The Mcm467 Complex of *Saccharomyces cerevisiae* Is Preferentially Activated by Autonomously Replicating DNA Sequences†

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ABSTRACT: We have analyzed the role of single-stranded DNA (ssDNA) in the modulation of the ATPase activity of Mcm467 helicase of the yeast *Saccharomyces cerevisiae*. The ATPase activity of the Mcm467 complex is modulated in a sequence-specific manner and that the ssDNA sequences derived from the origin of DNA replication of *S. cerevisiae* autonomously replicating sequence 1 (ARS1) are the most effective stimulators. Synthetic oligonucleotides, such as oligo(dA) and oligo(dT), also stimulated the ATPase activity of the Mcm467 complex, where oligo(dT) was more effective than oligo(dA). However, the preference of a thymidine stretch appeared unimportant, because with yeast ARS1 derived sequences, the A-rich strand was as effective in stimulating the ATPase activity, as was the T-rich strand. Both of these strands were more effective stimulators than either oligo(dA) or oligo(dT). The DNA helicase activity of Mcm467 complex is also significantly stimulated by the ARS1-derived sequences. These results indicate that the ssDNA sequences containing A and B1 motifs of ARS1, activate the Mcm467 complex and stimulate its ATPase and DNA helicase activities. Our results also indicate that the yeast replication protein A stimulated the ATPase activity of the Mcm467 complex.

Minichromosome maintenance proteins (Mcm) proteins are required for the initiation and subsequent stages of eukaryotic DNA replication (*1*). In the G_1-phase of the cell cycle, the origin recognition complex (ORC), Cdc6p, Cdt1p, Mcm2−7, and Cdc45p proteins bind sequentially to the origin of DNA replication to form a protein−DNA assembly known as the prereplication complex (pre-RC) (*1*−*3*). Once the cell cycle progresses to the G_1/S boundary, S-phase-specific cyclin-dependent protein kinases (Cdks) and particularly the Cdc7/Dbf4 kinase transform the pre-RC into an active replication complex (*4*). Other S- and M-phase Cdks block the rebinding of MCMs to the chromatin and prevent a new round of initiations until mitosis is completed (*2*, *5*). Finally, the initiation coincides with the association of polymerase α/primase (pol α/primase) complex with the RPA-coated unwound origins, leading to S-phase dependent DNA synthesis (*1*).

DNA replication in the yeast *Saccharomyces cerevisiae* initiates at specific sequences called autonomously replicating sequences (ARS), which have certain unique similarities to the 245 bp *Escherichia coli* origin of replication, *ori*C (*6*−*9*). ARS1 is a 200 bp sequence that contains four unique AT-rich elements: A, B1, B2, and B3 (*10*−*12*). The A and B1 elements are the recognition site for the initiator protein complex, ORC (*13*). In the case of chromosomal ARS1, ORC binds to double stranded A and B1 elements. ATP hydrolysis by ORC may unwind the strand containing the transition point or the start site for DNA replication involving B2 and B3 elements. RPA binds to this unwound region and likely prevents reannealing. Pol α-primase may also bind to the start site in an RPA-dependent manner. ORC is then displaced transiently from its binding site when Mcm2−7 complex binds to the A−B1 region, unwinds it, and translocates further toward the 5′ direction. Therefore, during initiation of DNA synthesis, Mcm467complex functions as the DNA helicase, and the helicase activity is dependent on the ssDNA-dependent ATP hydrolysis.

Initially, six Mcm proteins [Mcm2p (99 kDa), Mcm3p (108 kDa), Mcm4p (105 kDa), Mcm5p (86 kDa), Mcm6p (113 kDa), and Mcm7p (97 kDa)] associate with the pre-RC. A DNA helicase activity has been shown to be associated only with Mcm4, Mcm6, and Mcm7 proteins, which form a doughnut-shaped heterohexameric complex (Mcm467) (*14*, *15*). Almost all known DNA replication fork helicases studied to date have ring-shaped structures with central cavities for the single-stranded DNA (ssDNA) to bind (*16*, *17*). A similar model has been proposed for the Mcm467 complex (*18*, *19*). It has also been proposed that Mcm467 binds to the double-stranded DNA (dsDNA) and twists the dsDNA, thereby effecting its unwinding (*18*). The mechanisms of DNA unwinding by DNA helicases including the yeast Mcm467 helicase remain unclear. However, it appears that the mechanism of Mcm467 helicase is somewhat different from the bacterial replication fork helicases, including DnaB helicase. DnaB helicase unwinds DNA duplex with a 5′→3′ migration polarity that requires binding to the lagging strand of the replication fork. Mcm467 has been shown to unwind DNA with a 3′→5′ migration polarity, which requires it to bind to the leading strand (*14*, *20*). Nevertheless, one

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common aspect of both mechanisms of action is coupling of the energy of ATP hydrolysis to unwinding of the duplex DNA by breakage of hydrogen bonds in the base pairs. In *E. coli*, DnaC protein transfers DnaB helicase to the *E. coli* replication origin, *ori*C (*21*, *22*). Our recent results indicate that DnaC may form a hexameric assembly around ssDNA, which could attract DnaB to the origin and mediate DnaB loading. In eukaryotes, Cdc6p and/or Cdt1 may form such a heterohexameric complex on the ssDNA and could mediate the loading of the DNA helicase onto the unwound origin.

Mcm467 helicase is a heterohexameric complex. As a result, the properties of each subunit or subunit pair may differ from one another, and these could be analyzed separately to gain detailed insights. Mcm4p, Mcm6p, and Mcm7p proteins have Walker ATP binding motifs and are members of the AAA+ class of ATPases, which include the DnaC protein of *E. coli*. In this report, we have investigated the steady-state kinetics of ribonucleotide hydrolysis and ssDNA stimulation of the individual Mcm subunits as well as the Mcm467 protein complex. We have analyzed the modulation of the ATPase and DNA helicase activities of the yeast Mcm467 complex by synthetic and native ssDNA sequences, including those present in the ARS1 sequence. We have also analyzed the role of yeast replication protein A (RPA) in regulating the ATPase activity of the Mcm467 complex.

EXPERIMENTAL PROCEDURES

Nucleic Acids, Enzymes, and Other Reagents. Ultrapure deoxy- and ribonucleotides were obtained from AP Biotech (Piscataway, NJ) and were used without further purification. [α-^{32}P]- and [γ-^{32}P]ATP were obtained from AP Biotech (Piscataway, NJ). Yeast RPA was purified to homogeneity from wild-type yeast (>98% purity in SDS−PAGE) as described (*23*). All chemicals used to prepare buffers and solutions were of reagent grade and were purchased from Fisher Chemical Co. (Pittsburgh, PA). Polyethyleneimine−cellulose TLC plates were from J. T. Baker Chemical Co. HPLC ion exchange columns, ion exchange chromatography matrix, and the Bio-Cad 20 HPLC instrument were from Applied Biosystems Inc., Woburn, MA. Superose 6 and Superose 12 HPLC gel filtration columns were from AP Biotech Inc., Piscataway, NJ.

Buffers. Lysis buffer was made of 25 mM Tris pH 7.5, 10% sucrose, and 500 mM NaCl. Buffer A was made of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT. Buffer B was made of 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 1 mM DTT. Buffer C was made of 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.2 mg/mL BSA, and 5 mM DTT.

