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The Mcm Complex: Unwinding the Mechanism of a Replicative Helicase

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INTRODUCTION

Replicative DNA polymerases require a single-stranded DNA (ssDNA) template, yet many lack double-stranded DNA (dsDNA)-unwinding activity (reviewed in reference 127). Instead, ssDNA is generated by the replicative helicase, a motor protein that uses NTP-dependent conformational changes to unwind duplex DNA (197). Although in vivo evidence over the last 15 years has implicated the minichromosome maintenance (Mcm2-7) complex as the eukaryotic replicative helicase, the historical inability to observe in vitro helicase activity from this complex has provided a biochemical impasse toward an understanding of the mechanism and regulation of the eukaryotic replication fork. The recent demonstration that purified recombinant Mcm2-7 unwinds DNA in vitro (24) establishes that Mcm2-7, either alone or with additional positive in vivo activators (i.e., Cdc45 and the GINS complex [184]), is the eukaryotic replicative helicase. Combined with studies of the simpler archaeal Mcm complex, recent results suggest that contrary to other hexameric helicases, the six Mcm2-7 ATPase active sites contribute differentially to DNA unwinding. This review focuses on these recent biochemical advances; for additional background on the Mcm complex, the interested reader is directed to several previous reviews (9, 48, 80, 81, 169).

THE MCM HELICASE: AN AAA$^+$ PROTEIN ESSENTIAL FOR DNA REPLICATION

Discovery of the *MCM* Genes: Involvement in DNA Replication

The *MCM* genes were first identified in the yeast *Saccharomyces cerevisiae* by mutations in *MCM2*, *MCM3*, and *MCM5* that caused defective plasmid segregation (i.e., minichromosome maintenance) (161). *MCM4* (originally *CDC54*) and *MCM7* (originally *CDC47*) were isolated as cell cycle division mutants (102, 178), and *MCM6* was originally isolated in *Schizosaccharomyces pombe* as a chromosome segregation mutant (*mis5*) (243). Consistent with their involvement in a fundamental cellular process, all six of these genes are essential for viability (90, 218). To simplify and standardize the nomenclature, it was proposed that these six genes be renamed *MCM2* through *MCM7* (43); the name *MCM1* had already been given to a transcription factor (253).

Experiments with both yeast and *Xenopus laevis* demonstrated the involvement of the Mcm proteins in DNA replication. A cold-sensitive allele of *MCM4* that blocked DNA replication at nonpermissive temperatures was isolated (102). The identification of the six Mcm proteins as being active components of the *Xenopus* "replication licensing factor," a protein postulated to couple DNA replication to cell cycle progression (20), provided biochemical verification (41, 251).

The Eukaryotic *MCM* Genes Define Six Families of AAA$^+$ Motor Proteins

MCM genes are found in both eukaryotes and archaea (Fig. 1). In eukaryotes, each of the six *MCM2-7* genes defines a separate family (reviewed in references 80 and 155; M. Bochman, unpublished observations). However, the six eukaryotic Mcm proteins also share significant sequence similarity with one another, centering on but not restricted to a nearly 250-amino-acid region that encodes the ATPase active site (AAA$^+$ domain) (135). To date, all sequenced archaea contain at least one *MCM* homologue (128, 130), but all sequenced bacteria lack *MCM* genes. Interestingly, several bacteriophage genomes do contain *MCM* orthologues (Fig. 1). Based upon sequence similarity, two additional Mcm families have also been identified (Mcm8 [92, 118] and Mcm9 [271]) (Fig. 1). However, as they are not strictly conserved among eukaryotes (155) and appear to have controversial roles distinct from that of Mcm2-7 (e.g., see references 158 and 162), they will not be discussed here. There are also several additional genes identified in the same screen that are designated *MCM* but encode proteins with no homology to Mcm2-7 (161). These include Mcm10, another essential DNA replication factor (discussed further below), as well as others (e.g., Mcm17 [also known as Chl4] [211], Mcm21, and Mcm22 [199]) that function in chromosome segregation.

Both the archaeal Mcm proteins and the six subunits within Mcm2-7 are AAA$^+$ proteins (ATPases associated with a variety of cellular activities) (113, 135). This enzyme superfamily has functionally diverse roles that include not only RNA and DNA helicases, proteases, chaperones, and metal chelatases but many additional DNA replication factors such as processivity clamp loaders (e.g., replication factor C [38] and the γ complex [116]), initiator proteins (e.g., DnaA and the origin recognition complex [72]), and helicase loaders (e.g., DnaC [57] and Cdc6 [152]). The Mcm proteins, in common with many members of this group, oligomerize into ring-shaped (i.e., toroidal) complexes (23, 193) and use ATP binding and hydrolysis to manipulate a substrate (e.g., DNA) within their central channels. In contrast to the Mcm proteins, the other AAA$^+$ proteins involved in DNA replication lack a toroidal organization and function as molecular switches rather than molecular motors (for a detailed review of AAA$^+$ structural evolution, see reference 71).

AAA$^+$ proteins form ATPase active sites at clefts between two subdomains: one containing a series of loops connecting adjacent parallel β-strands (P loop) and a second positioned C terminal to the P-loop domain, called the lid (reviewed in reference 71). Both subdomains contain conserved active-site motifs: the P loop contains motifs involved in binding ATP (Walker A box) and orienting the nucleophilic water molecule (Walker B box and sensor 1), while the lid domain contains motifs that contact the γ-phosphate of ATP (arginine finger

