Fission Yeast Mcm10p Contains Primase Activity

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Although Mcm10p is a conserved essential component in eukaryotes required for both the initiation and elongation of DNA chains, its biochemical properties are unknown. Here, we report that the Schizosaccharomyces pombe fission yeast Mcm10 protein contains primase activity. Primases are enzymes that synthesize RNA primers on single-stranded DNA templates that are extended by DNA polymerases. In keeping with this property, Mcm10p supported oligoribonucleotide synthesis of short RNA primers (preferentially initiating synthesis on a dT template) that were extended with dATP by Escherichia coli DNA polymerase I. The C terminus of Mcm10p synthesized RNA, but less efficiently than the full-length protein at low rNTP levels. Mcm10p homologs contain a C-terminal motif found in proteins that polymerize nucleotides. A point mutant within this motif of S. pombe Mcm10p was defective in primer synthesis in vitro, and this mutant failed to support growth in vivo, suggesting that the primase activity of Mcm10p may be essential for cell viability.

DNA replication occurs through a complex series of reactions that are mechanistically coordinated at the replication fork. Studies of model DNA replication systems indicate that origin recognition by an initiator protein permits assembly and activation of the replicative helicase at the origin. Helicases unwind duplex DNA to generate a single-strand template on which primases can initiate synthesis of RNA primers that are 4–20 nucleotides in length and that are extended by DNA polymerases to make Okazaki fragments (1). Primases are physically coupled to the replicative helicases and DNA polymerases, which translocate together through the duplex, resulting in DNA unwinding coincident with synthesis of RNA primers and their extension with deoxynucleotides.

In eukaryotes, the Mcm proteins are essential replication factors that were identified as proteins required for minichromosome maintenance in a genetic screen for mutants defective in initiation of replication (2). Six of these members are sequence-related proteins, Mcm2-7, which interact to form a hexameric complex. Although a substantial body of data suggests that the Mcm2-7p complex acts as the replicative helicase (3), helicase activity has been detected only in the Mcm4-6-7 subcomplex (4–6). Prior to the initiation of replication, the Mcm2-7 complex associates with the initiator at replication origins. At the G1/S transition, both S phase cyclin-dependent kinase and Cdc7p-Dbf4p kinase activities rise, promoting the maturation of the pre-replicative complex to the preinitiation complex (7). Phosphorylation of the chromatin-bound Mcm2-7 complex by the Cdc7-Dbf4 kinase (8) is thought to trigger the binding of Mcm10p, Cdc45p, and the GINS complex with origins prior to the initiation of replication (9, 10).

Although Mcm10p was identified in the same genetic screen as the Mcm2-7 proteins, their amino acid sequences are unrelated. Studies in Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster, and Xenopus laevis indicate that Mcm10p is an essential conserved replication component. Mutations in mcm10 result in a marked reduction in the initiation of replication as well as a delay in S phase after hydroxyurea arrest, suggesting that Mcm10p is involved in initiation as well as elongation steps of replication (11–14). Mcm10p was shown recently to associate with origins in a cell cycle- and Mcm4p-dependent manner and with origin distal sequences during S phase (15). Mcm10p interacts genetically and biochemically with other replication factors, including the Mcm2-7 complex; origin recognition complexes Cdc45p; Dna2p; and the subunits of DNA polymerases (pol)3 α, δ, and ε; and S. pombe Dfp1p-Hsk1p (Cdc7p-Dbf4p) (14, 16–21). In both yeast and Xenopus, Mcm10p is required for the loading of Cdc45p onto chromatin (22–24).

Previously, we reported that, in vitro, S. pombe Mcm10p (amino acids 1–593; SpMcm10p) binds preferentially to single-stranded DNA (ssDNA) and to the large subunit of the pol α-prime complex and activates its DNA synthetic activity (25). In addition, we found that Mcm10p facilitates the binding of the pol α-prime complex to primed DNA and forms a stable ternary complex, suggesting that Mcm10p recruits the pol α-prime complex to template DNA. In keeping with these in vitro findings, Mcm10p was shown recently to stabilize the large subunit of the pol α-prime complex in S. cerevisiae (15) as well as the chromatin association of the pol α-prime complex in S. pombe (26).

In this study, we demonstrate that full-length SpMcm10p (amino acids 1–593) and its C-terminal fragment (amino acids 416–593) contain primase activity that catalyzes the synthesis of oligoribonucleotides that are extended by Escherichia coli pol I. Primase activity both co-sedimented and co-eluted with these full-length and truncated proteins, although their hydro-

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3 The abbreviations used are: pol, DNA polymerase; SpMcm10p, S. pombe Mcm10p; ssDNA, single-stranded DNA; NTD, nucleotide transfer domain; MES, 4-morpholineethanesulfonic acid.
dynamic properties differ significantly. The C-terminal domain of Mcm10p (and its homologs in other organisms) contains conserved acidic residues characteristic of proteins that polymerize nucleotides (the nucleotide transfer domain (NTD)) (27). A single amino acid substitution in one of these residues in full-length Mcm10p greatly reduced its primase activity (>10-fold). Further analysis of single amino acid substitutions of these conserved acidic residues suggests that they are essential for viability in yeast.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mcm10 Proteins**—Mcm10 proteins were overproduced in *E. coli* strain BL21(DE3)RIL containing various pET28a-MCM10 expression plasmids. Construction of plasmids, growth and lysis of cells, and purification of proteins by nickel-nitrilotriacetic acid affinity were described as (25) with the following exceptions. To obtain higher yields of protein, cells were grown in medium (4 liter) containing 100 ml of 10× M9 medium (10 g/liter NH4Cl, 30 g/liter KH2PO4, and 60 g/liter Na2HPO4), 200 ml of 5× medium (50 g/liter Tryptone, 25 g/liter yeast extract, and 25 g/liter NaCl), 20 ml of 20% glucose, 1 ml of 1 M MgSO4, and 40 μg/ml each kanamycin and chloramphenicol. Cell lysates were centrifuged at 10,000 rpm for 10 min; the supernatant was adjusted to 50% saturation with (NH4)2SO4, and the suspension was stirred for 20 min at 4 °C. The precipitate was collected at 18,000 rpm for 60 min and dissolved in lystate buffer. Mcm10p was bound to nickel-nitrilotriacetic acid beads, and the imidazole eluate was adjusted to 250 mM NaCl with Buffer A (25 mM MES-KOH (pH 6.5), 5% glycerol, 10 mM magnesium acetate, 0.1% Nonidet P-40, 2 mM EDTA, 1 mM dithiothreitol, 20 μM ZnSO4, and 1 mM phenylmethylsulfonyl fluoride). This material was loaded onto a 1-ml Mono S HR 5/50 column equilibrated with Buffer A containing 250 mM NaCl and eluted with a 6-ml gradient of 200–800 mM NaCl. Mcm10p eluted between ~400 and 500 mM NaCl (yield of 2.0 mg of protein). A portion of the purified Mono S fraction (0.40 mg) (see Fig. 1A, lane 1) was used for subsequent analysis. Mcm10p-(1–303) and Mcm10p-(416–593) were purified similarly, except that Mcm10p-(416–593) was isolated using a 1-ml Mono Q HR 5/50 column in place of the Mono S column. The yields of protein were 0.5 and 1 mg from 2 liter of medium, respectively.