Oligonucleotides. Oligonucleotides were synthesized by Sigma Genosys Inc. (Midland, TX). ARS-A (ARS1 A-rich strand) and ARS-T (ARS1 T-rich strand) oligonucleotides were 5′-CTTTTGAAAAGCAAGCATAAAAGATCTAAACATAAAATCTGTAAAATAAC-3′ and 5′-GTTATTTTACAGATTTTATGTTTAGATCTTTTATGCTTGCTTTTCAAAAG-3′, respectively, and the duplex ARS was generated by hybridizing the two oligonucleotides. The helicase substrates were designed to have identical 50 bp duplex regions with the sequence TCACGACGTTGTAAAACGACGGCCAGTGAA TTCGAGCTCGGTACCCGGGG and 50 bp single-stranded regions containing either ARS-A or ARS-T sequences (as shown above) or oligo(dA)$_{50}$ or oligo(dT)$_{50}$ sequences. ARS-MUTA and ARS-MUTT sequences were mutated in the A and B1 regions (italicized) and were CTTTTG*ATGATGATGATG*AAAAGATCT*TTTCATTTT*ATCTGTAAAATAAC and GTTATTTTACAGAT*AAAATGAAA*AGATCTTTT*CATCATCATCA*TCAAAAG, respectively.

Expression of MCM Genes. The MCM4, MCM6, and MCM7 genes were amplified from yeast chromosomal DNA by polymerase chain reaction using Ultra Pfu DNA polymerase and oligonucleotide primers. These primers were as follows.

MCM4: 5′-TCTTCTCCATGGCTCAACAGTCTAGCTCTCCAACA-3′, 5′-CTCCTCCTCGAGTAGACAGCATCAGACACG-3′.

MCM6: 5′-TCTTCTGGATCCGATGTCATCCCCTTTTCC-3′, 5′-CTCCTCGTCGACACTGCAAGTGGAAG CCAA-3′.
MCM7: 5′-TCTTCTGTCGACATGAATAATCTTTTTAATGAAAT-3′,

5′-CTCCTCGCGGCCGCGCCTTGGAAGTAAGTTACAGA-3′.

The amplified genes were digested with appropriate restriction enzymes and inserted in frame in the pET29 vector (Novagen Inc., Milwaukee, WI), a T7 expression plasmid vector, using a standard protocol. DNA sequences were verified by DNA sequencing.

Protein Expression and Extraction. E. coli BL21(DE3)RIL strain (Stratagene Inc., La Jolla, CA) was transformed with plasmids containing the desired MCM gene in the T7 expression vector pET29, using standard molecular biology protocols (*24*). The cells were grown in 2XYT medium containing 50 μg/mL kanamycin, 30 μg/mL chloramphenicol, and 10 μg/mL tetracycline at 37 °C until OD_{600} 0.5−0.7. Cells were cooled to 13−16 °C and protein expression was induced by adding 1 mm IPTG. Induction was carried out at 13−16 °C with shaking overnight (~16−18 h). Following induction, the cells were harvested and resuspended in lysis buffer. Protein extraction was carried out as described previously (*24*).

Mcm4p Subunit Purification. The protein extract (fraction I) containing Mcm4p was precipitated with 0.3 g/mL ammonium sulfate. The pellet was resuspended in buffer A to a volume such that the final conductivity was equivalent to 100 mM NaCl (fraction II). The protein was loaded on a 20 mL POROS HQ/H anion exchange column equilibrated with buffer A100. The column was washed successively with buffers A100 and A150 and the protein was eluted with 100−500 mM NaCl gradient in buffer A. Column fractions were analyzed by SDS−PAGE and the peak Mcm4p fractions were combined and dialyzed against buffer B100 (fraction III). The dialyzed pool was applied to a 7 mL POROS HS/M cation exchange column equilibrated with buffer B100. The column was eluted with a 100−1000 mM NaCl gradient in buffer B. Fractions containing Mcm4p were pooled and concentrated using ultrafiltration. At this stage, the Mcm4 protein fraction was ~75% pure (fraction IV). The concentrated sample was further purified by Superose 6 gel filtration column at 0.4 mL/min flow rate. It eluted at 14.8 mL, indicating that it is monomeric (fraction V).

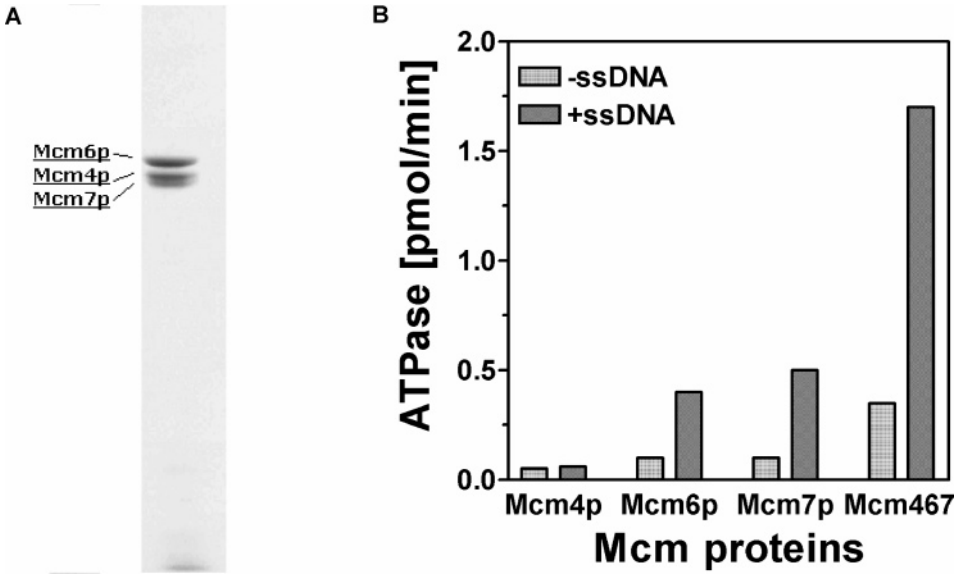

FIGURE 1: (A) SDS−PAGE of proteins utilized in this study. The recombinant Mcm467 complex (10 μg) was analyzed on a 5−18% SDS−PAGE gel and stained with Coomassie Blue R-250. (B) Influence of ssDNA effectors on Mcm Protein ATPase. Standard ATPase assays were carried out with Mcm protein complexes in the presence or absence of 200 pmol (as nucleotide) of oligo$(dT)_{50}$. In each set, the data points represent the mean of three separate experiments.

Mcm6p Subunit Purification. The protein extract (fraction I) containing Mcm6p was precipitated with 0.22 g/mL ammonium sulfate. The pellet was resuspended in buffer A to a volume such that the final conductivity was equivalent to 100 mM NaCl (fraction II). The protein was loaded on a 20 mL POROS HQ/M HPLC column equilibrated with buffer A100. The column was washed successively with buffers A100 and A225. The protein was eluted using a 60 mL gradient of buffer A225 to A450. Mcm6p eluted at ~350 mM NaCl concentration. Column fractions were analyzed by SDS−PAGE, the peak Mcm6p fractions were combined, and the conductivity was adjusted to 100 mM NaCl (fraction III). The diluted Mcm6p protein pool was applied to a 2 mL POROS HQ/H column. The column was washed successively with A100 and A150. The protein was eluted isocratically using buffer A350. Following HQ/H fractionation, the Mcm6p protein was ~90% pure (fraction IV). The fractions containing Mcm6p were pooled and concentrated. The concentrated sample was further purified by Superose 6 gel filtration chromatography (fraction V).