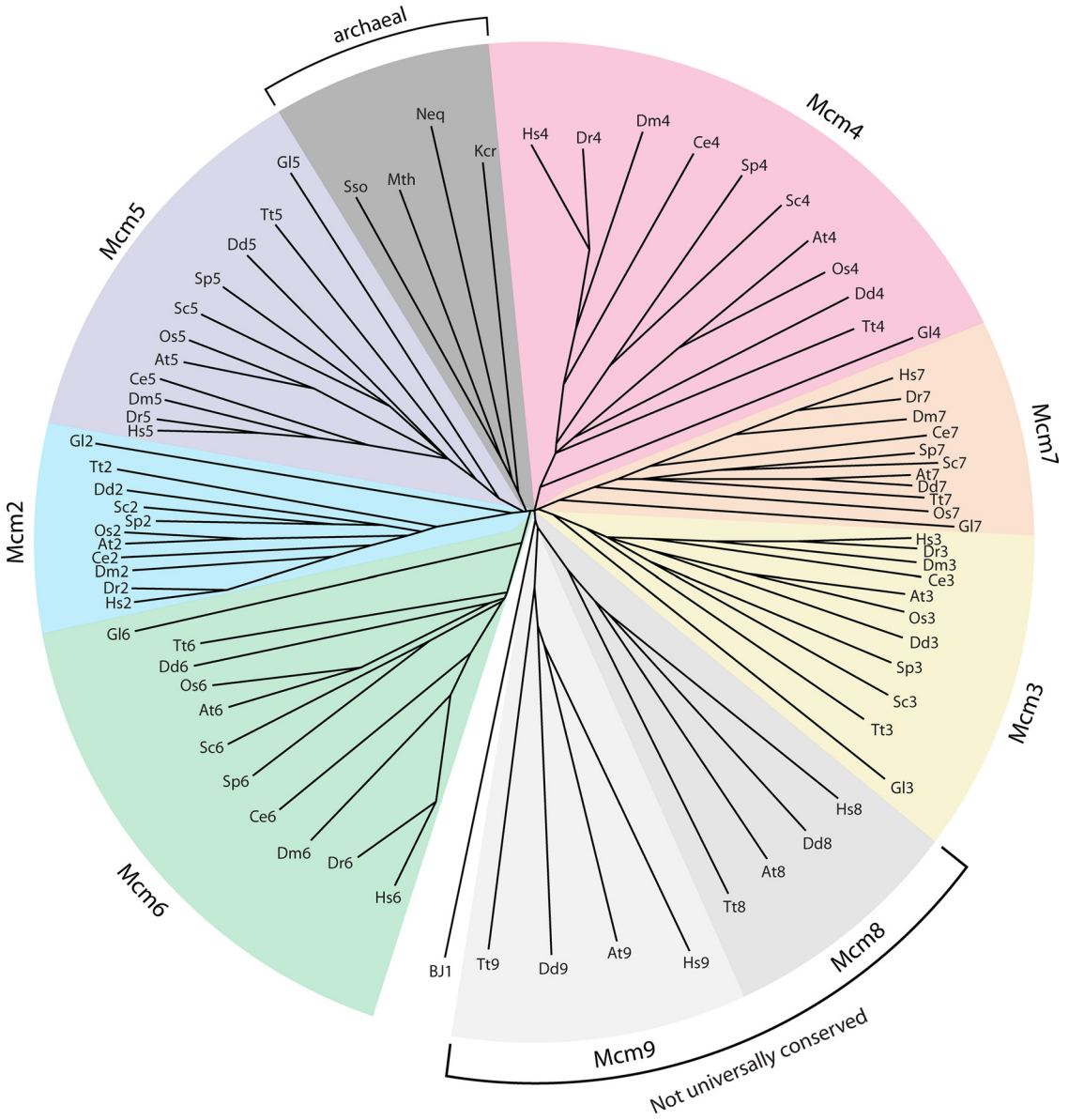

FIG. 1. Evolutionary relationship among the Mcm families. The indicated Mcm sequences were aligned using ClustalX (141), and the phylogenic relationships among them were drawn as an unrooted tree using the Drawtree interface of PHYLIP 3.67 (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=drawtree). It should be noted that although the archaeal Mcm proteins are frequently shown to be most closely related to Mcm4 (42, 129), our more extensive analysis using a larger number of Mcm sequences failed to duplicate these results. Mcm genes from the following organisms were used: *Homo sapiens* (Hs), *Danio rerio* (Dr), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Schizosaccharomyces pombe* (Sp), *Saccharomyces cerevisiae* (Sc), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Dictyostelium discoideum* (Dd), *Tetrahymena thermophila* (Tt), *Giardia lamblia* (Gl), *Sulfolobus solfataricus* (Sso), *Methanobacterium thermoautotrophicum* (Mth), "*Nanoarchaeum equitans*" (Neq), and "*Korarchaeum cryptofilum*" (Kc). Note that although bacteria lack the Mcm proteins, several phages possess an *MCM*-like gene (e.g., a *Bacillus cereus* prophage [173], the "*Haloarcula sinaiiensis*" archaephage HSTV-1 [R. Hendrix, personal communication], and the archaeal virus BJ1 [E. Pagaling et al., unpublished data; see http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=CAL92457]); the BJ1 Mcm homologue has also been included in Fig. 1. GenBank accession numbers are as follows: AtMcm2, NP_175112.2; AtMcm3, NP_199440.1; AtMcm4, NP_179236.3; AtMcm5, NP_178812.1; AtMcm6, NP_680393.1; AtMcm7, NP_192115.1; AtMcm8, NP_187577.1; AtMcm9, NP_179021.2; BJ1 Mcm, YP_919062; CeMcm2, NP_001022416.1; CeMcm3, NC_003283.9; CeMcm4, NP_490962.1; CeMcm5, NP_497858.1; CeMcm6, NP_001023011.1; CeMcm7, NP_504199.1; DdMcm2, XP_637579.1; DdMcm3, NC_007089.3; DdMcm4, NC_007088.4; DdMcm5, NC_007092.2; DdMcm6, NC_007088.4; DdMcm7, NC_007090.2; DdMcm8, XP_639313.1; DdMcm9, XP_637904.1; DmMcm2, NP_477121.1; DmMcm3, NP_511048.2; DmMcm4, NP_477185.1; DmMcm5, NP_524308.2; DmMcm6, NP_511065.1; DmMcm7, NP_523984.1; DrMcm2, NP_775364.1; DrMcm3, NP_997732.1; DrMcm4, NP_944595.1; DrMcm5, NP_848523.2; DrMcm6, NP_001076318.1; DrMcm7, NP_997734.1; GlMcm2, EAA40971.1; GlMcm3, EAA36979.1; GlMcm4, EAA40854.1; GlMcm5, EAA38067.1; GlMcm6, EAA40537.1; GlMcm7, EAA40792.1; HsMcm2, NP_004517.2; HsMcm3, NP_002379.2; HsMcm4, NP_005905.2; HsMcm5, NP_006730.2; HsMcm6, NP_005906.2; HsMcm7, NP_005907.3; HsMcm8, NP_115874.3; HsMcm9, NP_694987.1; Mcm_{Kc}, ACB08098.1; MthMcm, AAB86236.1; *Nanoarchaeum equitans* Mcm, AAR39132.1; OsMcm2, NP_001067910.1; OsMcm3, NP_001055835.1; OsMcm4, AP004232.4; OsMcm5, NP_001048396.1; OsMcm6, NP_001054989.1; OsMcm7, NP_001067020.1; ScMcm2, NP_009530.1; ScMcm3, NP_010882.1; ScMcm4, NP_015344.1; ScMcm5, NP_013376.1; ScMcm6, NP_011314.2; ScMcm7, NP_009761.1; SpMcm2, NP_595477.1; SpMcm3, NP_587795.1; SpMcm4, NP_588004.2; SpMcm5, XP_001713071; SpMcm6, NP_596614.1; SpMcm7, NP_596545.1; SsoMcm, AAK41071.1; TtMcm2, XP_001009217.2; TtMcm3, XP_001012838.1; TtMcm4, XP_001018113.1; TtMcm5, XP_001007780.1; TtMcm6, XP_001013258.1; TtMcm7, XP_001008253.2; TtMcm8, XP_001012433.2; TtMcm9, XP_001032062.2.

and sensor 2) (Fig. 2). Interestingly, AAA^+ active sites are not formed from subdomains on the same subunit but are rather formed at dimer interfaces, with one subunit contributing the P loop (*cis* motifs), while the adjoining subunit contributes the lid (*trans* motifs) (Fig. 2B). The purpose of nucleotide binding and hydrolysis in molecular motors is to promote conformational changes that perform mechanical work. Such a combinatorial active-site arrangement likely facilitates the coupling and propagation of conformational changes among the six active sites of toroidal hexamers (reviewed in reference 71), making the identification and study of *trans*-acting motifs of particular mechanistic interest.