**Primase Assay of Oligoribonucleotide Synthesis**—Primase activity was determined by measuring the amount of oligoribonucleotide synthesized in the presence of a DNA template. The yields of protein were 0.5 and 1 mg from 2 liter of medium, respectively. The reactions in Fig. 4 were stopped at the times indicated by heat inactivation at 95 °C for 5 min and chilled on ice. Calf intestine alkaline phosphatase (1 unit) was added, and the mixture was incubated for 30 min at 37 °C. Reactions were terminated by the addition of 3 μl of sequencing dye (98% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, and 0.1% bromphenol blue), heated to 98 °C for 5 min, and separated on a 25% denaturing polyacrylamide sequencing gel containing 7 M urea. Labeled oligoribonucleotide products were visualized using a Fuji BAS 1000 bioimaging analyzer and quantitated from a phosphorimage of the gel.

** Primer-dependent DNA Synthesis Assay**—Oligoribonucleotides synthesized by full-length Mcm10p, Mcm10p-(416–593), or Mcm10p-(1–303) were assayed by measuring their ability to serve as primers for the *E. coli* pol I large fragment (Klenow). The conditions used were the same as those described for the primase-catalyzed oligoribonucleotide synthesis assay, except for the following additions: 0.1 mM dATP, 0.08 unit of Klenow fragment, 1.5 μCi of [α-32P]dATP in place of radiolabeled ATP, and 0.1 μM (dT)100 in place of (dT)50. The longer dT template supported ~10 times more dAMP incorporation than the shorter template. Reaction mixtures were incubated for 40 min at 37 °C, after which half of the reaction mixture was mixed with 10 μl of alkaline loading dye (0.5 mM NaOH, 7 mM EDTA (pH 8.0), 0.5% Ficoll, 0.1% xylene cyanol, and 0.1% bromphenol blue), and DNA products were separated on 2% alkaline agarose gels. The amount of nucleotide incorporated into DNA was measured as described (25) or by using a phosphorimage of the gel.

**Computer-assisted Analysis of Amino Acid Sequences**—The data used in this study were from the NCBI Database; the protein sets were encoded in publicly available completely sequenced genomes. Initially, we used P-BLAST to identify Mcm10p amino acid sequences from different organisms with blast hits above a certain expectation value (e-value). Eleven full-length Mcm10p amino acid sequences from different divergent organisms found by the BLAST search were then aligned using the ClustalW program. This program produces biologically meaningful multiple sequence alignments of divergent sequences. The ClustalW program allows putative motifs to be identified because of the calculated best match for a large group of related selected sequences and lines them up so that the identities, similarities, and differences can be seen. The following is a list of the Mcm10 protein sequences used in the alignment and their accession numbers: *X. laevis* (frog) Mcm10p (AAG38358), *Homo sapiens* MCM10 (BAB18723), *S. cerevisiae* (budding yeast) Mcm10p (CAA86128), *Rattus norvegicus* (rat) MCM10 (XP_225570), *D. melanogaster* (fly) MCM10 (AAF53976), *Neurospora crassa* (bread mold) Mcm10-like protein (EAA31137), *Mus musculus* (mouse) MCM10 (NP_081566), *Caenorhabditis elegans* (worm) MCM10-like protein (CA57990), *Anopheles gambiae* (malaria mosquito) Mcm10-like protein (EAA10289), and *Plasmodium yoelii* yoelii (rodent malaria) Mcm10-like protein (EAA17050).

**Construction of Mcm10p Point Mutations**—The MCM10 shuffle strain used in this study was ILY251 (MATa leu2-3,112, ura3-52, mcm10::hisG (pRS315-MCM10-URA3)) (a gift from Bik Tye), and the wild-type *S. cerevisiae* Mcm10p strain used was JPY9 (MATa his3-D200, ura3-52, leu2-D1, trp1D63, gal4D11). Plasmids pET-Mcm10p(E586A), pET-Mcm10p(E586G), pET-Mcm10p(E586R), pET-Mcm10p(E586Q), pET-Mcm10p(E586D), pET-Mcm10p(E586K), pET-Mcm10p(E586S), pET-Mcm10p(E586N), pET-Mcm10p(E586C), pET-Mcm10p(E586L), pET-Mcm10p(E586V), pET-Mcm10p(E586I), pET-Mcm10p(E586W), pET-Mcm10p(E586F), pET-Mcm10p(E586H), pET-Mcm10p(E586T), pET-Mcm10p(E586P), pET-Mcm10p(E586A) were transformed into the MCM10 shuffle strain. 