Mcm7p Subunit Purification. The protein extract (fraction I) containing Mcm4p was precipitated at 0.3 g/mL ammonium sulfate for 1 h while being stirred on ice followed by centrifugation at 34,000*g* for 30 min. The pellet was resuspended in buffer A to a volume such that the final conductivity was equivalent to 100 mM NaCl (fraction II). The protein was loaded on 20 mL POROS HQ/H column equilibrated with buffer A containing 100 mM NaCl. The protein was eluted with a 180 mL gradient of buffer A100 to buffer A1000. The fractions were analyzed for protein content and by SDS−PAGE. Mcm7p eluted between 200 and 300 mM NaCl. Fractions containing Mcm7p were combined and were precipitated using 0.3 g/mL ammonium sulfate (fraction III). The pellet was resuspended in buffer A and applied to a preparative (2.5 × 80 cm, Bio-Rad TSK250) HPLC gel filtration column and 80 1-min fractions were collected in buffer A100 at 2.5 mL/min flow rate. We identified two peaks; one peak (minor) corresponded to Mcm7p dimer and the other (major) to the Mcm7p monomer (fraction IV).

Reconstitution of Mcm467 Complex. Mcm467 complex was reconstituted by mixing Mcm4p, Mcm6p, and Mcm7p in equimolar amount at 30 °C for 5 min. The complex was then further purified by anion exchange chromatography.

ATPase Assay. The ATPase assays were carried out as previously described (*25*). The amount of protein used in the assays was selected such that the rate of hydrolysis would be linear under the reaction conditions. Unless otherwise indicated, 1 pmol of total Mcm protein (as monomer) were used in each assay.

A standard 10 μL reaction mixture contained 10 mM $MgCl_2$, 200 pmol ssDNA (in terms of nucleotides), 100 μM [α-^{32}P]ATP (1000−2000 cpm/pmol), and Mcm proteins in buffer C. Reactions were incubated at 30 °C for the indicated times and terminated by the addition of 2 μL of 200 mM EDTA followed by chilling on ice. Two microliter aliquots were applied to polyethyleneimine−cellulose strips, which were prespotted with ADP−ATP markers. The strips were developed with 1 M formic acid, 0.5 M LiCl and dried. The ADP−ATP spots were located by UV fluorescence. The portions containing ATP and ADP were excised and counted in a liquid scintillation counter using a toluene-based scintillator.

In kinetic analysis of the ATPase activity, a given set of reactions was carried out in a single tube, in an appropriate reaction volume, and was initiated by the addition of Mcm proteins. At the indicated time points, 10 μL aliquots were removed and transferred to tubes containing 2 μL of 200 mM EDTA at 0 °C to stop the reaction. The reaction rates were plotted against the substrate concentrations and fitted using nonlinear regression analysis of enzyme kinetics using the Michaelis−Menten equation [$V = (V_{max}[S])/(K_m + [S])$] and PRISM 3.03 software. The two-site-binding equation used in this study was derived from the Michaelis−Menten equation.

DNA Helicase Assay. DNA helicase assays were carried out with ARS and non-ARS substrates similar to that described earlier (*25*).

Other Methods. Protein concentrations were determined by the method of Bradford (*26*) using BSA as a protein standard. SDS−PAGE analysis of proteins was carried out according to Laemmli (*27*).

RESULTS

The Mcm6p and Mcm7p subunits of Mcm467 Complex are DNA Stimulated ATPases. The Mcm467 helicase of *S. cerevisiae* is a heterohexameric protein complex comprised of two subunits each of Mcm4p, Mcm6p, and Mcm7p proteins. Each of these subunits contains an AAA+ ATPase signature domain as well as Walker nucleotide binding motifs. Therefore, the intrinsic ATPase activities reported by other laboratories were not surprising (*28*−*30*). The dynamics of the ATPase activity and steady-state kinetic parameters of the ATP hydrolysis as well as the ssDNA modulation of the ATPase activities of each of these subunits need to be analyzed. Here we have examined the kinetics of ATP hydrolysis and DNA dependence of the ATPase activity of each individual subunit of Mcm467 helicase. We have expressed and purified each of the three subunits to homogeneity (Figure 1A). First, each individual subunit was examined for ATPase activity in the presence or absence of ssDNA. The results of these analyses are presented in Figure 1B. All three subunits showed similar intrinsic ATPase activities in the absence of DNA. However, Mcm6p and Mcm7p, but not Mcm4p, could be significantly stimulated with ssDNA. Mcm6p was stimulated 2.5-fold and Mcm7p was stimulated 4-fold by ssDNA. Mcm4p displayed a low ATPase activity in the presence or absence of ssDNA. Therefore, it appears that either Mcm4p does not bind ssDNA or ssDNA binding does not induce the conformational changes that are required to stimulate its ATPase activity.

Kinetics and ssDNA Dependence of the ATPase Activity of the Mcm467 Complex. Our results clearly indicate that, compared to the individual subunits, the complete Mcm467 complex is the most active ssDNA-dependent ATPase (Figure 1B). We have carried out a comprehensive steady-state kinetic analysis of the ATPase activity of Mcm467 complex and the modulation by ssDNA. Primarily, our goal was to delineate the characteristics of ATP binding, the ATPase activity, and the modulation of these parameters by various single-stranded DNAs. Earlier, it was shown that thymine-rich sequences and oligonucleotides stimulate the mouse Mcm467 complex (*30*). It has been proposed that the thymine-rich sequences could mimic the AT-rich sequences in the replication origin. Consequently, we have examined ssDNA allosteric effectors of thymine-rich as well as non-thymine-rich sequences for stimulation of the ATPase activity of the yeast Mcm467 complex. It should be noted that the cellular ATP concentration in the log-phase *S. cerevisiae* is ≥1 mM, which is substantially higher than the determined K_m values in this paper (Table 1).

In the absence of ssDNA, Mcm467 complex displayed a low ATPase activity (Figure 2A). The rate of ATP hydrolysis increased with increasing concentration of ATP and appeared to saturate above 0.5 mM ATP. The K_m for ATP in the absence of DNA was 170 μM and the V_{max} was 0.52 pmol/min. In the presence of oligo(dT)$_{35}$, the rate of ATP hydrolysis increased significantly (Figure 2B). The K_m for ATP decreased to 58 μM, indicating a possible increase in the nucleotide binding affinity (Figure 2B, inset). The V_{max} increased to 1.68 pmol/min.