In addition to the canonical active-site motifs found in all AAA^+ proteins, the Mcm proteins contain sequence insertions that are either unique to the Mcm proteins or specific to their particular AAA^+ clade (helix 2 insert clade) (Fig. 2A and C) (113). These additional insertions form β-hairpin "fingers," three of which protrude into the central channel of the complex (N terminal, helix 2 insert, and presensor 1 hairpins), while a fourth is purported to lie on the outside of the complex (external hairpin) (30, 76, 153, 174). At least some of these hairpins are likely to couple ATP binding and hydrolysis to DNA unwinding; structural analysis of the simian virus 40 (SV40) large T antigen (TAg) (a related AAA^+ hexameric helicase) reveals a 17-Å movement of the presensor 1 hairpin in response to the nucleotide occupancy of the adjacent active site (84). In addition, the Mcm proteins contain zinc finger motifs near their N termini (76, 78, 269) that are not directly involved in DNA binding but rather stabilize the folding of their N-terminal domain (see below).

Although in other AAA^+ proteins, sensor 2 functions in *cis*, the Mcm proteins contain a sequence insertion N terminal to sensor 2 (presensor 2) that allows sensor 2 to function in *trans* (30, 71, 180). This presensor 2 insert is one of the few regions of significant sequence divergence within the ATPase domain among the six eukaryotic Mcm proteins (Fig. 3). This insertion can be conveniently viewed as having two parts: a conserved region that contains limited similarity among the Mcm families (but high sequence conservation within any single Mcm family) and a highly charged spacer region (Bochman, unpublished). Although this spacer region demonstrates little or no sequence conservation either among or between Mcm families, the length of the spacer is reasonably well conserved and varies between Mcm families. Except for *Giardia* sequences (which align poorly within this region), spacer lengths range from ~5 amino acids (Mcm6 and Mcm7) to >50 amino acids (Mcm3). While the specific sequence is not conserved, Mcm3 does have a conserved patch of positively charged residues (basic patch) within the presensor 2 insert. These group-specific differences in the presensor 2 insert may account at least in part for the active-site-specific differences within Mcm2-7 that will be discussed later in this review.

Mcm2-7 as the Replicative Helicase

In common with bacterial and viral replicative helicases, Mcm2-7 is required for both initiation and elongation, with its regulation at each stage being a central feature of eukaryotic DNA replication. During the G_1 phase of the cell cycle, Mcm2-7 is transported to origins of replication in an inactive state by Cdt1 to form the prereplication complex (pre-RC) (reviewed in reference 14), a higher-order assembly that additionally contains the AAA^+ proteins Cdc6 and the heterohexameric origin recognition complex (Orc1-6) (Fig. 4A) (4, 14). All six Mcm subunits colocalize to origins of replication during pre-RC formation (3, 4, 148, 266); moreover, the inactivation or loss of any of the six Mcm subunits during G_1 phase blocks pre-RC formation in vivo in yeast (140) and in vitro with *Xenopus* extracts (170, 201). The loading of Mcm2-7 onto DNA is an active process requiring ATP hydrolysis by both Orc1-6 and Cdc6 (26, 205). Significantly, the purpose of pre-RC formation is to load the Mcm proteins onto DNA; once accomplished, Orc1-6 and Cdc6 are no longer required for Mcm2-7 retention at the origin (26, 65, 67), and they are dispensable for subsequent DNA replication (106, 210).

Upon entry into S phase, the activity of the cyclin-dependent kinases (CDKs) and the Dbf4-dependent kinase (DDK) Cdc7 promotes the assembly of replication forks (14, 190), likely in part by activating Mcm2-7 to unwind DNA (Fig. 4B). Pre-RC activation by these kinases results in the loading of additional replication factors of currently unknown function, such as Cdc45 (120), the GINS complex (245), and Mcm10 (207). Following DNA polymerase loading (171), bidirectional DNA replication commences. As demonstrated by chromatin immunoprecipitation experiments, all six Mcm subunits colocalize with the DNA polymerases during elongation (3, 4, 148, 266). Moreover, the inactivation of any of at least five of the six Mcm subunits (the participation of Mcm5 could not be studied for technical reasons) during S phase quickly blocks ongoing elongation (140). As a critical mechanism to ensure only a single round of DNA replication, the loading of additional Mcm2-7 complexes into pre-RCs is inactivated by redundant means after passage into S phase (Fig. 4C) (reviewed in reference 6).

Mcm2-7 activity can also be regulated during elongation. The loss of replication fork integrity, an event precipitated by DNA damage, unusual DNA sequence, or insufficient deoxyribonucleotide precursors, can lead to the formation of DNA double-strand breaks and chromosome rearrangements (156, 163, 195, 235, 250). Normally, such replication problems trigger an S-phase checkpoint that minimizes genomic damage by blocking further elongation and physically stabilizing protein-DNA associations at the replication fork until the problem is fixed (29, 156, 248). Although the mechanistic details are unknown, this stabilization of the replication fork requires the physical interaction of Mcm2-7 with Mrc1, Tof1, and Csm3 (M/T/C complex) (Fig. 4C). In the absence of these proteins, dsDNA unwinding and replisome movement powered by Mcm2-7 continue, but DNA synthesis stops at least in part due to the dissociation of polymerase ε from the replication fork (10, 85, 126).

As many aspects of eukaryotic DNA replication remain experimentally intractable, some details have been inferred using the simpler archaeal system. In addition to the Mcm proteins, the archaea encode homologues of additional eukaryotic replication factors (11, 255). Among pre-RC components, all currently sequenced archaeal genomes contain between one and three genes that code for proteins equally related to both the eukaryotic Orc1 and Cdc6 proteins. Structural work has shown that these proteins generally have N-terminal AAA^+ domains and C-terminal winged-helix domains, and both appear to aid

