNA complex fully retarded in the gel (supplemental Fig. S.2B). At a critical concentration of ScMcm10, a doubling of the amount of ScMcm10 in the reaction caused ~10-fold increase in the amount of Mcm10-DNA complex formed (supplemental Fig. S.2B). This quantitative relationship between the amount of fusion protein added and nascent nucleoprotein complex formed suggests that the binding of multiple copies of ScMcm10 to a single DNA molecule may be cooperative. This cooperativity implies that a DNA-bound ScMcm10 molecule may facilitate the binding of the next one, perhaps by protein–protein interactions. Alternatively, the interaction of ScMcm10 with the DNA may cause a conformational change in a proximal DNA sequence for which ScMcm10 may have an enhanced affinity.

If multiple copies of the ScMcm10 bind to the DNA as suggested by the experiment in supplemental Fig. S.2A, a substantial increase in mass of the nucleoprotein complex is expected. To test this prediction, we performed the experiment shown in supplemental Fig. S.2C. In this experiment 32P-labeled ARS1–100 was first incubated with ScMcm10 and then fractionated by gel filtration (Bioigel A-15m). DNA alone (in the absence of ScMcm10) migrated on the Biogel-A15m column as a broad, diffused band (supplemental Fig. S.2C). However, when first incubated with ScMcm10, the DNA eluted as a sharp peak that migrated faster than nX174 superhelical RFI DNA (3.55 × 106 kDa), thyroglobulin (660
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kDa), or catalase (232 kDa). This suggests a large Stokes radius for the DNA in the nucleoprotein complex, implying a significant increase in mass and perhaps a conformational change of the DNA in the complex.

ScMcm10 Binding to dsDNA Is DNA Sequence-independent—
In the DNA binding assays described above we used DNA substrates corresponding to \textit{S. cerevisiae} replication origins, ARS1501 and ARS1. To determine whether ScMcm10 binding requires a specific DNA sequence, we performed DNA competition experiments. In Fig. 2A, DNA binding assays were carried out in the presence of increasing amounts of competitor dsDNA. As expected, increasing amounts of a plasmid containing ARS1501 (pARS1501) effectively competed with the \textsuperscript{32}P-labeled ARS1501 substrate for the ScMcm10 protein (Fig. 2A, lanes 3–6). However, unrelated DNA, superhelical øX174 RFI, was equally effective in competing for ScMcm10 (Fig. 2A, lanes 7–10). In addition, to test the possibility that DNA topology may influence DNA binding, we performed the competition experiment with relaxed øX174 RFI, which yielded similar results (Fig. 3A, lanes 11–14). Taken together, these results suggest that binding of ScMcm10 protein to DNA is DNA sequence- and topology-independent.

ScMcm10 Binding to DNA Analyzed by SPR—SPR technology is designed for the study of macromolecular interactions by quantitatively defining both the kinetics and affinity of binding. To analyze ScMcm10 binding to DNA by SPR, biotinylated ARS1–400 DNA (harboring an Abf1p-binding site), ARS1–25, and ARS1–50 were prepared as described under “Experimental Procedures.” The amount of DNA ligand retained on the surface of the chip (RI, measured in RU units) was 296, 29, and 56 for ARS1–400, ARS1–25, and ARS1–50, respectively. B, real-time kinetics of Abf1p binding to ARS1–400. In this experiment we generated kinetic curves by applying different concentrations (0.1 to 50 nM, see “Experimental Procedures”) of Abf1p flowing through the chip containing ARS1–400 DNA (red curves). These curves were fitted with kinetic curves generated by an evaluation model (Langmuir) relying on a 1:1 interaction at surface (black curves). C, represents kinetic analysis of ScMcm10 binding to ARS1–25, as in B. D, represents kinetic analysis of ScMcm10 binding to ARS1–50, essentially as described in B. E, kinetic parameters calculated by a computer program based on the “fitted” kinetic curves (black) shown in B–D. The stoichiometry of Abf1p or ScMcm10 bound to DNA was calculated by the formula described under “Experimental Procedures.” A computer program calculated the amounts of Abf1p and ScMcm10 at saturation. These amounts (expressed as \( R_{\text{max}} \) units) were 98.3 in B, 62.7 in C, and 154.5 in D.