Table 1: Kinetic Parameters of the ATPase Activity of the Yeast Mcm467 Complex in the Presence of Various DNA Effectors

DNA	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m
no DNA	170 ± 6	0.52 ± 0.09	0.003
oligo(dT)	58 ± 8	1.68 ± 0.06	0.029
oligo60	55 ± 9	1.09 ± 0.04	0.02
M13mp19	434 ± 24	3.77 ± 0.26	0.0067
ARS-A	163 ± 19	8.9 ± 0.2	0.055
ARS-T	139 ± 11	8.3 ± 0.9	0.06
ARS1 dsDNA	76 ± 9	4.4 ± 0.8	0.057
ARS-MUTA	76 ± 9	1.65 ± 0.09	0.022
ARS-MUTT	63 ± 5	1.47 ± 2	0.023

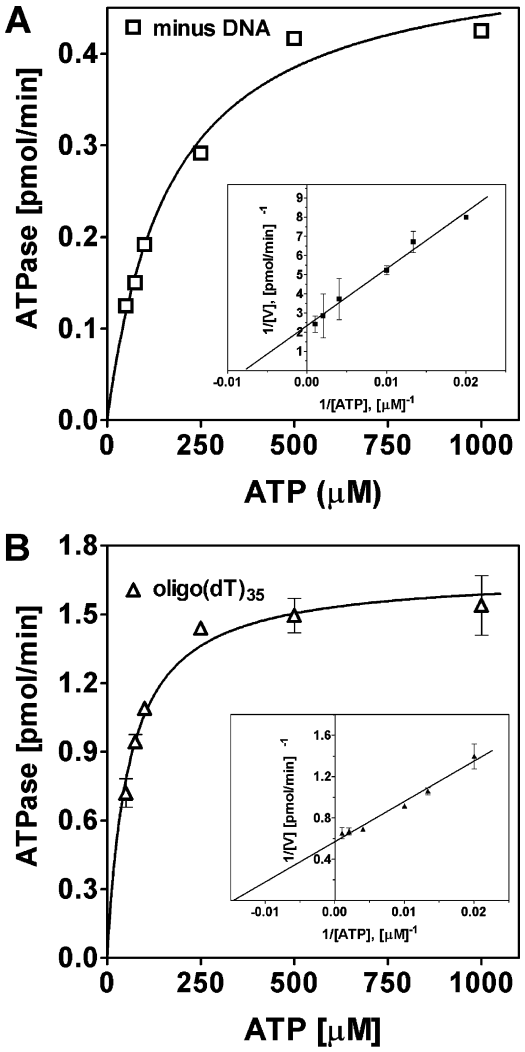

FIGURE 2: Kinetic analysis of Mcm467 ATPase in the absence of DNA effectors (A) and in the presence of oligo(dT)$_{35}$ (B). The ATPase assays were carried out as described in Materials and Methods in the presence of 200 pmol DNA effector. The curves were generated using a nonlinear regression analysis of the data. In each set, the data points represent the mean of three separate experiments. The assays were carried out for 30 min at 30 °C and this time point was chosen by a time-course analysis of the ATPase activity of Mcm467. Under these conditions, it was determined to be the steady state and total ATP hydrolysis was less than 10%.

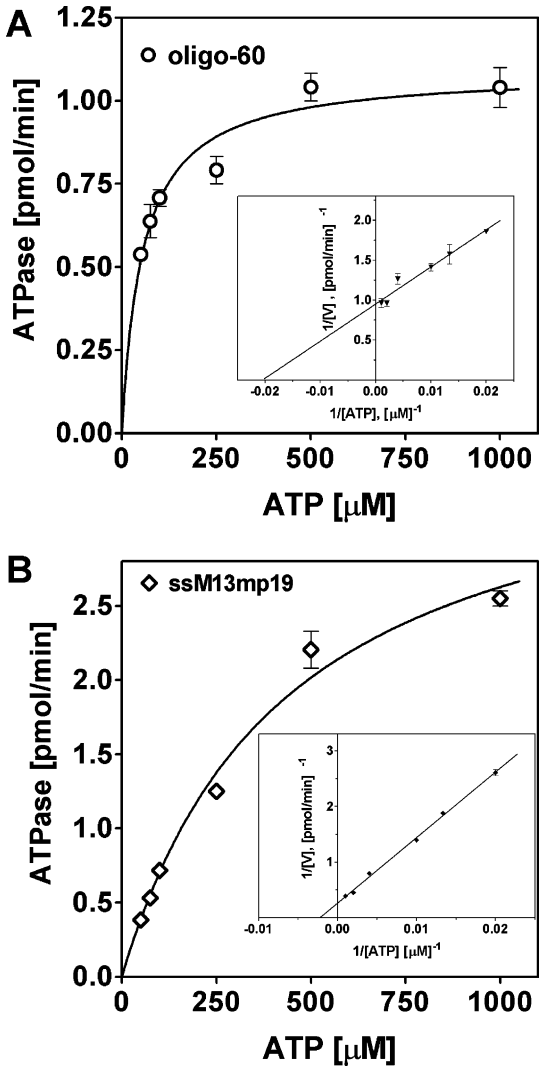

FIGURE 3: Kinetic analysis of Mcm467 ATPase in the presence of (A) a random 60mer oligonucleotide and (B) closed circular M13mp19 ssDNA. The ATPase assays were carried out in the presence of (A) 200 pmol of a 60mer oligonucleotide derived from a naturally occurring DNA sequence found in M13mp19 bacteriophage ssDNA or (B) 200 pmol of M13mp19 closed circular ssDNA. The assays were allowed to proceed for 60 min. The curves were generated using a nonlinear regression analysis of the data. In each set, the data points represent the mean of three separate experiments.

As oligo(dT)$_{35}$ is a thymine-rich sequence, similar to that observed in the origins of eukaryotic DNA replication, we examined a naturally occurring DNA sequence derived from the M13mp19 bacteriophage ssDNA. This 60-mer oligonucleotide (oligo60) did not contain a thymine or a pyrimidine string. It would be reasonable to assume that Mcm467 complex would encounter similar sequences during processive helicase unwinding. We hoped that this substrate would allow us to determine any significant change of DNA-stimulated ATPase with nucleotide sequence. The 60-mer oligonucleotide significantly stimulated the ATPase activity, except that the ssDNA stimulation was attenuated (Figure 3A). The K_m was 55 μM, which was comparable to that observed with oligo(dT)$_{35}$ (Figure 3A, inset). However, the V_{max} was 1.09 pmol/min, which was lower than that observed with oligo(dT)$_{35}$.

To determine the effect(s) of long single-stranded DNA on the ATPase activity of Mcm467, we have further analyzed

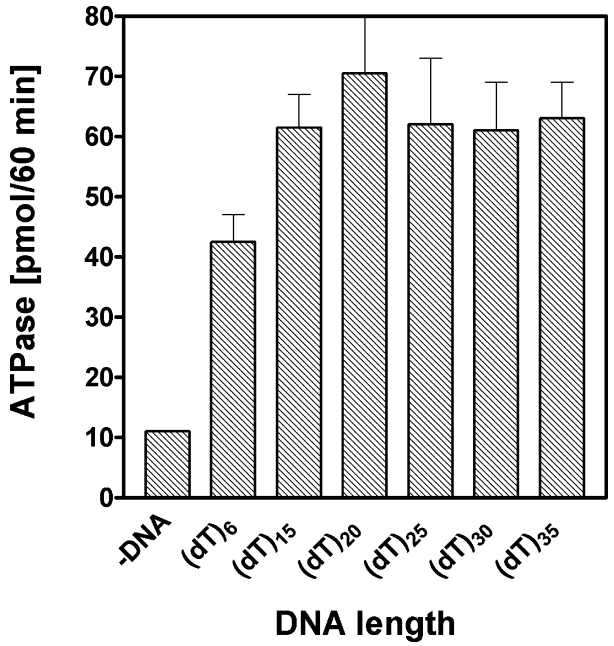

FIGURE 4: Analysis of oligonucleotide length required for activation the ATPase activity of Mcm467 complex. The ATPase assays were carried out in the presence of 200 pmol (as nucleotides) of oligo(dT) effectors of increasing length, from 6 to 35 nucleotides. In each set, the data points represent the mean of three separate experiments.