**Mcm10p Possesses Primase Activity**
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A

B

C

poly(dT)₅₀

(1-593) pmol

ATP pmol

AMP pmol

inc. lanes

- 2 5

- 2 5

- 2 5

100μM 100μM 10nM

0 0 0

0 0 0

1 2 3 4 5 6 7 8 9

poly(dT)₅₀

(1-593) pmol

(1-203) pmol

2.5 25 100 100

ATP (μM)

1 2 3 4 5 6 7 8 9

Lanes
Mcm10p(D587A), and pET-Mcm10p(D588A), expressing Mcm10p containing Ala in place of Glu at position 586 or in place of Asp at position 587 or 588, respectively, were constructed by PCR mutagenesis of the pET28a-MCM10 plasmid. The presence of the mutated site was screened by restriction digestion with BstUl and by sequencing to verify specific changes. Mutated plasmids were digested either with NdeI and NotI or with BamHI and XhoI, and 1.7-kb fragments were cloned either into the yeast- E. coli shuttle vector pRSSG5415 (LEU2) containing the SGS1 promoter (a gift from Steve Brill) and digested with NdeI and NotI or into the vector pRSADH425 (LEU2) containing the strong alcohol dehydrogenase promoter and digested with BamHI and XhoI. Both shuttle vectors expressing mutant and SpMcm10p were examined in the plasmid shuffle assay.

RESULTS

Mcm10p Catalyzes Template-dependent Synthesis of Oligoribonucleotides—We examined whether SpMcm10p and its N- and C-terminally truncated derivatives, Mcm10p-(1–303) and Mcm10p (416–597), catalyze template-dependent oligoribonucleotide synthesis. The purity of these preparations, which were expressed in E. coli and purified, is shown in Fig. 1A (lanes 1–3). These protein preparations were screened for their ability to synthesize small oligoribonucleotides using [α-32P]ATP and single-stranded (dT)50 as the template. In the presence of ATP (100 μM), (dT)50, and full-length SpMcm10p, RNA products 4–18 nucleotides in length were produced (Fig. 1B, lanes 5 and 6). In the absence of an ssDNA template or in the presence of a low level of ATP (0.01 μM), oligoribonucleotide synthesis was not observed (Fig. 1B, lanes 2 and 3 and lanes 8 and 9, respectively). Quantitation of the amount of RNA formed indicated that the purified SpMcm10p supported incorporation of 0.3 pmol of AMP/min/pmol of protein at 37 °C. This level of activity is comparable with that observed with the phage T7 primase (30) and found that the activity curve was sigmoidal with respect to protein concentration, in contrast to that observed with full-length Mcm10p. The possible significance of this result is discussed further below.

Oligoribonucleotide synthesis catalyzed by Mcm10p-(416–593) (Fig. 2A). The primase activity of the Mcm10p derivatives (1), lanes 7–9, required added ssDNA for RNA synthesis (lanes 15–17). Whereas full-length Mcm10p supported oligoribonucleotide synthesis in the presence of ATP at 2.5 μM and higher, the truncated protein Mcm10p-(416–593) required at least 25 μM ATP. Significant differences in the length of RNA products formed by full-length Mcm10p and Mcm10p-(416–593) were noted. At all levels of protein tested, full-length Mcm10p synthesized products 5–18 nucleotides in length, whereas high levels of Mcm10p-(416–593) accumulated significantly longer products (Fig. 1C). Quantitative analysis of the RNA products formed at different levels of added ATP indicated that full-length Mcm10p had a higher specific activity compared with Mcm10p-(416–593) in the presence of 2 pmol of protein (supplemental Fig. 1A), whereas in the presence of 5 pmol of protein, the truncated derivative was five times more active than full-length Mcm10p (Fig. 1, compare B, lane 6, and C, lane 13). These results indicate that the residues between positions 416 and 593 of Mcm10p encode an RNA polymerase activity, but that the N-terminal regions of Mcm10p may play a role in regulating both the length and efficiency of oligoribonucleotide synthesis at low ATP levels. A recent study with the E. coli DnaG primase catalytic domain showed similarly that this region synthesizes primers whose abundance and lengths differ from those synthesized by the full-length primase (29). We titrated Mcm10p-(416–593) in the direct primase assays and found that the activity curve was sigmoidal with respect to protein concentration, in contrast to that observed with full-length Mcm10p. The possible significance of this result is discussed further below.

RNA Primers Formed by Mcm10p Support DNA Synthesis—The biological role of oligoribonucleotides synthesized by primase is to serve as primers for DNA polymerases that are incapable of initiating DNA chains de novo. To evaluate whether oligoribonucleotides formed by Mcm10p support DNA synthesis, we used the E. coli pol I-primase-coupled assay (30). In this assay, which was carried out with (dT)300p, the Klenow fragment-catalyzed incorporation of [α-32P]dATP is totally dependent on the oligoriboadenylate primers formed by primase. As shown in Fig. 2 (A and B), both full-length Mcm10p and the truncated derivative supported dAMP incorporation and formation of DNA chains 1–1.5 kb in length. Poly(dA) synthesis required the addition of ATP, (dT)300p either full-length Mcm10p or Mcm10p-(416–593), and the Klenow fragment (data showing the latter requirement not shown). In keeping with the results shown in Fig. 1C, full-length Mcm10p (amino acids 1–593) supported significantly more DNA synthesis at −10-fold lower levels of ATP compared with Mcm10p-(416–593) (Fig. 2B). The primase activity of the Mcm10p derivatives and the S. pombe p48–58 primase complex (normally associ-
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FIGURE 2. Mcm10p supports primer-dependent DNA synthesis catalyzed by the Klenow fragment. A, influence of ATP concentration on the size and amount of poly(dA) synthesized. The reaction mixtures used to measure primer-dependent DNA synthesis (see "Experimental Procedures") and containing (dT)\textsubscript{300} and either Mcm10p (2 pmol) or Mcm10p-(416–593) (2 pmol) and various levels of ATP as indicated were subjected to electrophoresis on a 2% alkaline agarose gel. B, quantitation of the level of dAMP incorporation (inc.) observed in A. Analysis was carried out as described under "Experimental Procedures." C, comparison of the level of primer-dependent replication activity observed in the presence of increasing levels of Mcm10p, Mcm10p-(416–593), and the p48-p58 complex. Reaction conditions were as described for A.

ated with the p180-p70 complex of pol α-primase) were compared using the coupled Klenow extension assay. As shown in Fig. 2C, full-length Mcm10p and Mcm10p-(416–593) were 5- and 12-fold less active, respectively, than the p48-p58 complex. These findings indicate that the Klenow fragment extends oligoriboadenylate chains formed by the Mcm10 proteins. When the extension assay was carried out in reactions containing the S. pombe pol α-primase subcomplex and the S. pombe pol δ or δ holoenzyme (including S. pombe replication factor C and S. pombe proliferating cell nuclear antigen) in lieu of the E. coli Klenow fragment, low but significant DNA synthesis was detected (supplemental Table 1).