FIGURE 2. ScMcm10 binding to dsDNA analyzed by DNA competition experiments and by SPR. A, DNA binding reactions were performed as described under “Experimental Procedures.” The DNA substrate used was \textsuperscript{32}P-labeled ARS1501–300. To test DNA binding specificity, increasing amounts of competing DNA were added to the reaction mixtures prior to incubation and their loading on agarose gels. Competing DNA used was plasmid pARS1501 harboring ARS1501 DNA and øX174 RFI and RFI. B–E represent real-time kinetic analysis using SPR designed to determine the ScMcm10:DNA stoichiometry in the nucleoprotein complex. Sensor chips containing ARS1–400 DNA (harboring an Abf1p-binding site), ARS1–25, and ARS1–50 were prepared as described under “Experimental Procedures.” The amount of DNA ligand retained on the surface of the chip (RI, measured in RU units) was 296, 29, and 56 for ARS1–400, ARS1–25, and ARS1–50, respectively. B, real-time kinetics of Abf1p binding to ARS1–400. In this experiment we generated kinetic curves by applying different concentrations (0.1 to 50 nM, see “Experimental Procedures”) of Abf1p flowing through the chip containing ARS1–400 DNA (red curves). These curves were fitted with kinetic curves generated by an evaluation model (Langmuir) relying on a 1:1 interaction at surface (black curves). C, represents kinetic analysis of ScMcm10 binding to ARS1–25, as in B. D, represents kinetic analysis of ScMcm10 binding to ARS1–50, essentially as described in B. E, kinetic parameters calculated by a computer program based on the “fitted” kinetic curves (black) shown in B–D. The stoichiometry of Abf1p or ScMcm10 bound to DNA was calculated by the formula described under “Experimental Procedures.” A computer program calculated the amounts of Abf1p and ScMcm10 at saturation. These amounts (expressed as \( R_{\text{max}} \) units) were 98.3 in B, 62.7 in C, and 154.5 in D.
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The Stoichiometry of ScMcm10 Bound to dsDNA Determined by SPR Analysis—Previous studies showed that ScMcm10 forms a large complex with ARS1 (supplemental Fig. S.2), which suggested that multiple copies of ScMcm10 bind to the DNA. To understand the nature of this interaction we carried out a kinetic analysis of ScMcm10 binding to dsDNA of increasing size (supplemental Fig. S.4, a, c, e, and g). This analysis indicated that the theoretical curves obtained by the Langmuir 1:1 binding “fitting” model (supplemental Fig. S4, b, d, f, and h) did not fit well the ScMcm10 binding curves, suggesting that binding of ScMcm10 to dsDNA is more complex than a simple 1:1 interaction.

Additionally, we determined the stoichiometry of protein to DNA in the nucleoprotein complex, as before (Fig. 2). This examination revealed that the amount of ScMcm10 bound to dsDNA at saturation is directly proportional to the length of the dsDNA (Fig. 3A). These results suggest a tight packing of ScMcm10 on dsDNA fragments that are equal to or longer than 100 bp. Each ScMcm10 molecule appears to occlude on average 10 bp of DNA. The assay became saturated (or was close to saturation) when 25–50 nM ScMcm10 was used. The calculated kinetic parameters suggest slower association and dissociation rates for ScMcm10 relative to Abf1p. Based on a similar computation used in Fig. 2B a 0.6:1 and 1.3:1 stoichiometry of ScMcm10 to dsDNA-25 and dsDNA-50, respectively, was determined (Fig. 2, C and D). We therefore infer that a 25- to 50-bp-long duplex DNA fragment can sustain the binding of only one ScMcm10 molecule. However, the nucleoprotein complex formed with the dsDNA-50 ligand is substantially more stable than the complex formed with the shorter dsDNA-25 fragment as indicated by the apparent dissociation constants shown in Fig. 2E.

In this experiment we generated kinetic curves by applying different concentrations of Abf1p to a chip containing ARS1–400 DNA (Fig. 2B, red curves). These curves were fitted with kinetic curves generated by an evaluation model (Langmuir) relying on a 1:1 interaction at surface (Fig. 2B, black curves). The “fitting” model was used to calculate the kinetic parameters shown in Fig. 2E. The shape of the kinetic curves in Fig. 2B and the calculated apparent rate constants (k_{app} and k_{dapp}) suggest relatively fast association and dissociation rates for Abf1p with the DNA and the nucleoprotein complex, respectively. Additionally, the maximal amount of Abf1p bound to DNA at saturation, expressed as R_{max} was used to calculate the stoichiometry of protein to DNA in the nucleoprotein complex (see “Experimental Procedures”). The ARS1 DNA-400 contains a single Abf1p DNA-binding site, thus predicting a 1:1 stoichiometry. Indeed, the results shown in Fig. 2B revealed that Abf1p binds to ARS1–400 as predicted, confirming the validity of the approach and methodology we used in this analysis.