the stimulation of the ATPase activity of Mcm467 complex in the presence of closed circular M13mp19 bacteriophage ssDNA. The kinetics of ATP hydrolysis with M13mp19 bacteriophage ssDNA was significantly different than that observed with small oligonucleotides (Figure 3B). In a nonlinear regression analysis using the Michaelis−Menten equation, K_m was found to be 434.4 μM, which was 10-fold higher than that observed with shorter oligonucleotides (Figure 3A). The V_{max} increased to 3.8 pmol/min, which was also significantly higher than that observed with shorter oligonucleotides. This significant increase in the K_m could be related to the difficulty associated with the binding of Mcm467 complex to a closed circular ssDNA.

An oligomer of 15 Deoxynucleotides Efficiently Stimulated the ATPase Activity of the Mcm467 Complex. To determine the minimum size of oligomer that is required for the optimal stimulation of the ATPase activity of the Mcm467 complex, we have examined oligodeoxythymidylates that ranged in size from six to 35. Surprisingly, oligodeoxythymidylates as small as a hexamer can stimulate the ATPase activity of Mcm467 complex 4-fold (Figure 4). With oligo(dT)$_{35}$, the stimulation of ATPase activity was approximately 6-fold. Larger oligothymidylates such as oligo(dT)$_{35}$ did not increase stimulation further. A 15-mer oligonucleotide such as oligo(dT)$_{15}$ could be sufficient or close enough to span the entire ssDNA binding site(s) in Mcm467.

Replication Origin Sequences Were Most Effective in Stimulating the ATPase Activity of Mcm467 Complex. To test the hypothesis that the sequences involved in the origin of DNA replication may be the most effective in stimulating the Mcm467 complex, we have synthesized 50 nucleotides sequences containing the A and B1 elements of yeast ARS1 and mutated ARS1 sequences with mutations in the A and B1 domains of ARS1. The A and B1 elements were chosen for our initial analysis on the basis of the assumption that Mcm2−7 and perhaps Mcm467 complex may bind to this

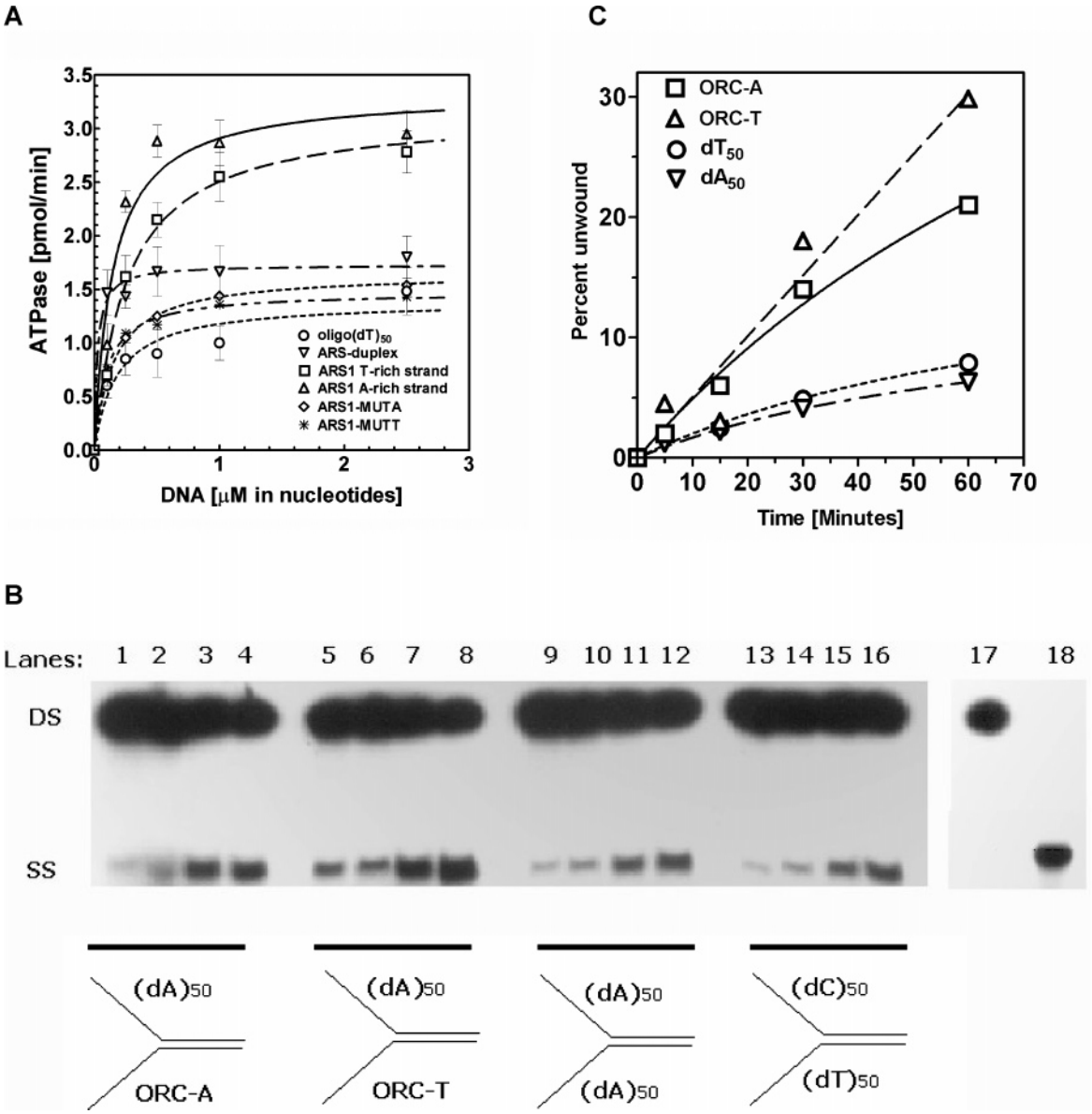

FIGURE 5: Analysis of ARS1 A-rich and T-rich sequences in the stimulation of ATP hydrolysis and DNA unwinding by Mcm467 complex. (A) The ATPase assays were carried out in the presence of increasing amounts of (□) 50 bp ARS1(A-B1) T-rich ssDNA, (△) ARS1(A-B1) A-rich ssDNA, (▽) ARS1 duplex DNA, (○) oligo(dT)$_{50}$, (◇) ARS-MUTA, or (*) ARS-MUTT. (B) The DNA helicase activity of Mcm467 complex (250 ng) with substrates containing either ARS-A ssDNA (lanes 1−4), ARS-T ssDNA (lanes 5−8), oligo(dA) $_{50}$ ssDNA (lanes 9−12), or oligo(dT)$_{50}$ ssDNA sequences for 5, 15, 30, and 60 min at 30 °C or without Mcm467 protein for 60 min at 30 °C as negative control (17) and the substrate after heat denaturation (18). (C) Plot of percent unwinding with each of the four substrates in B, quantitated by densitometry scanning.

region (*31*). Both single-stranded A-rich and T-rich ssDNA sequences were synthesized and a duplex of the two strands was created by hybridization. We compared stimulation of the ATPase activity of Mcm467 complex by the A-rich, T-rich duplex ARS1 sequences and oligo(dT)$_{35}$. The results of our analysis are shown in Figure 5 and Table 1. The single-stranded A-rich and T-rich strands stimulated at the maximal level, whereas the duplex DNA was effective in stimulating the ATPase to a lower extent. In parallel comparison, oligo(dT)$_{35}$ was far less effective (Figure 5, Table 1). Between the A-rich and T-rich strands, we did not observe any specific preference, even though a difference between the two strands was anticipated from our studies with oligo(dT)$_{35}$ and other oligonucleotides. Therefore, the observed preference for oligo(dT)$_{35}$ over other oligonucleotides was fortuitous and not related to ARS.