Physical Properties of Full-length Mcm10p, Mcm10p-(1–303), and Mcm10p-(416–593)—To characterize the hydrodynamic properties of full-length Mcm10p and its truncated derivatives and to establish whether they contain intrinsic primase activity, purified full-length Mcm10p, Mcm10p-(1–303), and Mcm10p-(416–593) were subjected to Superdex 200 gel filtration chromatography and glycerol gradient centrifugation. Fractions eluted from the size column were examined for primase activity in the coupled polymerase assay. Both full-length Mcm10p and Mcm10p-(416–593) eluted as a single protein peak that was largely coincident with the peak of primase activity, whereas fractions containing Mcm10p-(1–303) were devoid of this activity (Fig. 3A). The results obtained with glycerol gradient fractions indicated similarly that full-length Mcm10p and Mcm10p-(416–593) both eluted with a single peak of activity that was coincident with the peak of primase activity, whereas fractions containing Mcm10p-(1–303) were devoid of activity (Fig. 3B) (data not shown). These findings suggest that both full-length Mcm10p and Mcm10p-(416–593) intrinsically contain primase activity.
Full-length Mcm10p displayed distinct properties in the two different sizing procedures used. The activity eluted from the gel filtration column with a relative molecular mass of 220 kDa, whereas the activity sedimented through a glycerol gradient at 70 kDa. The reason for the differential behavior during these two sizing steps is likely due to an irregularity in shape, which can cause a protein to be more excluded than spherical standards during gel filtration and centrifugation.

**FIGURE 3. Analysis of the hydrodynamic properties of Mcm10p and truncated derivatives.**

A, gel exclusion chromatography of various Mcm10p preparations. Purified fractions (200 μl, 2 mg/ml) of full-length SpMcm10p, Mcm10p-(1–303), or Mcm10p-(416–593) were subjected to Sephadex 200 gel filtration (see "Experimental Procedures"). Even-numbered fractions 34–60 (0.5 ml) were assayed for primer-dependent DNA synthesis (upper panel) and analyzed by SDS-PAGE (lower panel). The peak positions at which standard protein markers eluted from the column are indicated at the bottom: thyroglobulin (Thy; 670 kDa), ferritin (Fer; 440 kDa), catalase (Cat; 220 kDa), bovine serum albumin (BSA; 66 kDa), and ovalbumin (Ova; 45 kDa). B, glycerol gradient centrifugation of full-length SpMcm10p. Mcm10p was sedimented through a 15–35% glycerol gradient. Even-numbered fractions (0.5 ml) collected from the bottom of the gradient were assayed for primer-dependent DNA synthesis (upper panel) and subjected to SDS-PAGE analysis (10 μl) (lower panel). The sedimentation positions of standard protein markers are indicated at the top: catalase, aldolase (Aldo), bovine serum albumin, ovalbumin, and cytochrome c (CytC). Ld, load. C, summary of hydrodynamic properties. The Stokes radii and sedimentation values were determined from the elution properties of Mcm10p, Mcm10p-(1–303), and Mcm10p-(416–593) from a Sephadex 200 PC 3.2/30 column (see A) and after glycerol gradient sedimentation. The sedimentation profile of Mcm10p is shown (B), whereas those observed with Mcm10p-(1–303) and Mcm10p-(416–593) are not shown. The Stokes radii of the indicated proteins were determined by plotting the (−log Kav)1/2 versus the Stokes radius (Å). The molecular mass markers shown in A are listed, and their Stokes radii were as follows: thyroglobulin (85 Å); ferritin (65 Å), catalase (52 Å), bovine serum albumin (35 Å), and ovalbumin (28 Å). The Siegel-Monty equation (31), which depends on the s value and the Stokes radius, was used to calculate the apparent molecular mass of each protein.
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Sediment more slowly during centrifugation (31). The Siegel-Monty equation (31), which employs both the sedimentation value and Stokes radius, was used to calculate the apparent molecular mass of Mcm10p as well as its truncated derivatives (Fig. 3C). The apparent molecular masses of Mcm10p (120 kDa) and Mcm10p-(1–303) (74 kDa) are close to the values expected for a dimer (133 and 70 kDa, respectively), whereas that of Mcm10p-(416–593) (26 kDa) was
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Mcm10p-catalyzed Oligoribonucleotide Synthesis Is Influenced by the Template—Primases usually synthesize oligoribonucleotides complementary to template ssDNAs (1). However, prokaryotic primases support higher rates of oligoribonucleotide synthesis in the presence of specific DNA sequences called primase recognition sites. Although eukaryotic primases lack stringent requirements for such sites, they display sequence preferences. Primase initiation site selection depends on the reaction conditions used and appears to be more random at high rNTP concentrations and/or in the presence of Mn2+. For this reason, oligoribonucleotide synthesis with Mcm10p and the S. pombe p48-p58 primase complex was examined in the presence of (dT)50, (dC)50, and M13mp18 ssDNA at various rNTP levels (only with Mg2+). We examined the rate of oligoriboadenylate synthesis in the presence of (dT)50 and the S. pombe p48-p58 primase complex or full-length Mcm10p (Fig. 4A). In these experiments, the concentration of ATP was 100 μM, and the products formed were subjected to calf intestine alkaline phosphatase digestion. This treatment altered both the size and distribution of the oligoriboadenylate products formed (compare Figs. 1B and 4A) and prevented the detection of products smaller than 8 nucleotides. The aberrant migration of small RNAs due to calf intestine alkaline phosphatase treatment has been noted previously (32) (Fig. 4F). These conditions facilitated the synthesis by the p48-p58 primase complex of short RNA products, which were not detected in reactions containing 25 μM ATP (compare Fig. 4A and supplemental Fig. 1B). The rate of oligoribonucleotide formation by Mcm10p was slower than that by p48-p58 (Fig. 4, A and C). In the presence of p48-p58, the synthesis of oligoriboadenylate was detected 1 min after enzyme addition and plateaued after 10 min of incubation. In contrast, product formation with Mcm10p showed a pronounced lag of 5 min and then increased linearly up to 40 min. The size distribution of the products formed differed as well; products formed with Mcm10p were more varied in length than those synthesized by the p48-p58 complex.