We then applied this approach to determine the stoichiometry of ScMcm10 binding to dsDNA-25 and dsDNA-50 fragments (Fig. 2, C and D). A known amount of DNA ligand, biotinylated at a single 5’ terminus, was immobilized on a streptavidin-coated sensor chip. The amounts of DNA ligand and protein analyte retained on the surface of the chip were determined and expressed as RUs (“Experimental Procedures” and Fig. 2B). The assay became saturated (or was close to saturation) when 25–50 nM ScMcm10 was used. The calculated kinetic parameters suggest slower association and dissociation rates for ScMcm10 relative to Abf1p. Based on a similar computation used in Fig. 2B a 0.6:1 and 1.3:1 stoichiometry of ScMcm10 to dsDNA-25 and dsDNA-50, respectively, was determined (Fig. 2, C and D). We therefore infer that a 25- to 50-bp-long duplex DNA fragment can sustain the binding of only one ScMcm10 molecule. However, the nucleoprotein complex formed with the dsDNA-50 ligand is substantially more stable than the complex formed with the shorter dsDNA-25 fragment as indicated by the apparent dissociation constants shown in Fig. 2E.
If cooperative oligomerization were true, pre-binding of ScMcm10 to the ARS1–400 DNA template, which contains an Abf1p DNA-binding site, should inhibit the binding of the Abf1p protein. Indeed, as shown in supplemental Fig. S.5(A and B), prior injection of ScMcm10 strongly inhibited the binding of Abf1p. When the order of injection was reversed, Abf1p injected first followed by ScMcm10, no inhibition of ScMcm10 binding to DNA was observed (supplemental Fig. S.5C).

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**ScMcm10 Interacts with DNA**

Studies in other systems suggested that Mcm10 can also bind ssDNA (16, 18, 19). To determine whether ScMcm10 binds to ssDNA, we performed DNA competition experiments (A). The DNA substrate used was 32P-labeled ARS1501-300. The competing DNA used were øX174 ssDNA, ssDNA-60, and ssDNA-21 oligonucleotides. Analysis of direct binding to ssDNA was performed by SPR. B–G represent real-time kinetics of ScMcm10 binding to ssDNA oligonucleotides of different length. In this experiment we generated kinetic curves by applying different concentrations (0.1 to 50 nm, see “Experimental Procedures”) flowing over a chip containing immobilized ssDNA-8 oligonucleotide (red curves). Similar experiments are presented in C–F, except that ssDNA-12, ssDNA-20, ssDNA-30, and ssDNA-40 were captured on the chips, respectively. These curves were fitted with kinetic curves generated by an evaluation model (Langmuir) relying on a 1:1 interaction at surface (black curves), as in Fig. 2. The amounts of captured ssDNA on a chip, measured in RU units, were as follows: 8, 8, 11, 17, and 23 in B–F, respectively. A computer program calculated the amounts of ScMcm10 bound at saturation. These amounts (expressed as R_max units) were 114 in C, 404 in D, 347 in E, and 451 in F. G, kinetic parameters calculated by a computer program based on the “fitted” kinetic curves shown in C–F. The stoichiometry of ScMcm10 bound to DNA was calculated by the formula described under “Experimental Procedures.”

**FIGURE 4. Assembly of a ScMcm10 multimeric complex on ssDNA.** To determine whether ScMcm10 binds to ssDNA, we performed DNA competition experiments (A). The DNA substrate used was 32P-labeled ARS1501-300. The competing DNA used were øX174 ssDNA, ssDNA-60, and ssDNA-21 oligonucleotides. Analysis of direct binding to ssDNA was performed by SPR. B–G represent real-time kinetics of ScMcm10 binding to ssDNA oligonucleotides of different length. B, in this experiment we generated kinetic curves by applying different concentrations (0.1 to 50 nm, see “Experimental Procedures”) flowing over a chip containing immobilized ssDNA-8 oligonucleotide (red curves). Similar experiments are presented in C–F, except that ssDNA-12, ssDNA-20, ssDNA-30, and ssDNA-40 were captured on the chips, respectively. These curves were fitted with kinetic curves generated by an evaluation model (Langmuir) relying on a 1:1 interaction at surface (black curves), as in Fig. 2. The amounts of captured ssDNA on a chip, measured in RU units, were as follows: 8, 8, 11, 17, and 23 in B–F, respectively. A computer program calculated the amounts of ScMcm10 bound at saturation. These amounts (expressed as R_max units) were 114 in C, 404 in D, 347 in E, and 451 in F. G, kinetic parameters calculated by a computer program based on the “fitted” kinetic curves shown in C–F. The stoichiometry of ScMcm10 bound to DNA was calculated by the formula described under “Experimental Procedures.”