Kinetic analysis of the ATPase activities of Mcm467 complex in the presence of single- and double-stranded ARS1 DNA at various ATP concentrations was carried out, and the results are presented in Figure 6A,B. The double-stranded ARS1 sequence stimulated the ATPase activity (Figure 6A). However, the *V* vs [S] plot in Figure 6A did not appear to fit to a single binding event; rather, it could be fitted only to a double binding event as shown in Figure 6A. The molecular basis of the complex kinetics involving dsDNA is not clear. The kinetics of ATP hydrolysis appeared significantly different from those observed with all single stranded DNA, including the ARS1 ssDNAs. The single-stranded A-rich strand of ARS1 DNA stimulated the ATPase activity of Mcm467 complex to a much higher degree (Table 1). Similar to other single stranded DNA effectors, as described above, the *V* vs [S] plot fitted to a single binding kinetics. The

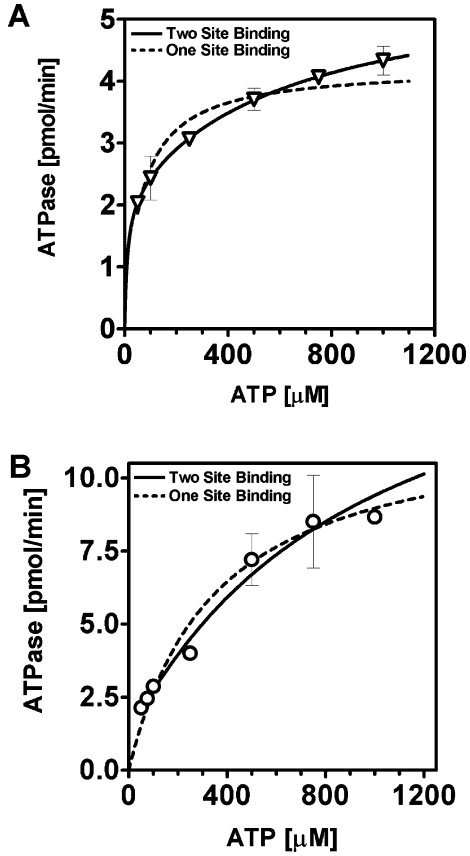

FIGURE 6: Kinetic analysis of Mcm467 ATP hydrolysis in the presence of ARS1 DNA effectors. The ATPase assays were carried out in the presence of (A) 200 pmol (as nucleotides) of ARS1 dsDNA and (B) 200 pmol (as nucleotides) of ARS1 A-rich ssDNA. The assays were allowed to proceed for 60 min. The curves were generated using a nonlinear regression analysis of the data using built in single and double binding event equations. In each set, the data points represent the mean of three separate experiments.

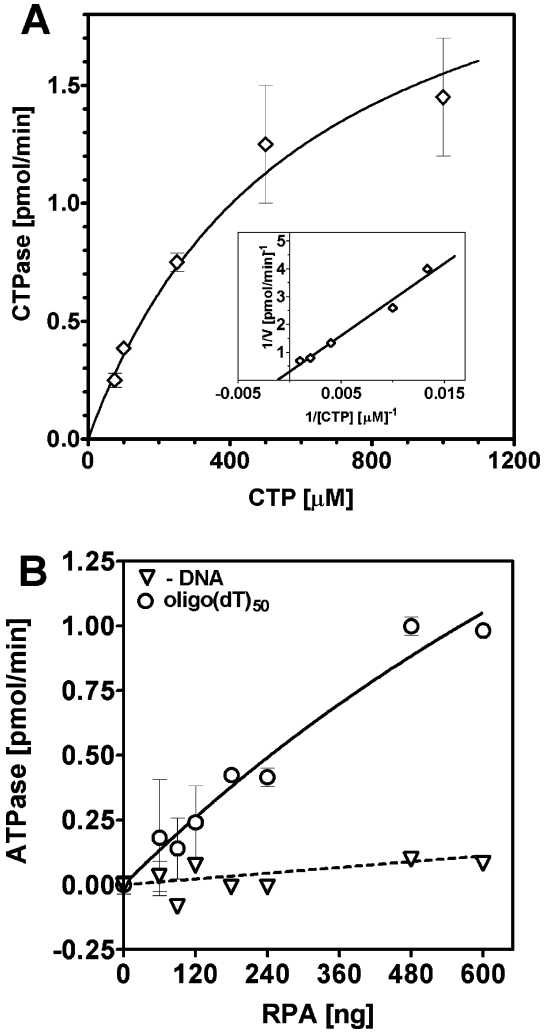

FIGURE 7: (A) Kinetic analysis of Mcm467 CTP hydrolysis. The CTPase assays were carried for a period of 60 min in the presence of 200 pmol (as nucleotides) oligo(dT)$_{35}$. The curves were generated using a nonlinear regression analysis of the data. In each set, the data points represent the mean of three separate experiments. (B) Modulation of Mcm467 ATPase activity by replication protein A. Standard ATPase assays were carried out in the presence of increasing amounts of yeast RPA. Assays were carried out in the absence (▽) of DNA effector or in the presence (○) of 200 pmol oligo(dT)$_{50}$ as nucleotide or 4 pmol of oligonucleotides. In each set, the data points represent the mean of three separate experiments. The values in the absence of RPA were subtracted and the values were 0.3 ± 0.15 and 0.9 ± 0.1 pmol/min in the absence and presence of DNA, respectively.

kinetic parameters involving stimulation by the A-rich, T-rich, and double stranded ARS1 DNA are presented in Table 1. With mutated A- and T-rich ARS1 single stranded sequences, the kinetic parameters were comparable to oligo60 and oligo(dT)$_{35}$ sequences and not ARS1 sequences (Table 1). Therefore, the A and B1 domain sequences are involved in the modulation of the ATPase activities of Mcm467 complex.

Using the ratio of V_{max}/K_m, we have measured the alterations of the catalytic efficiencies of the complex in the presence of various effectors. As shown in Table 1, the catalytic efficiency is very low in the absence of DNA and increases significantly in the presence of DNA. With non-ARS DNA as effector, the catalytic efficiency ranges from 0.02 to 0.029. Whereas, in the presence of ARS related DNA, the catalytic efficiency was approximately 0.06. As stated earlier, the cellular ATP concentration in *S. cerevisiae* is ≥1 mM; as a result, the rates of ATP hydrolysis of the Mcm467 complex would be close to the observed V_{max} values, as the observed K_m values involving all of these DNA effectors were much lower than the cellular ATP concentrations (Table 1). Consequently, ARS1-derived sequences appear to have significant influence in the ATPase activity of the Mcm467 complex.