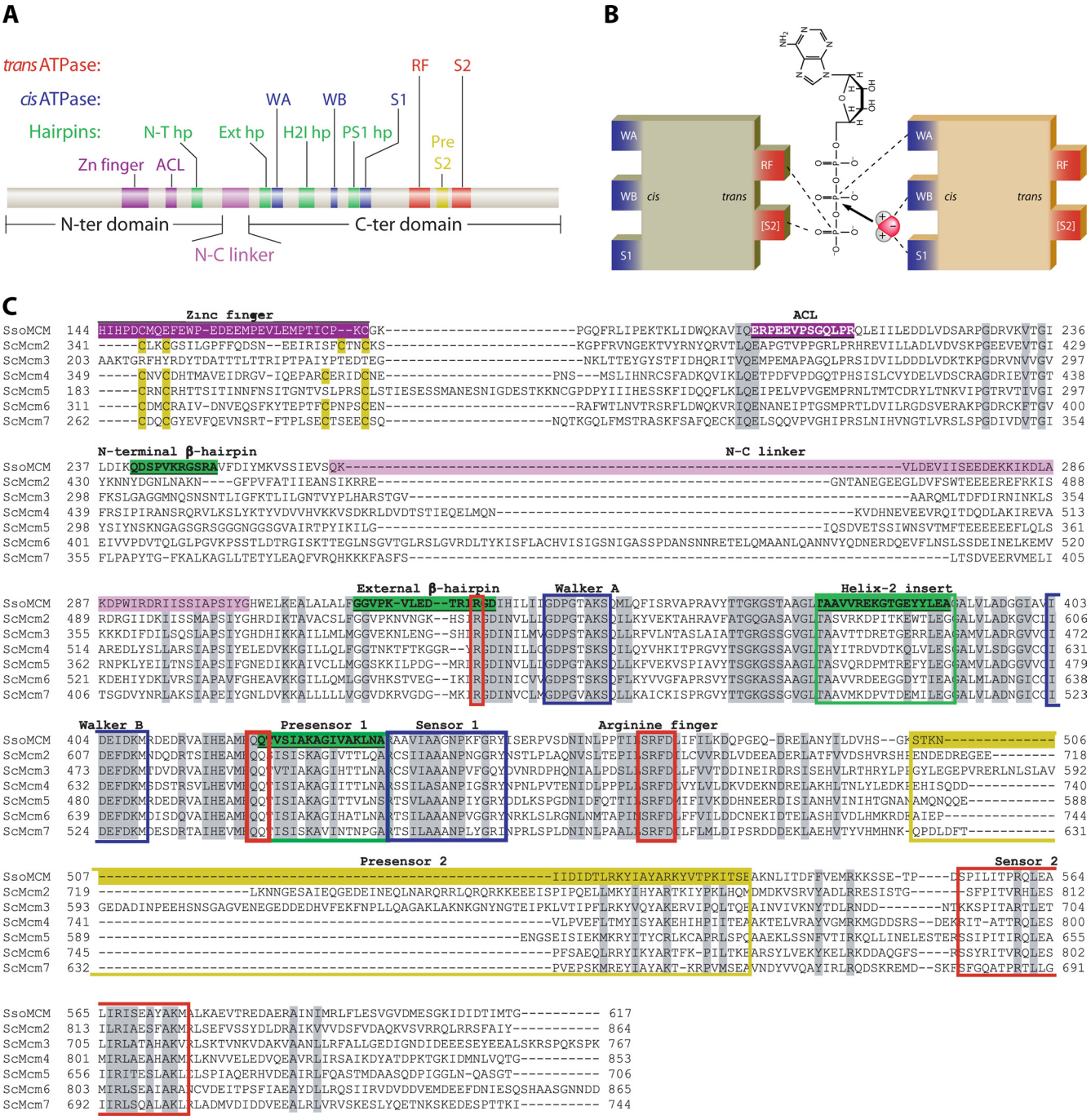

FIG. 2. Organization of the Mcm structural motifs. (A) Cartoon showing the domain structure and linear organization of SsoMcm. Purple denotes structural elements, green denotes β-hairpins, blue denotes *cis*-acing ATPase elements, red denotes *trans*-acting ATPase elements, and yellow denotes the presensor 2 insertion. N-T hp, N-terminal β-hairpin; Ext hp, external β-hairpin; WA, Walker A motif; H2I, helix 2 insert β-hairpin; WB, Walker B motif; PS1 hp, presensor 1 β-hairpin; S1, sensor 1; RF, arginine finger motif; Pre-S2, presensor 2 insertion; S2, sensor 2. (B) AAA^+ active sites are formed at the interface between adjacent subunits. The Walker A, sensor 1, and Walker B motifs act in *cis* while the arginine finger and sensor 2 motifs act in *trans* to hydrolyze ATP. The nucleophilic water molecule is oriented by sensor 1 and the Walker B motif. Note that the *trans* arrangement of the sensor 2 motif appears to be specific for the Mcm subclade of AAA^+ proteins. (C) Shared motifs among the Mcm proteins. Abbreviations and color coding are the same as described above (A). The *S. solfataricus* (SsoMcm), *G. lamblia* Mcm2-7, *S. cerevisiae* Mcm2-7 (ScMcm2-7), and human Mcm2-7 protein sequences used to generate Fig. 1 were aligned with CLUSTALW (141). The *G. lamblia* and human sequences, the remaining gaps shared by the seven displayed sequences, and the nonconserved N- and C-terminal regions were then removed due to spatial constraints. Residues within the zinc finger predicted to be important for coordinating Zn^{2+} are highlighted in yellow. Residues conserved among the 19 sequences in the original alignment are shaded in gray.

FIG. 3. Alignment of Mcm presensor 2 insertions. The presensor 2 inserts (defined in the legend of Fig. 2C) were excised from the Clustal alignment of all 60 eukaryotic Mcm proteins used to generate Fig. 1. The *G. lamblia* (Gl) proteins align poorly with the related Mcm proteins in this region, and thus, the corresponding Mcm2 and Mcm3 sequences have been truncated for presentation purposes (excised regions marked with the number of amino acids removed in parentheses). Vertical green bars denote conserved amino acid identities (conserved, 9 of 10 sequences within any Mcm group), and yellow corresponds to conserved amino acid similarities (again, at least 9 out of 10 sequences) according to the following groups: aromatic (F, T, and W), hydrophobic (L, I, and V), hydrophilic (S, T, and C), basic (K, R, and H), and acidic (E and D). Within the spacer region, red corresponds to acidic residues, while blue corresponds to basic residues. Asterisks indicate residues with either conserved identities or similarities shared among all six Mcm groups.