**ScMcm10 Binds ssDNA**—Studies in other systems suggested that Mcm10 can also bind ssDNA (16, 18, 19). To determine whether ScMcm10 can also bind ssDNA, we used DNA competition as shown in Fig. 4A. Results in Fig. 4A, lanes 3–6, clearly demonstrated that øX174 viral ssDNA circles effectively competed with ARS1501-300 dsDNA for the binding of the ScMcm10 protein. Furthermore, ssDNA-60 (60 nucleotides long) also inhibited the binding of ScMcm10 to the ARS1501-300 dsDNA (Fig. 4A, lanes 7–10). However, a short oligonucleotide containing 21 nucleotides, ssDNA-21, was not an effective competitor. (Fig. 4A, lanes 11–14). These results suggest that ScMcm10 may not be able to interact stably with short oligonucleotides.

We decided to test the binding of ScMcm10 to short oligonucleotides of increasing size by using SPR as a direct DNA binding assay. Biotinylated oligonucleotides were immobilized on sensor chips and subjected to a flow of ScMcm10 at different concentrations. As shown in Fig. 4B, ScMcm10 binding to an
immobilized ssDNA-8 ligand was barely detectable. Increasing the size of the ligand by four nucleotides, ssDNA-12, sustained the binding of ScMcm10 (Fig. 4C). The calculated stoichiometry of ScMcm10 in the nucleoprotein complex was 0:8:1, suggesting a 1:1 interaction. However, the complex formed was relatively unstable based on the increased slope of the kinetic curves during the dissociation phase of the kinetic analysis and the larger apparent dissociation constant relative to the dissociation constants of complexes with longer ssDNA ligands (Fig. 4G). The most stable complexes were formed when ScMcm10 interacted with ssDNA containing more than 20 nucleotides (Fig. 4G). Remarkably, in contrast to ssDNA-12, the ScMcm10:DNA stoichiometry in the nucleoprotein complexes containing longer oligonucleotides (ssDNA-20, ssDNA-30, ssDNA-40, and ssDNA-50) was ~3:1 (Fig. 4, D–F, and supplemental Fig. S6). The stoichiometry of ScMcm10:ssDNA changed to ~6:1 for ssDNA-100 (100 nucleotides long). We infer from these results that ssDNA containing at least 20 nucleotides induces the assembly of a multimeric (at least trimeric) complex of the ScMcm10 protein.

**DISCUSSION**

The three-dimensional structure of the ssDNA-binding domain of XMcm10 has recently been determined by crystallography to be located within 200 conserved residues of the internal domain (30). This DNA binding surface forms an OB-fold/zinc loop that contains clusters of basic residues. To better understand how Mcm10 functions in DNA replication we undertook a biochemical characterization of the ScMcm10 protein. We showed that ScMcm10 has distinct DNA binding properties for single- and double-stranded DNA. DNA competition experiments suggested that the DNA-binding domains for ssDNA and dsDNA are likely overlapping.

The physical characterization of the protein showed that ScMcm10 is a monomer in solution and that the purified protein binds DNA in a sequence-independent manner. Two approaches, EMSA and SPR, were used for the analysis of ScMcm10-DNA interactions. Kinetic analysis suggested a relatively slow off rate for the ScMcm10 protein when bound to the DNA, consistent with the stability of this complex in EMSA. Both, EMSA and SPR analysis suggested that ScMcm10 molecules cooperate to form a tightly packed nucleoprotein complex on dsDNA that may involve protein-protein interactions. Interactions between Mcm10 molecules have been documented in previous studies (6, 24). The direct and tight correlation between the length of the dsDNA and the number of ScMcm10 molecules bound suggests that the protein may align along the helical axis of the dsDNA.