Even at a high GTP concentration (100 μM), reactions that contained (dC)50 produced low levels of oligomers in the presence of Mcm10p (Fig. 4, B and D), in keeping with observations made at lower nucleotide levels (supplemental Fig. 1B). However, at the higher GTP concentration, p48-p58 produced substantial levels of oligomer considerably longer in length than that formed at lower GTP levels (compare Fig. 4D and supplemental Fig. 1B). These data indicate that Mcm10p initiates primase synthesis preferentially with ATP rather than GTP. In general, primases do not initiate RNA chains with pyrimidine nucleotides, and in keeping with this property, dTTP incorporation was not observed in the coupled DNA synthesis reactions using poly(dA) as the template (in the presence of UTP and [α-32P]dTTP) with either Mcm10p or p48-p58 (data not shown). We found that the primers synthesized by Mcm10p were not efficiently extended farther in the presence of dATP and the S. pombe pol α-primase p180-p70 subcomplex (supplemental Fig. 1B and supplemental Table 1), whereas the oligo(G) product formed by the p48-p58 complex was extended by dGTP addition in the presence of the S. pombe pol α-primase p180-p70 subcomplex. These results suggest that primases may hand off primers more efficiently to one DNA polymerase compared with another.

We also examined RNA products formed in reactions containing M13mp18 ssDNA as the template in the presence of the four rNTPs ([α-32P]ATP) at various rNTP levels. In these experiments, all products formed were subjected to calf intestine alkaline phosphatase digestion (Fig. 4, E and F). The major products synthesized by Mcm10p at a high concentration of NTP (100 μM) were 50–100 nucleotides long, although small levels of products ~9 nucleotides in length were detected (Fig. 4E, lanes 2–4). Maximal RNA synthesis required all four rNTPs. Omission of UTP and CTP, UTP, or CTP in the presence of 2 pmol of Mcm10p reduced the total level of RNA formed by 3.7-, 4.5-, and 25-fold, respectively.

We examined oligoribonucleotide synthesis by Mcm10p (5 pmol) in the presence of M13mp18 ssDNA at a low rNTP level (10 μM), conditions that select for strong primase initiation sites. Using these conditions, two small RNAs and oligomers 25–100 nucleotides in length were formed (Fig. 4F, lane 2). In the absence of CTP and UTP, UTP, or CTP, the synthesis of RNA chains 25–100 nucleotides in length was reduced (Fig. 4F, lane 5).

**FIGURE 4. Oligoribonucleotide synthesis catalyzed by full-length Mcm10p (amino acids 1–593), S. pombe p48-p58, and various templates.** A, rate of oligoribonucleotide synthesis catalyzed by full-length Mcm10p and S. pombe p48-p58 at high levels of NTP. The kinetics of poly(A) formation by SpMcm10p and the S. pombe p48-p58 primase complex in the presence of 0.1 μM ATP and (dT)50 are shown. Reactions were similar to those described in the legend to Fig. 1B and contained SpMcm10p (5 pmol) or p48-p58 subunits (1 pmol). Labeled oligoribonucleotide products were separated on a 25% denaturing polyacrylamide sequencing gel. B, rate of poly(A) formation in the presence of SpMcm10p or p48-p58. Reactions were as described for A and contained the indicated proteins, (dC)50, and 0.1 mM GTP. C, quantitation of the poly(A) synthesis observed in Fig. 3A. D, quantitation of the poly(G) synthesis observed in Fig. 3B. E, oligoribonucleotide synthesis catalyzed by Mcm10p in the presence of the M13mp18 DNA template. Reactions contained the indicated levels of Mcm10p; 0.2 pmol of M13mp18 ssDNA; 25 μCi of [α-32P]ATP; and 100 μM all four NTPs (lanes 1–4), ATP and GTP (lanes 5–8), no GTP (lanes 9–12), or no UTP (lanes 13–16). F, oligoribonucleotide synthesis catalyzed by full-length Mcm10p (0 and 5 pmol) in the presence of the M13mp18 ssDNA template at low rNTP levels. Reactions contained 5 pmol of Mcm10p where indicated; 0.2 pmol of M13mp18 ssDNA; and 10 μM all four rNTPs (lanes 1 and 2). ATP and GTP (lane 3), no ATP (lane 4), or no UTP (lane 5). Molecular size markers (M; kDa) are indicated. G, quantitation of the AMP incorporated into oligoribonucleotides observed in E. Shown is a summary of the [32P]AMP incorporated into oligoribonucleotides in the presence of Mcm10p (2 pmol) and all 4 NTPs or with various rNTPs omitted as indicated.
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compare lane 2 with lanes 3–5, similar to the results obtained at higher concentrations of nucleotide (Fig. 4E). In the absence of UTP and CTP, both small oligoribonucleotide products were not detected (Fig. 4F, lane 3), whereas the omission of either CTP or UTP resulted in the selective formation of only one of the two products (lanes 4 and 5). Although these small RNA products (shown in Fig. 4F) migrated faster than the various dinucleoside monophosphate markers, it is likely that these small dephosphorylated oligomers fractionated by both charge and molecular sieving in the gel system employed. Such aberrant migrations were noted previously with pentameric and tetrameric products formed with the phage T4 gp61 primase protein in the presence of the T4 gp41 helicase (32) and T7g4p (which contains both primase and helicase activity) (33).