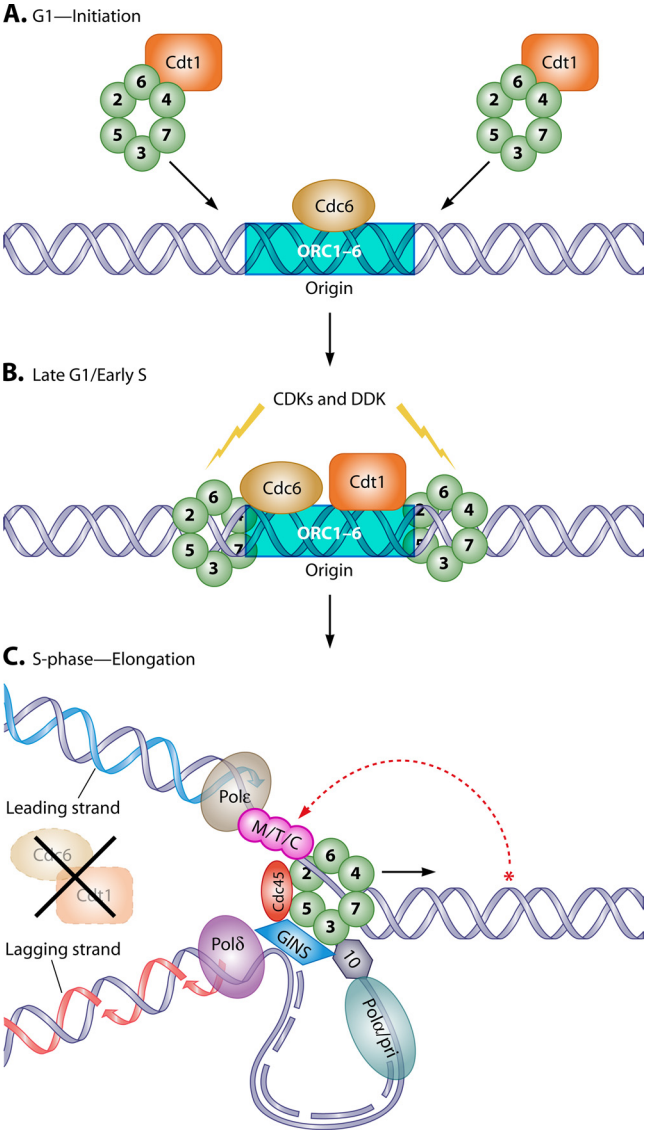

FIG. 4. Mcm involvement in eukaryotic DNA replication initiation and elongation. (A) During G_1 phase, Cdc6 and Cdt1 recruit and load Mcm2-7 to origins of replication (marked by the binding of Orc1-6) to form a stable and inactive complex called the pre-RC. (B) In late G_1/early S phase, the pre-RC is somehow activated for DNA unwinding by the CDKs and DDK. This facilitates the loading of additional replication factors (e.g., Cdc45, Mcm10, GINS, polymerase α/primase, and DNA polymerases δ and ε) and unwinding of the DNA at the origin (not shown). (C) During S phase, Cdc6 and Cdt1 are degraded or inactivated (represented by dashed outlines) to block additional pre-RC formation, and bidirectional DNA replication ensues. For diagrammatic purposes, only one replication fork is shown, and many replication factors (e.g., the processivity clamp [PCNA], its loader [replication factor C], and factors required for Okazaki fragment processing, etc.) are omitted. When the replication fork encounters lesions in the DNA (red asterisk), the S-phase checkpoint response (via the Mrc1/Tof1/Csm3 [M/T/C] complex) slows or stops fork progression and stabilizes the association of Mcm2-7 with the replication fork during DNA repair.

in local DNA unwinding/melting at origins of replication (33, 66, 86, 94). These proteins have variously been referred to as Orc, Cdc6, or Orc/Cdc6 (with prefixes and suffixes to indicate species and numbering, respectively), but for the sake of simplicity, we will subsequently refer to them as Cdc6 homologues.

Mechanistic Considerations: How Helicases Unwind DNA

To understand current Mcm research, knowledge of general helicase properties is a useful prerequisite. As this topic has been the subject of several recent excellent reviews (70, 232), only a brief summary of the relevant information will be provided here.

Subunit oligomerization and DNA substrate requirements. Helicases are a common and very diverse group of enzymes (16, 232); *S. cerevisiae*, for example, has 134 open reading frames (2% of its genome) that encode proteins containing helicase structural motifs (230). Most helicases are monomeric or dimeric, demonstrating that DNA unwinding per se does not require a toroidal hexamer. As replicative helicases are typically hexameric (but not necessarily, e.g., the dimeric herpesvirus helicase [36]) and bind DNA within their central channel, this ring-shaped topology likely assists in lengthy chromosome unwinding without dissociation (i.e., increased processivity). In contrast, monomeric/dimeric helicases that are dedicated to the repair of short DNA lesions have little need for extended DNA associations. Helicases also differ in their intrinsic abilities to oligomerize. Some hexameric helicases (i.e., DnaB [276]) oligomerize in solution and thus require specific factors to load them onto chromosomes, while others oligomerize only upon binding ATP (i.e., SV40 TAg [84]) and are thus able to self-assemble around DNA (59).

As a fundamental aspect of DNA unwinding, helicases translocate along either single-stranded or double-stranded substrates. Those that bind ssDNA typically demonstrate a preferred directionality (either 3′→5′ or 5′→3′); for replicative helicases, this corresponds to specific translocation along either the lagging (i.e., DnaB) or leading (e.g., SV40 TAg) strand (197). In contrast, dsDNA is structurally symmetric, and helicases that transverse this substrate often lack a preferred directionality (197).

Mechanistic issues specific to hexameric helicases. Except for the eukaryotic Mcm2-7 complex, all currently known hexameric helicases are formed from six copies of an identical subunit (homohexamers), a feature leading to the understandable notion that each component active site participates equally in helicase activity. In contrast to monomeric/dimeric helicases, the presence of multiple active sites immediately suggests that their regulated and coordinate involvement might be required for DNA unwinding. However, despite several high-resolution crystal structures of a variety of homohexameric helicases (1, 18, 21, 30, 69, 149, 172, 176, 197, 232, 260), there is yet no universal agreement on the mechanistic details needed for DNA unwinding.

(i) Coordination between ATP hydrolysis and DNA unwinding. As molecular motors, helicases unwind nucleic acids by coupling the conformational changes caused by nucleotide binding and hydrolysis to the physical manipulation of nucleic acids. The toroidal nature of hexameric helicases suggests the possibility that they could function by coupling an endless sequential wave of ATP hydrolysis to conformational changes around the ring. Thus, the study of ATP hydrolysis provides important mechanistic clues about helicase function. As most helicases exhibit ATP-dependent ssDNA binding, ATP binding apparently causes a conformational change that facilitates DNA binding. In contrast, the hydrolysis of ATP or its dis-