ARS1 AB1 Sequences Stimulate DNA Helicase Activity of Mcm467 Complex. We have compared DNA helicase substrates containing single-stranded oligo(dA), oligo(dT) sequences or ARS1 AB1 sequences. ARS1 substrates were better substrates than the dA$_{50}$ or dT$_{50}$ substrates in the time course analysis (Figure 5B,C). It is likely that, in vivo, ARS sequences may have a far greater impact in the DNA helicase function of Mcm467 than the in vitro DNA helicase assays.

Mcm467 Complex Can Hydrolyze CTP at an Attenuated Level. To determine the substrate specificity of the ATPase activity of Mcm467 complex, we have analyzed the hydrolysis of other ribonucleotides by the complex. Results presented here suggest that CTP is a substrate of Mcm467 complex. However, nonlinear regression analysis of the data using the Michaelis−Menten equation demonstrated that K_m was ~598 μM and V_{max} was 2.475 pmol/min (Figure 7A). Even though CTP appeared to be a substrate, the affinity of

Mcm467 complex for CTP was far less than that for ATP. We did not observe any appreciable nucleotide hydrolysis with either GTP or UTP (data not shown). Therefore, ATP and CTP hydrolysis activities appear to be quite specific. Incidentally, the yeast origin recognition complex, ORC, also exhibits a similar low-level CTPase activity, but not GTPase or UTPase activity (*32*). Therefore, the ability to hydrolyze CTP appears to be a common characteristic of certain eukaryotic DNA-dependent ATPases. Alternatively, the CTPase activity of Mcm467 complex could be an example of enzymatic infidelity involving these ATPases (*33*).

Replication Protein A Significantly Stimulates ATPase Activity of Mcm467 Complex. In addition to DNA, other replication proteins may also regulate the ATPase activity of the Mcm467 complex (*34*). With *E. coli* DnaB helicase, the cognate single-stranded DNA binding protein (SSB) stimulated its DNA helicase activity but suppressed the ATPase activity (*34*). Therefore, we examined the effect, if any, of yeast replication protein A (RPA) on the ATPase activity of Mcm467 complex (Figure 7B). In the absence of ssDNA, RPA does not have any effect on the basal ATPase activity of Mcm467 complex. Consequently, our results do not suggest a direct physical interaction between these two proteins. However, in the presence of ssDNA, RPA stimulated the ATPase activity approximately 2-fold. RPA has been shown to have strong DNA binding affinity for ssDNA, particularly the pyrimidine-rich DNA such as oligo(dT)$_{35}$. Therefore, we considered the possibility that RPA may inhibit the binding of Mcm467 complex to oligo(dT)$_{35}$. However, these results clearly demonstrate a lack of inhibition and a significant stimulation of the ATPase activity.

DISCUSSION

To understand the mechanism of energy transduction in the DNA unwinding by Mcm467 helicase, we have analyzed the DNA-dependent ATPase activities of the Mcm467 complex, its subunits, and its subcomplexes and regulation by various single-stranded DNA sequences. We have determined the kinetics of ATP hydrolysis by these polypeptides and their assemblies and the modulation of their activities by ssDNA.

Single-Stranded DNA Templates Modulate the ATPase Activity of Mcm6p and Mcm7p Subunits: Individually, all three subunits of Mcm467 complex have attenuated but measurable ATPase activity (Figure 1B). The activities were in the range of 0−12 pmol/h; however, the activity of the Mcm4p subunit was within the experimental error. However, the ATPase activities of both Mcm6p and Mcm7p were more pronounced. Earlier, Davey et al. also found a similar ATPase activity of the Mcm6p in the absence of DNA (*28*). In the presence of ssDNA, the ATPase activity of Mcm4p did not increase and remained within the experimental error. Therefore, Mcm4p does not appear to have any ATPase activity in the presence or absence of DNA. However, both Mcm6p and Mcm7p exhibited significant ssDNA stimulation of ATPase activity. The maximum stimulation was observed with Mcm6p. Therefore, both Mcm6p and Mcm7p appear to be able to interact with or bind ssDNA.

The ATPase Activity of Mcm467 Complex Is Regulated by DNA. Mcm467 complex showed ATPase activity in the absence of DNA, unlike its individual subunits or the subunit pairs (Figure 2). DNA binding appears to have a dual regulatory effect on the ATPase activity of Mcm467 complex. Oligo(dT)$_{35}$ containing a thymidine stretch appears to be somewhat superior to an oligonucleotide with a natural or random sequence. These results are similar to that observed by You et al. (*30*) with mouse Mcm467 complex, although we did not find such a drastic effect with yeast Mcm467. This difference could be due to mechanistic differences between mouse and yeast or due to differences in the expression systems, baculovirus and *E. coli*.

Closed Circular ssDNA Is Less Efficient in Stimulating the ATPase Activity. We did not observe a major difference in the stimulation by oligonucleotides; however, a significant difference was observed with closed circular ssDNA from M13mp19 bacteriophage ssDNA. The K_m for ATP increased 4-fold and the V_{max} increased 8-fold. It is not clear why the K_m for ATP increased so dramatically. It could be due to a very different mode of interaction of Mcm467 with a closed circular long ssDNA such as M13mp19 bacteriophage ssDNA.

It has been proposed earlier by You et al. (*35*) that small oligonucleotides such as oligo(dT)$_{35}$ can insert through the opening in the ring-shaped structure of Mcm467 complex. Similarly, we observed ssDNA stimulation of the ATPase activity involving small oligonucleotides, oligo(dT)$_{35}$ and oligo60. The binding of closed circular long ssDNA (ssDNA$_{cc}$) to Mcm467 may require more complex DNA binding mechanism. Previously, we have proposed a mechanism of loading of DnaB hexamer to ssDNA by the DnaC helicase loader of *E. coli* (*25*). At the origin of eukaryotic DNA replication, the Mcm467 complex may require a similar loader, such as the Cdc6p/Cdt1p protein complex. In the absence of a loading mechanism, Mcm467 complex may not readily form a productive Mcm467−ssDNA$_{cc}$ complex. However, the V_{max} in the presence of ssDNA$_{cc}$ clearly indicates that, once an appropriate complex is formed, the ATPase activity is significantly enhanced. This enhancement could be due to a processive migration of Mcm467 along a long ssDNA, which is an unlikely event on a short oligonucleotide.

Overall, the DNA-dependent ATPase activity of Mcm467 is low. However, Mcm467 helicase is likely the most evolved replicative DNA helicase. Therefore, we do not find it surprising that Mcm467 DNA helicase does not appear to use a lot of ATP to carry out its function. In terms of DNA helicase activity, it appears efficient. This efficiency and conservation of nucleotides could be important for the cell, as the size of the eukaryotic genome is large and the lengths of time replicative helicases have to work in each cell cycle is limited.