The properties of ScMcm10 binding to ssDNA suggest the assembly of a distinct structure, different from the nucleoprotein complex with dsDNA. The shortest oligonucleotide we identified to form a semi-stable complex with ScMcm10 was 12 nucleotides long. In agreement with our study, 10 nucleotides was the reported minimal length of ssDNA required for binding of the XMcm10 internal domain (30). Although it is possible to observe interactions of ScMcm10 with the ssDNA-12 substrate, we found that the most stable nucleoprotein complex is formed with ssDNA ligands longer than 20 nucleotides (Fig. 4). The analysis of ScMcm10:ssDNA stoichiometry was most revealing. While it appears that only one copy of ScMcm10 binds to a single ssDNA-12 oligonucleotide, ssDNA-20 and longer DNA templates form a more stable complex that sustain the binding of three copies of ScMcm10. In contrast to ssDNA, only one ScMcm10 molecule bound to a 25- or 50-bp double-stranded DNA. Therefore, it is spatially unlikely that the three copies of Mcm10 align along the length of the 20 nucleotides of ssDNA. More likely, ScMcm10 forms a multimeric complex of three subunits on ssDNA.

What might be the significance of the two different binding modes of Mcm10 for single- and double-stranded DNA? We speculate that the distinct properties of ScMcm10 binding to dsDNA and ssDNA may be directly linked to its multiple roles in DNA replication. Based on the findings described in this report and other published results, we propose a working hypothesis for how ScMcm10 may function in DNA replication (Fig. 5). Previous studies have shown that ScMcm10 is recruited to replication origins in a cell cycle-dependent manner at the G1 to S transition, following the assembly of the Pre-RC complex (12). The ordered assembly of the pre-RC begins with the ORC protein binding to its recognition site at the origin followed by the recruitment of the Mmc2–7 replication helicase (9, 10). It is unclear at what point and via what mechanism Mmc2–7 becomes topologically linked to origin DNA to initiate DNA unwinding.
The mechanism for the recruitment of Mcm10 in S. cerevisiae is also unclear. Because Mcm10 protein does not recognize specific DNA sequences, the origin-specific recruitment of Mcm10 is likely mediated through its interaction with either Mcm2–7 or ORC. Upon initiation of DNA replication, Mcm10 and Mcm2–7 colocalize to replication forks in vivo (12), suggesting that Mcm10 enters the chromatin at origins of replication via its interaction with Mcm2–7. We propose that, at the origin of replication, ScMcm10 binds both the Mcm2–7 and DNA, bridging the helicase to the DNA. We also suggest that additional ScMcm10 molecules are targeted to the origin by virtue of the bound ScMcm10, leading to the oligomerization of ScMcm10 on the DNA as shown in Fig. 5.

In bacteria, the initiator protein DnaA recognizes specific sequences at OriC and assembles to form a multimeric complex (20–40 molecules). In the presence of ATP, torsional strain is introduced by wrapping DNA around this complex thereby unwinding the flanking AT-rich 13-mer repeats to form a replication bubble (31–33). The S. pombe ORC protein has been suggested to fulfill an analogous function for DnaA (34), although similar evidence is lacking for ScORC (10). Regardless of the role of ORC in this unwinding process, synergistic torsion of origin DNA may be necessary for its eventual melting. We speculate that the cooperative assembly of a multimeric ScMcm10 complex along the length of origin DNA may provide multiple contacts to destabilize the duplex DNA with its basic charges. It is possible that the incoming Cdc45 and GINS complex may aid ScMcm10 in this process as well (12). Consistent with this hypothesis is the timing of the entry of ScMcm10 into chromosomal sites at replication origins. This entry follows the assembly of the pre-RC complex and precedes the unwinding of replication origins (12) as reported for XMcm10 in the early stage of origin unwinding during the replication of a plasmid in a Xenopus egg-cell extract (4).

It is well established that the Mcm2–7 complex is localized to the replication forks during chromosomal replication (12, 35). However, a key point, poorly understood, is the mechanism of the initial delivery of Mcm2–7 to the open initiation bubble. We suggest that ScMcm10 in a complex with Mcm2–7 may fulfill this function by virtue of its affinity for single-stranded DNA (Fig. 5). We imagine that ScMcm10 plays a critical role in both the melting of origin DNA to form a single-stranded bubble and the delivery of the Mcm2–7 helicase to the unwound DNA. We suggest that, during this transition, ScMcm10 assembles and functions at the replication fork as a multimeric complex containing at least three subunits. We imagine that upon transfer of the ScMcm10-Mcm2–7 complex to the replication forks, recruitment of DNA polymerase-α and replisome assembly ensues and initiation of DNA synthesis takes place. The predictions of this working hypothesis are testable by future genetic and biochemical experiments.

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