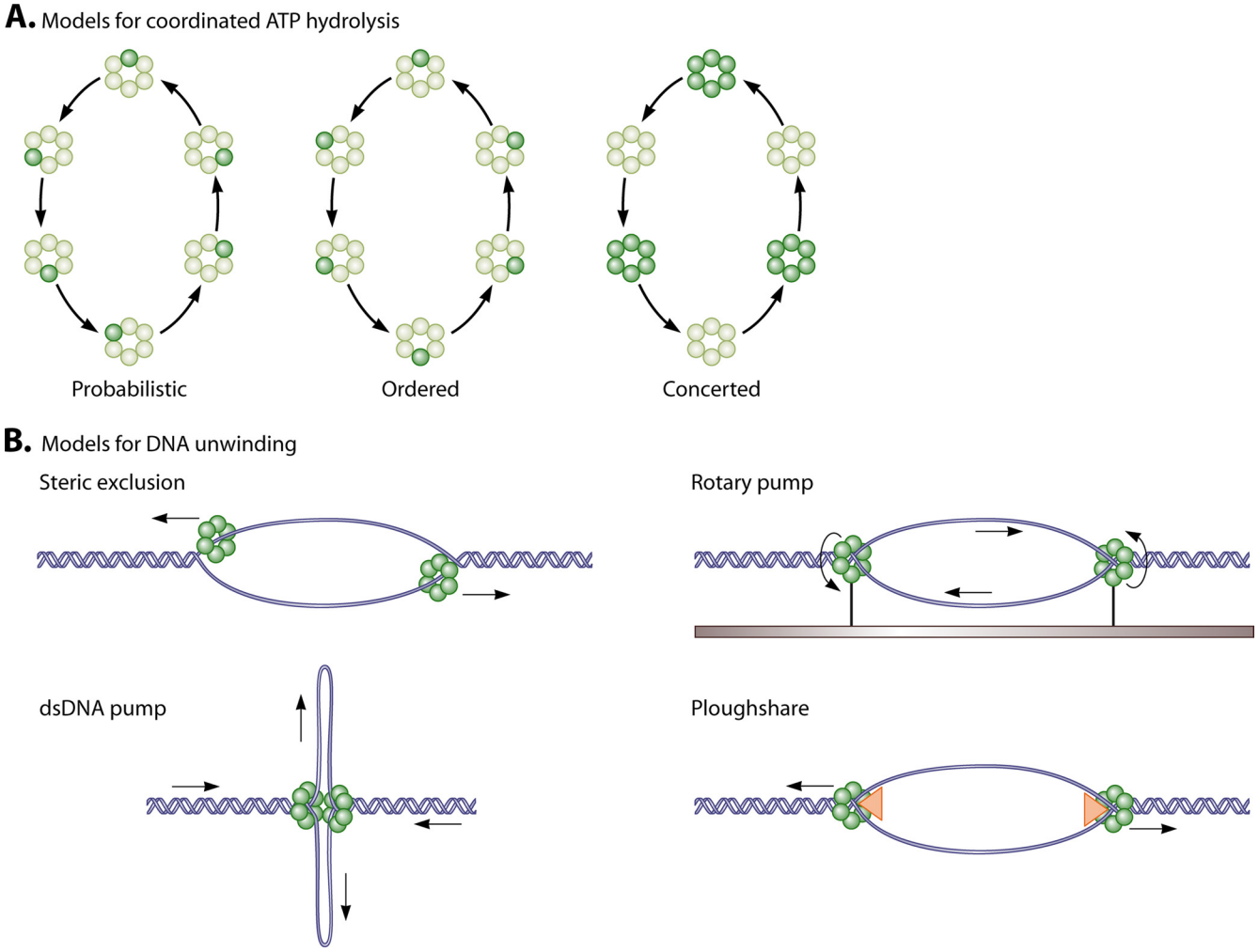

FIG. 5. Mechanistic models of hexameric helicase function. (A) Coordination of ATP hydrolysis. In each model, circles represent ATPase active sites, and the alternating position of the dark green circles represents the location of ATP hydrolysis as a function of time within the respective hexamers. (B) Models for DNA unwinding. (Adapted from reference 244 with permission from Elsevier.) In the steric-exclusion model, the helicase encircles and translocates along one strand of ssDNA and unwinds the duplex DNA by the exclusion of the other strand. In the rotary-pump model, the helicases load at origins and translocate away from them, where they are eventually anchored (dark vertical lines). They then rotate the intervening DNA in opposite directions, causing the unwinding of the origin. In the dsDNA pump model, two helicases form a head-to-head complex and pump dsDNA toward the origin, where it is extruded as single strands. In the ploughshare model, the helicase encircles dsDNA and, after local melting of the DNA duplex at the origin, "drags" a rigid protein or protein domain (triangle) that acts as a wedge to separate the DNA strands. Arrows indicate the direction of DNA and/or helicase movement.

placement from the active site leads to a conformation that is less able to bind DNA. Consistent with these findings, the addition of DNA to many helicases stimulates their ATP hydrolysis (197).

Although some AAA^+ proteins demonstrate little coordination of ATP hydrolysis among component active sites (probabilistic model, e.g., see reference 166) (Fig. 5A, left), this is believed to be uncommon with helicases; studies of ATP hydrolysis as a function of ATP concentration often demonstrate that nucleotide binding or hydrolysis is cooperative (e.g., see reference 31). In addition, "mixing experiments" with wild-type and mutant subunits often demonstrate that helicase activity or ATP hydrolysis can be "poisoned" by the inclusion of a single inactive subunit (53). Additionally, available helicase-nucleotide costructures have a defined arrangement of nucleotide occupancy (e.g., T7 helicase [233]). These studies suggest a specific ATP hydrolysis order within the hexamer, usually postulated to be either sequential (Fig. 5A, middle) (239) or concerted (Fig. 5A, right) (84) in nature.

(ii) Mechanism of DNA unwinding. A further consideration is how a hexameric helicase physically unwinds DNA. To explain a large variety of both in vitro and in vivo data, two broad types of models have been proposed: (i) "steric" models, in which the helicase tightly translocates along one strand of DNA while physically displacing the complementary strand, and (ii) "pump" models, in which pairs of hexameric helicases unwind duplex DNA by either twisting it apart (rotary models) or extruding it through channels in the complex (dsDNA pump) (Fig. 5B) (discussed in reference 244).

(*a*) *Steric models*. Steric models represent the more tradi-

tional view of how a hexameric helicase may function (Fig. 5B) (122). Most hexameric helicases bind ssDNA with higher affinity than dsDNA and can unwind DNA using this method in vitro (197). Moreover, the recent cocrystal structure of the papillomavirus E1 helicase with ATP and ssDNA strongly suggests that E1 couples sequential ordered ATP binding and hydrolysis to the sequential "escort" of individual bases through the central channel of the hexamer (69). A variation of the steric model is the ploughshare model, which is based partially upon studies of the trimeric RecBCD helicase (212, 231). This model postulates that the helicase encircles dsDNA and, after local melting of the duplex DNA at the origin, translocates away from the origin, dragging a rigid proteinaceous "wedge" (either part of the helicase itself or another associated protein) that separates the DNA strands (Fig. 5B).

(*b*) *Pump models*. In contrast to the steric models, recent experiments suggested that hexameric helicases may directly rotate DNA. Mechanistic similarities between hexameric helicases and the F_1-ATPase, an unrelated hexameric ATPase that couples ATP binding and hydrolysis to the rotation of a component protein within its central channel, have been observed (104, 218). Structural work, particularly with SV40 TAg (84, 149), verifies that the central channel of some hexameric helicases is wide enough to accommodate dsDNA. In addition, six channels of sufficient diameter to enclose ssDNA lay roughly perpendicular to the main channel in TAg and connect the central channel to the exterior of the protein, raising that possibility that ssDNA could be extruded through them (84, 149). Experiments using cleverly constructed substrates indicate that such helicases are capable of traversing dsDNA (122, 124, 227). Moreover, data from in vivo experiments suggest that helicases may unwind DNA at sites distant from where they are bound (142).