Residues Required for RNA Synthesis Are in the C Terminus of Mcm10p—Previous studies with primases demonstrated that an acidic patch of residues clusters together at the center of the active site to bind two metal ions (36). This acidic patch contains the residues from conserved primase motifs IV–VI exemplified by the T7g4p sequence (see Fig. 6). Primase motif IV resembles the motifs involved in Mg$^{2+}$-mediated NTP binding by ATPases. Primase motifs V and VI (the putative NTD) contain the sequence (GS)X(DhD) (where X and h indicate any amino acid and a hydrophobic amino acid, respectively) in addition to a third negatively charged residue spatially juxtaposed to cooperate in the binding of the NTP substrate (27). To identify such motifs within Mcm10p, we used the ClustalW program, a general-purpose multiple sequence alignment program, to align 11 amino acid sequences from divergent organisms using full-length Mcm10p in each case (see “Experimental Procedures”). The ClustalW program allows putative motifs to be identified for a large group of related selected sequences and aligns them so that the identities, similarities, and differences can be observed. The alignment of several Mcm10p orthologs from divergent species identified two conserved C-terminal acidic patches. We designated one acidic patch between SpMcm10p amino acids 462 and 477 as a primase motif IV (supplemental Fig. 2A). The other patch resembles the NTD. We chose to make mutations in this second putative NTD motif. Shown in Fig. 5A is the putative NTD (in green) in the Mcm10p homologs of frog, human, yeast, rat, fly, mouse, and worm.

To determine whether these residues influence the SpMcm10p primase activity, we made three point mutants: E586A and D587A, which reside near the putative NTD of SpMcm10p, and D588A, which lies within the putative NTD (Fig. 5A, orange). The wild-type and mutant proteins were overproduced to the same extent in E. coli and behaved similarly during their purification. Glycerol gradient fractions of mutants E586A, D587A, and D588A and the wild-type protein were tested for their ability to synthesize primers using the coupled Klenow assay (Fig. 5B). The primase activity of mutant E586A was similar to that of wild-type Mcm10p, whereas mutants D587A and D588A exhibited 3- and 15-fold lower activities compared with the wild-type protein, respectively. These findings indicate that Asp$^{588}$, which lies within the putative NTD of Mcm10p, and D587A, which lies outside of this motif, are both defective in primase activity. These data demonstrate that the primase activity is intrinsic to the Mcm10p that was mutated.

In Vivo Analysis of Mcm10p C-terminal Point Mutants—We examined whether mutants E586A, D587A, and D588A support the functions of wild-type Mcm10p in vivo using the plasmid shuffle assay. We used an S. cerevisiae strain (ILY251) in which the MCM10 gene was deleted and viable only when harboring a plasmid expressing functional S. cerevisiae or S. pombe Mcm10p (34). Immunoblot analysis showed that the levels of the mutant and wild-type Mcm10 proteins expressed in vivo were comparable (Fig. 5D). The viability experiment showed that all three plasmids containing the mcm10 E586A, D587A, and D588A mutations, respectively, did not support cell growth on 5-fluoroorotic acid plates, whereas the plasmid expressing wild-type SpMcm10p did (Fig. 5C). Thus, a mutation in Mcm10p at Glu$^{586}$, Asp$^{587}$, or Asp$^{588}$ blocked an essential function of Mcm10p in vivo. We used the same plasmid shuffle assay to determine that Mcm10p-(416–593) and Mcm10p-(1–303) were unable to support cell growth in the absence of wild-type Mcm10p (data not shown).

A comparison of the mutational analysis data indicated that the requirements for viability and in vitro primase activity differ. Mutant D588A (located in the putative NTD) possessed significantly lower primase activity and lost Mcm10p activity in vivo, indicating that the primase activity may be required for cell viability. Mutant E586A (located at a residue adjacent to the putative catalytic site) had similar activity compared with wild-type Mcm10p. However, this mutant lost Mcm10p activity in vivo. The data for E586A suggest that Mcm10p activity can be lost without losing the in vitro Mcm10p primase activity, possibly because of changes in protein structure or failure to interact with partner proteins. A previous in vivo study demonstrated that, when the C-terminal 170 amino acids of SpMcm10p are cleaved off by tobacco etch virus protease, cells are unable to grow and that the Mcm10p replication function is abolished (26). Together, these data demonstrate that both point mutations within the C terminus and deletion of the C terminus of Mcm10p are lethal.