Preferential Activation of the ATPase and Helicase Activities of Mcm467 Complex by the ARS Sequences. ARS sequences are defined sequences where the pre-RC is known to form (*36*, *37*). As proposed by Bielinski and Gerbi (*13*, *31*), ORC protein complex binds to the A−B1 motifs in ARS, that may create a partially unwound structure similar to DnaA protein initiator of *E. coli* (*9*), and recruits other pre-RC proteins, including Mcm complexes and RPA. Mcm complex appears to bind to the single-stranded A−B1 motifs and initiates further duplex unwinding through its ATPase and DNA helicase activities (*13*). Therefore, it was important to understand the role of these sequences in the activation of

the ATPase activity of Mcm467 complex. Unlike higher eukaryotes, in the yeast *S. cerevisiae*, the ARS sequences are well-defined and their roles in activating Mcm467 complex can be examined precisely. Therefore, we examined both the A- and T-rich sequences spanning the A and B1 regions of ARS1. We also found that both A- and T-rich sequences are comparable in stimulating the ATPase activity of Mcm467 complex. Consequently, the preference for T-rich sequences observed here with yeast Mcm467 complex and earlier with mouse Mcm467 complex (*30*) is a phenomenon that could be due to factors unrelated to the origin function.

The DNA helicase activity of the Mcm467 complex was significantly stimulated by the A-rich ARS-A and the T-rich ARS-T containing duplexes compared to the $(dT)_{50}$ or $(dA)_{50}$ containing duplexes. Therefore, the ARS sequences activate both ATPase and DNA helicase activities of the Mcm467 complex. We have analyzed the nucleotide sequences of both strands of the 50 bp AB1 region of yeast ARS1, oligo(dT) sequences, and the 60 bp oligo(60) oligonucleotide, used in this study, for secondary structures or a fork-like structure that could explain the observed activation. We have examined these oligonucleotides using the Sybyl 6.7 molecular modeling program (TRIPOS associates, St Louis, MO). We did not find any unusual secondary structure in these oligonucleotides. Therefore, the nucleotide sequence is primarily responsible for such activation of the ATPase activity of the Mcm467 complex.

The ATPase activity of Mcm467 complex was also modulated significantly by the double-stranded ARS sequence (Figure 5). However, the extent of stimulation was much lower than the single-stranded ARS sequences. In addition, the kinetics was biphasic, whereas the kinetics observed with all ssDNAs were monophasic and distinctly different.

ARS sequences appear to be a better substrate for the DNA helicase activity of Mcm467 complex than an oligo(dA) sequence (Figure 5B). The origin of eukaryotic DNA replication has been defined clearly only in the yeast *S. cerevisiae*. In higher eukaryotes, the origin sequences are less pronounced and their role in such stimulation could be difficult to assess. Therefore, our results using the *S. cerevisiae* origin sequences, as presented here, are significant and applicable in understanding the roles of similar origin sequences from higher eukaryotes. However, further detailed studies are required to delineate the true impact of these sequences on the DNA helicase activity of Mcm467 complex.

In the Replication Fork, RPA May Regulate the ATPase Activity. Replicative DNA helicases work in concert with a large number of other replication proteins. Frequently, the enzymatic activities of these helicases are modulated, at least in part, by other replication proteins. Most importantly, the single-stranded DNA binding proteins, such as *E. coli* SSB, are known to modulate their cognate DNA helicases. In the case of *E. coli* DnaB helicase, SSB uniquely modulates its ATPase and DNA helicase activities (*34*). SSB inhibits the ssDNA-dependent ATPase activity of DnaB but stimulates its DNA helicase activity. Yeast RPA appears to stimulate the DNA-dependent ATPase activity of Mcm467, which is relatively weak, and thus, could be very significant (Figure 7B). Unlike Mcm467, DnaB has a strong ssDNA-dependent ATPase activity in the absence of its helicase function; therefore, the SSB attenuation of this idle ATPase activity of DnaB appears appropriate. On the other hand, Mcm467, with weak ssDNA-dependent ATPase activity, may require a stimulus from RPA, particularly in the replication fork during origin (or ARS) activation. Therefore, RPA- or RPA-coated ssDNA may actually promote ATPase activity and the movement of Mcm467 helicase on the ssDNA, perhaps to locate the nearest replication fork. Clearly, these two helicases, *E. coli* DnaB and yeast Mcm467, and their cognate SSBs appear to work somewhat differently in terms of their mechanisms of action.

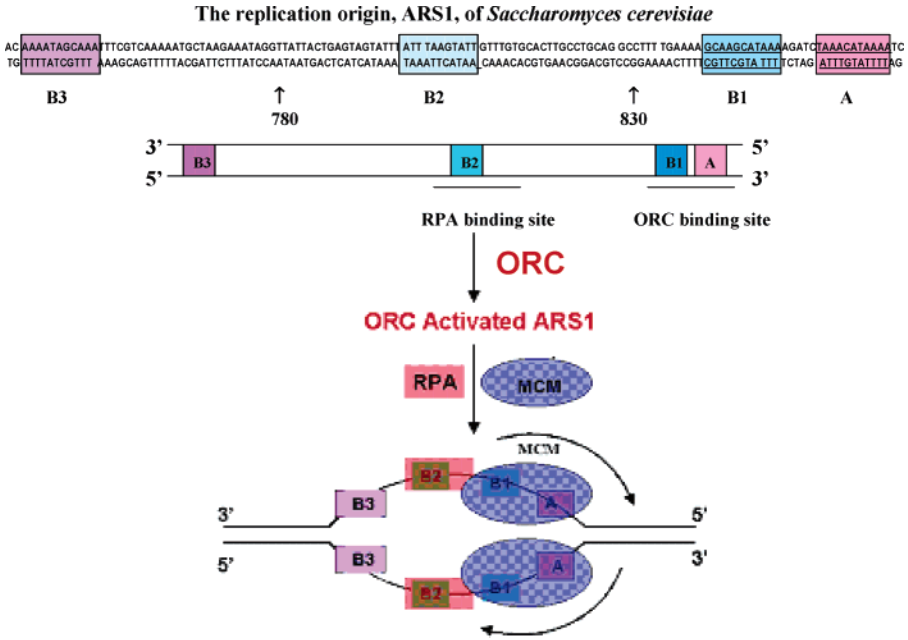

FIGURE 8: Scheme depicting the activation of the yeast origin of DNA replication, ARS1, and the role of Mcm467: (1) The ARS1 containing four A/T-rich elements (A, B1, B2, and B3) binds to ORC; (2) the ARS1 is then activated due to partial unwinding; (3) partially unwound A and B1 sequences allow Mcm complexes to bind, and RPA binds to the B1/B2 region; (4) ARS1 ssDNA sequences activate the ATPase activity of Mcm467; and (5) RPA activates the ATPase activities further and turns on the DNA helicase action of Mcm467.

CONCLUSIONS

The *S. cerevisiae* Mcm467 complex is activated by ssDNA, particularly those derived from the autonomously replicating sequences. Subunits of Mcm467 complex have ssDNA-dependent ATPase activities, with the exception of the Mcm4 subunit. Its cognate ssDNA binding protein, RPA, significantly stimulates its ATPase activity. ORC activation of the ARS origins of DNA replication leads to the binding of the MCM proteins to the unwound origin (*13*); ssDNA sequences of the unwound origin activate the enzymatic activities of the Mcm467 complex; and RPA bound to the unwound origin provides additional activation of the ATPase activity of Mcm467 complex (Figure 8).

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