Several specific models have been proposed to account for these observations (Fig. 5B). The rotary-pump model postulates that multiple helicases load at replication origins, translocate away from one another, and in some manner eventually become anchored in place (142). They then rotate dsDNA in opposite directions, resulting in the unwinding of the double helix in the intervening region. Experimental evidence consistent with this model includes the finding that pre-RCs may contain up to 50-fold more Mcm2-7 complexes than Orc1-6, a finding at odds with the standard one-helicase-per-fork steric model (26, 67, 205).

In contrast, considerable biochemical data (74), "bunny ear" electron micrographs (262), and a high-resolution X-ray crystal structure of SV40 TAg favor the dsDNA pump model (256). This model postulates that two helicases form a head-to-head complex and, through a concerted all-or-nothing mode of ATP hydrolysis, translocate dsDNA through their central channels, followed by the extrusion of ssDNA from their side channels (84, 149). This model is based upon the mechanism of RuvAB, a prokaryotic AAA^+ enzyme and close relative to the Mcm complex that facilitates the branch migration of Holliday junctions (268). The in vitro ability of *Escherichia coli* DnaB and a specific Mcm subcomplex (i.e., Mcm467 [see below]) to unwind synthetic Holliday junction substrates provides support for this model (122, 123).

(iii) Considerations for DNA loading and helicase activation. Although the structure and unwinding mechanism of hexameric helicases is clearly important, an equally important but somewhat neglected issue concerns the manner in which they load onto DNA and assume an unwinding-competent conformation. This event requires at least two steps: (i) the origin DNA is melted to form ssDNA from dsDNA, and (ii) the helicase must be modeled/remodeled so that DNA is moved either into its central channel or possibly through various side channels. The details of these events differ among helicases. Although some helicases catalyze both steps (i.e., E1 [216] and SV40 TAg [reviewed in reference 73]), most others, such as the *E. coli* DnaB helicase (reviewed in reference 182), require help from additional proteins to localize the helicase to replication origins and melt duplex DNA (i.e., initiator proteins) and to then remodel the helicase to allow it to be loaded around the DNA (i.e., helicase loaders). Although replication origins differ widely between prokaryotes and eukaryotes, the base composition itself contributes to helicase loading, as most origins contain A-T-rich regions that likely facilitate initial DNA melting (55, 182, 187).

Viral E1 helicase represents a well-understood paradigm for a self-loading hexameric helicase. E1, like the related helicase SV40 TAg, forms a double hexamer at the viral replication origin but does not exist as stable toroidal hexamers in solution (216). In addition to binding DNA within its central channel following hexamerization (in this case, largely through the presensor 1 hairpin [154]), each E1 monomer also binds DNA in a sequence-specific manner using a different domain on the protein, an ability that channels the loading of E1 onto viral replication origins. The process of E1 loading and activation occurs in two stages: (i) the loading of a (double) trimer of E1 monomers around the DNA at origins in an ATP-dependent manner, followed by (ii) the formation of the final (double) hexamer upon ATP hydrolysis, with local DNA unwinding occurring coincident with (double) hexamer formation (216). Thus, the oligomerization of E1 into a toroidal hexamer neatly accomplishes both the transfer of DNA to the central channel of the hexamer as well as the melting of the origin of replication. Origin melting during initiation and DNA unwinding that occurs during elongation appear to be separable events with this enzyme insofar as mutations in the presensor 1 hairpin of E1 that have specific defects in melting but not DNA unwinding exist (154).

Although more complex, the loading of the *E. coli* DnaB replicative helicase has been extensively studied. Unlike E1, DnaB forms stable toroidal hexamers under dilute conditions (215, 265), necessitating the need for both a helicase loader (DnaC) and an initiator protein (DnaA) in DNA loading. Although the biochemistry of *E. coli* replication is well known (reviewed in reference 136), recent structural work has provided new mechanistic details concerning both helicase activity and DNA remodeling at the *E. coli* origin of replication (*oriC*). Both DnaA and DnaC are closely related AAA^+ proteins; however, unlike other AAA^+ proteins, they contain an additional alpha helix (113), named the initiator/loader-specific (ISM) motif (66), that is found at a position in these proteins that roughly corresponds to the location of the helix 2 β-hairpin in the Mcm proteins. Both DnaA and DnaC function as oligomers (83, 132), and their structural analysis has shown

that the ISM motif forms a "steric wedge" that causes both proteins to oligomerize into a right-handed helical filament rather than a closed toroidal structure as commonly observed for other AAA^+ proteins (72, 183).

The resulting DnaA or DnaC filament in each case has possible biological consequences: (i) with DnaA, the binding of a right-handed DnaA filament to dsDNA induces negative writhe and thus local DNA unwinding (72), an activity catalyzed at *oriC* by DnaA (27), and (ii) likewise, the binding of a helical DnaC oligomer to a hexameric DnaB ring might force DnaB to change conformation from a closed toroid to an open lockwasher, facilitating ssDNA passage into its central channel (183). These observations for bacteria may also extend to eukaryotic DNA replication, as Orc1-6 and Cdc6 contain the ISM and are orthologous to both DnaA and DnaC (113). Moreover, structural modeling based upon electron micrographs suggests that the Orc1-6/Cdc6 complex can also assume a right-handed helical form (45, 236). However, despite the obvious structural similarities and functional analogies between prokaryotic and eukaryotic DNA replication, relatively few mechanistic features of Mcm2-7 loading are known (see below).

In the absence of compelling experimental data, models for Mcm2-7 loading must again be considered. It should be noted that the DNA-unwinding scenarios presented in Fig. 5B imply differences in helicase loading and activation. Since both the steric and rotary-pump models are predicted to productively bind only one form of DNA (ssDNA for the steric model and dsDNA for the rotary pump), Mcm2-7 could be minimally loaded onto the correct DNA substrate in a single step. As Mcm2-7 forms hexamers in solution (reviewed in reference 14), both models would likely require a helicase loader, an activity putatively contributed by Cdc6. Additionally, for the steric model, ssDNA would need to be formed prior to Mcm2-7 loading. To date, however, Orc1-6-dependent DNA melting has not been observed in vitro (206), and the demonstration of ssDNA prior to the onset of elongation in vivo has been largely inconclusive (however, see reference 88). This failure to observe Orc1-6-dependent DNA melting could reflect a limitation of current assays, may indicate that other replication factors (possibly even Mcm2-7 itself) facilitate DNA melting, or may suggest that unlike DnaB, Mcm2-7 loads onto dsDNA.

In contrast to either the steric-exclusion or rotary-pump models, Mcm loading and activation according to both the dsDNA pump and ploughshare models would likely require additional steps. These two models predict functional interactions between the helicase and both ssDNA and dsDNA. Moreover, these interactions are predicted to require different channels within the Mcm complex. This complicated juxtaposition between helicase and DNA would necessitate both a considerable degree of subunit rearrangement to allow the appropriate DNA strand(s) to be threaded into the appropriate channels and a concomitant input of additional energy. Conceivably, the necessary subunit remodeling could be contributed by known or currently unknown components of the pre-RC, or alternatively, protein chaperones might function in the subunit rearrangements. Although there are no current indications of a requirement for protein chaperones in eukaryotic DNA replication, such components are required for other DNA replication systems (e.g., *E. coli* Hsp70 is needed for phage λ DNA replication [280]).