DISCUSSION

Mcm10p is required during late G1 phase at the origin of replication as well as during the elongation of DNA chains. It binds to the pre-replicative complex in a cell cycle-regulated manner, dependent on the Mcm2-7 complex, and participates in the conversion of the pre-replicative complex to the preinitiation complex. Phosphorylation of the Mcm2 subunit and possibly other members of the Mcm2-7 complex by the Cdc7p-Dbf4p complex at the G1/S transition phase is critical for this conversion. Biochemical data indicate that phosphorylation of the Mcm2 subunit within the Mcm2-7 complex is facilitated by Mcm10p because of its interaction with both Dbf4p and the Mcm4, Mcm6, and Mcm7 subunits. In accord with this temporal order of events, Mcm10p is required for the loading of Cdc45p and the GINS complex onto DNA, a step that follows the Cdc7p-Dbf4p-mediated phosphorylation of the pre-replicative complex. How Mcm10p participates in these loading steps is unknown. Our previous data indicate that Mcm10p may contribute to the recruitment and activation of pol $\alpha$-pri-
FIGURE 5. Mutations in the NTD domain of Mcm10p affect its primase activity in vitro and biological activity in vivo. A, sequence alignment of a C-terminal region of the X. laevis (xl), H. sapiens (hs), S. cerevisiae (ss), S. pombe (sp), R. norvegicus (rn), D. melanogaster (dm), M. musculus (mm), and C. elegans (ce) Mcm10 homologs. Conserved amino acids within the homologs found by the ClustalW alignment program are highlighted in green. The putative minimal NTD residues found in Mcm10p homologs are shown in orange, and Glu586, Asp587, and Asp588 of SpMcm10p are designated with asterisks. B, in vitro primase activity of mutant and wild-type Mcm10p preparations. Increasing levels of glycerol gradient fractions of wild-type (WT) Mcm10p and point mutants were assayed for their ability to support primer-dependent DNA synthesis as described in the legend to Fig. 2A. Aliquots of the peak glycerol gradient fractions (5 μl) were subjected to SDS-PAGE analysis and visualized by Coomassie Blue staining as shown in the inset. C, in vivo biological activity of the plasmid Mcm10p derivatives. The plasmid shuffle complementation assay was used to analyze the growth properties of S. pombe mcm10 point mutants. As shown, growth was measured on Leu- and Leu- /fluoroorotic acid (FOA-/) plates after 5 days of incubation at 30 °C for transformants with the MCM10 strain ILY251 (see “Experimental Procedures”) containing plasmids expressing wild-type S. pombe mcm10 or the indicated S. pombe mcm10 variants or with strain JPY9 expressing wild-type S. cerevisiae MCM10 as shown in the schematic. D, in vivo expression of wild-type and mutant proteins. Cell-free extracts (50 μg of protein) prepared from the strains expressing the indicated MCM10 genes were analyzed by immunoblotting using the polyclonal antibody specific for SpMcm10p. ADH, aldehyde dehydrogenase.
mase in the preinitiation complex via its interaction with the p180 catalytic subunit of pol α (25). Chromatin immunoprecipitation analyses indicated that Mcm10p, like the putative replicative helicase Mcm2-7, Cdc45p, and replicative polymerases, is a component of the replication fork (15).

During investigation of the biochemical properties of SpMcm10p, we noted that this protein contains primase activity. In the presence of a DT template, full-length Mcm10p catalyzed the synthesis of oligoriboadenylate chains that varied from 20 nucleotides to chains as short as dimers. This activity appears to reside at the C terminus of the protein because Mcm10p-(416–593) supported oligoriboadenylate synthesis. No activity was detected with the N-terminal fragment (amino acids 1–303). Two different assays were used to measure primase activity. These included the direct synthesis of small RNA using labeled rNTPs and the synthesis of DNA from ssDNA templates by E. coli pol I in a coupled polymerase-priming reaction. The levels of primase activity detected with purified Mcm10p and its C-terminal fragment were unaffected by rifampicin (an inhibitor of E. coli RNA polymerase), and we did not detect the presence of either DnaG or T7 RNA polymerase with antibodies specific for the E. coli primase or the bacteriophage RNA polymerase, respectively, by Western blot analysis.

(data not shown). In addition, the template specificity of an RNA polymerase and the product length synthesized by these enzymes are their distinctive “fingerprint.” The T7 RNA polymerase synthesizes poly(rG) five times more efficiently than poly(rA) on homopolymeric templates (37). Mcm10p synthesized poly(rA) 10 times more efficiently than poly(rG) (Fig. 4C). The majority (~90%) of products synthesized by T7 RNA polymerase on the non-promoter templates were dinucleotides. The products formed by Mcm10p were between 4 and 12 nucleotides in length. Together, these data suggest that it is unlikely that the observed primase activity is due to this contaminant. Additional evidence substantiating the conclusion that primase activity is associated intrinsically with Mcm10p or Mcm10p-(416–593) included their coincident elution during size column separation and sedimentation in glycerol gradients. Point mutations introduced at two C-terminal acidic residues located in the NTD region reduced the primase activity of the altered Mcm10p, further supporting this conclusion.

We would like to propose a model in which the Mcm10p and DnaG-type primases (such as T7g4p) are functional homologs and share similar enzymatic functions. There are six conserved bacterial primase motifs, I–VI (35), that are signature sequences present in prokaryotic primases. Primase motifs II and IV of T7g4p function as Mg2+–dependent nucleotide-binding regions (blue and purple ellipses). A motif of similar amino acid sequence corresponding to motif IV was found in yeast Mcm10p (purple ellipse) (see supplemental Fig. 2A). Primase motif III (pink ellipse) corresponds to a group of conserved lysines present in DnaG-type primases. Primase motifs V and VI (green ellipse; the catalytic center of T7g4p) correspond to a functionally homologous region of SpMcm10p found in the C termini of the yeast, human, fly, rat, and frog Mcm10 proteins (see Fig. 5A). Residues of the RNA polymerase catalytic center of T7g4p and residues required for the similar function of Mcm10p are designated as the NTD. T7g4p consists of a primase and helicase domain, and Mcm10p consists of a DNA polymerase recruitment and RNA polymerase domain; these domains are connected in both proteins to their cognate helicase via a conserved linker region (orange ellipse). Sites marked by asterisks denote amino acids that affect the activities of T7g4p and Mcm10p (in both S. pombe and S. cerevisiae) when mutated.

Model of the modular structure of Mcm10p and the functional T7g4p homolog. Shown is a linear comparison of the organization of the primase and helicase domains of T7g4p and the DNA polymerase recruitment and RNA polymerase domains of SpMcm10p. The colored ellipses identified by Roman numerals depict conserved primase motifs I–VI in T7g4p (35, 36). The similar primase motifs identified in SpMcm10p are designated by the same colored ellipses. As shown, primase motif I corresponds to the zinc finger regions (gold ellipse) in T7g4p and was identified in Mcm10 proteins of all species. Primase motifs II and IV of T7g4p function as Mg2+–dependent nucleotide-binding regions (blue and purple ellipses). A motif of similar amino acid sequence corresponding to motif IV was found in yeast Mcm10p (purple ellipse) (see supplemental Fig. 2A). Primase motif III (pink ellipse) corresponds to a group of conserved lysines present in DnaG-type primases. Primase motifs V and VI (green ellipse; the catalytic center of T7g4p) correspond to a functionally homologous region of SpMcm10p found in the C termini of the yeast, human, fly, rat, and frog Mcm10 proteins (see Fig. 5A).

During investigation of the biochemical properties of SpMcm10p, we noted that this protein contains primase activity. In the presence of a DT template, full-length Mcm10p catalyzed the synthesis of oligoriboadenylate chains that varied from 20 nucleotides to chains as short as dimers. This activity appears to reside at the C terminus of the protein because Mcm10p-(416–593) supported oligoriboadenylate synthesis. No activity was detected with the N-terminal fragment (amino acids 1–303). Two different assays were used to measure primase activity. These included the direct synthesis of small RNA using labeled rNTPs and the synthesis of DNA from ssDNA templates by E. coli pol I in a coupled polymerase-prime reaction. The levels of primase activity detected with purified Mcm10p and its C-terminal fragment were unaffected by rifampicin (an inhibitor of E. coli RNA polymerase), and we did not detect the presence of either DnaG or T7 RNA polymerase with antibodies specific for the E. coli primase or the bacteriophage RNA polymerase, respectively, by Western blot analysis.
Mg$^{2+}$-dependent nucleotide binding. There is little shared amino acid sequence among the prokaryotic and phage primases in the region of primase motifs II and III. However, primase motif IV shares similar amino acids with the yeast, *Xenopus*, rat, and human Mcm10 proteins and is located between amino acids 462 and 475 of SpMcm10p (Fig. 6 and supplemental Fig. 2A). Based on the crystal structure of T7g4p, primase motifs V–VI, which include acidic residues Asp$^{267}$, Asp$^{269}$, and Asp$^{237}$, bind metal ions and are part of the primase catalytic center (36). We noted the presence of the conserved NTD at the C terminus of Mcm10p (Fig. 5, A and B), which, when mutated, is defective for primer synthesis. Prokaryotic primases contain a Topoprim domain located within motifs IV–VI of *E. coli* DnaG and T7g4p (36, 38). In the absence of additional biochemical data and a three-dimensional structure of SpMcm10p, it is difficult to evaluate whether Mcm10p homologs are related evolutionarily or structurally to the prokaryotic primases. However, we suggest that primase motifs I and IV and critical residues of the NTD (primase motifs V and VI) in SpMcm10p and T7g4p, which are essential for the primase activity of the phage protein, contribute to the primase activity of the yeast protein.

In addition to synthesizing RNA primers, primases contribute to the extension of these primers by DNA polymerases. In T7, primase remains associated with the tetraligoligoribonucleotide products on the DNA template and participates in the interaction between the primer and DNA polymerase (39). This mechanism prevents the dissociation of the short RNA primer from the template and stabilizes the primed template in the active site of the DNA polymerase. In *E. coli*, the primed template is transferred from the DnaG primase to DNA polymerase III by the combined actions of the β clamp, the clamp loader, and ssDNA-binding protein (40). A similar conserved mechanism occurs in eukaryotes (41). Our finding that the N terminus of Mcm10p stabilized the interaction of the pol α-primase complex with primed DNA templates suggests that Mcm10p may also affect this type of transfer. We noted that RNA primers formed by Mcm10p (or the C-terminal fragment) were extended, albeit poorly, by the eukaryotic *S. pombe* pol α-primase or pol δ systems (supplemental Table 1). The role of additional proteins in such reactions remains to be examined.

A variety of replicative helicases form intermolecular complexes with primases that have critical functions in priming DNA replication synthesis. In accord with this relationship, the central region of Mcm10p interacts genetically and biochemically with the Mcm2-7 helicase. This region aligns and shares sequence similarities with the T7g4p linker domain that connects its primase and helicase regions (supplemental Fig. 2B). In phage T7, both helicase and primer activities are contained within a single polypeptide. These two functions are joined covalently by a 26-amino acid linker domain that is essential for the oligomerization of T7g4p to a hexamer, the active form of the helicase (42). Notably, two amino acids that are essential for phage growth and linker function, T7g4p Ala$^{257}$ and Asp$^{263}$ (43), are conserved in yeast Mcm10p. Temperature-sensitive *mcm10* mutants are located in a region that aligns with the T7 linker domain. The residues at these mutant sites (P269L and V265I) are conserved with similar residues in T7g4p. The positions of mutants *mcm10-1* (P269L), *cdc23-M36* (D232G and V265I), and *cdc23-1E2* (C239Y), which genetically interact with the Mcm2-7 helicase (16), are within the minimal region required for its physical interaction with the Mcm4-6-7 subcomplex (supplemental Fig. 2B) (21). One such mutant, *mcm10-1* (P269L), is suppressed by mutants *mcm5-1* and *mcm7-1*, suggesting that this region of Mcm10p is important for its interaction with the Mcm2-7 complex (16). Together, these data suggest that, like T7g4p, Mcm10p contains a linker region that connects it to the replicative helicase. This relationship, coupled with the detection of conserved primase motifs within Mcm10p, adds more credence to the biochemical finding that SpMcm10p contains primase activity. To date, however, we have not found that the *S. pombe* Mcm2-7 complex influences the primase activity of Mcm10p (data not shown). Because the Mcm2-7 complex lacks DNA helicase activity, its ability to stimulate the primase activity of Mcm10p may require additional factors that promote its cryptic unwinding properties.

Although the biological role of the Mcm10p primase activity is presently unclear, the findings that point mutations in the NTD region of SpMcm10p reduced its primase activity and rendered cells inviable suggest that this function is important. Because genetic and biochemical data indicate that the eukaryotic DNA primase associated with pol α is essential for viability, we consider it unlikely that the Mcm10p primase acts at the replication fork in lieu of the pol α-primase p48-p58 complex. We speculate that interactions between Mcm10p and the replicative Mcm2-7 helicase, analogous to those observed with the replicative helicase (DnaB) and primase (DnaG) in prokaryotes, may support priming events catalyzed by the Mcm10p primase rather than the pol α-primase complex. However, experiments to test this notion will require the isolation of a Mcm2-7 complex possessing helicase activity. Although we have failed to observe any significant effect of *S. pombe* Mcm2-7 on the Mcm10p primase activity, it is possible that additional factors that lead to a helicase active form of the Mcm2-7 complex may lead to different results.

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