BIOCHEMISTRY OF THE ARCHAEAL Mcm HELICASE

Largely due to the complicated nature of the eukaryotic Mcm2-7 complex, the most detailed examinations of Mcm biochemistry have almost exclusively come from the more facile homohexameric archaeal *Methanothermobacter thermoautotrophicus* Mcm (MthMcm) and *Sulfolobus solfataricus* Mcm (SsoMcm) complexes. As DNA unwinding is the culmination of a variety of simpler biochemical activities—oligomerization, ATP binding and hydrolysis, DNA binding, and translocation (reviewed in reference 197)—archaeal Mcm studies that focus on these activities provide important mechanistic details and serve as a field guide for the current in vitro exploration of Mcm2-7. These results will be discussed below and are summarized and compared to results for Mcm2-7 in Table 1.

ATP Hydrolysis and Allosteric Interactions

Many groups have shown that both the MthMcm (77, 78) and SSoMcm (13, 34, 174, 203, 204) complexes hydrolyze ATP. As is typical for DNA helicases, but in contrast to Mcm2-7 (22, 58, 218), ATP hydrolysis is stimulated by the addition of either ssDNA or dsDNA (42, 125, 174).

Subunit-mixing experiments have successfully identified the functional ATPase motifs within the SsoMcm complex as well as their contribution to DNA unwinding. Prior studies indicated that free archaeal Mcm subunits are in equilibrium with the hexameric form (174), providing an experimental method to easily generate hexameric complexes containing various combinations of wild-type or mutant subunits. Mixing a population of subunits containing *cis*-acting mutations (e.g., Walker A or B mutants) with a population containing *trans*-acting mutations (e.g., arginine finger mutants) will reconstitute both doubly mutant active sites as well as wild-type ATPase active sites, resulting in measurable ATP hydrolysis that is absent in either single mutant population. In combination with known *cis* or *trans* ATPase mutations, this experimental approach provides a way to operationally identify new *cis* or *trans* motifs. Moreover, combining mutant and wild-type subunits over a range of ratios provides information on the number of functional active sites required for DNA unwinding or ATP hydrolysis.

This approach has been used to study the SsoMcm ATPase active sites (180). Consistent with other AAA^+ ATPases (113), mutant analysis indicates that the Walker A and B and sensor 1 motifs function in *cis* and that the arginine finger motif functions in *trans*; all are similarly essential for ATP hydrolysis (Fig. 6) (180). However, additional *trans*-acting motifs that are essential to ATP hydrolysis were identified. These include sensor 2 and conserved amino acids that map immediately N terminal to the Walker A and presensor 1 insert motifs (SsoMcm R331 [within the external β hairpin] and QQ423/24, respectively) (Fig. 2C). By reference to the structure of SV40 TAg (84) as well as a recent structure of the SsoMcm complex (30), these additional *trans* motifs not only are well positioned to catalytically assist ATP hydrolysis but also are good candi-

TABLE 1. Comparison of the biochemical activities of the archaeal Mcm and eukaryotic Mcm467 and Mcm2-7 complexes[a]

Activity	Description or value (reference[s] or source)		
	Archaeal Mcm proteins	Mcm467 (hexamer)	Mcm2-7
Oligomerization	Homohexamer for AfuMcm (95), ApeMcm (7), PfuMcm (272), SsoMcm (34), and TacMcm (100); homododecomer for MthMcm (42, 129)	Heterotrimer (M. Davey, personal communication), dimeric heterotrimer (109, 144)	Heterohexamer (2, 144, 218)
ATP hydrolysis	SsoMcm K_m = 280 ± 50 nM; turnovers/min = 3.1 ± 0.2 (30); SsoMcm K_m = 51 ± 9 μM (203)	K_m = 170 ± 6 μM; turnover/min = 0.3 ± 0.05 (19)	*S. cerevisiae*, 3 hydrolysis modes (218); K_m = 85 ± 10 μM and turnovers/min = 20 ± 3 for 1st mode, K_m = 0.9 ± 0.04 mM and turnovers/min = 48 ± 1 for 2nd mode, and K_m = 5–20 mM and turnovers/min = 156 ± 50 for 3rd mode
Stimulation of ATP hydrolysis by ssDNA or forked DNA substrate (fold increase)	SsoMcm, 1.3–1.8 (30); no stimulation (34, 203)	~4 (109)	None (58, 218, 273)
	TacMcm, 1.5–2 (98)	~3–18 dependent upon ssDNA base content (19)	
	MthMcm, 4–13 (125, 129, 200)	~2 (121)	
Stimulation of ATP hydrolysis by dsDNA (fold increase)	TacMcm, none (98)	~2–5 (109, 145)	None (A. Schwacha, unpublished data)
	SsoMcm, none (203)	~8, sequence dependent (19)	
	MthMcm, 4–11 (42, 129, 200)		
K_d (nM)			
ssDNA binding	SsoMcm, 150–200 (13); 800 ± 200 (174); 300 ± 130 (203)	~35 ± 15 (23); 2 (214)	~35 ± 15 (23)
	TacMcm, 117 (98)		
	MthMcm, ~65 (115)		
dsDNA binding	MthMcm, >1 (115)	5.6 ± 0.6 (23)	2.12 ± 0.2 (23)
	SsoMcm, 0.51 (153)		
ssDNA binding $k_{1/2}$ as a function of ATP (μM)	ND	177 ± 82 (23)	248 ± 150 (23)
dsDNA translocation	MthMcm, yes (125, 227)	Yes (122, 227)	ND
Helicase polarity	MthMcm, 3′→5′ (98, 129)	3′→5′ (109, 144)	CMG complex, 3′→5′ (184)
	SsoMcm, 3′→5′ (174)		
ATP necessary for helicase activity (mM)	SsoMcm, 7.5 = optimal activity (180)	ND	$k_{1/2}$ = ~1.8 (24)
	TacMcm, ~4 = optimal activity (98)		
Preferred helicase substrate	MthMcm, various (227)	Forks or bubble (122, 144, 274)	ND
	SsoMcm, various (13)		
Estimated helicase processivity (bp unwound)	MthMcm, 200–500 (42, 129)	~35–50 (109, 274); +RPA, ~450 (227); −SSB, ~300; +SSB, ~600 (145)	100–200 (Bochman, unpublished)

[a] ND, not determined; AfuMcm, *Archaeoglobus fulgidus* Mcm; ApeMcm, *Aeropyrum pernix* Mcm; TacMcm, *Thermoplasma acidophilum* Mcm; RPA, replication protein A; SSB, single-strand break.

dates for propagating conformational changes through the complex to facilitate DNA unwinding.

Extensive interdependence among the six component ATPase active sites is not required for DNA unwinding. If all six ATPase active sites were essential, one mutant subunit would be sufficient to poison the activity of the entire hexamer. Experiments that mix specific ratios of wild-type and mutant SsoMcm subunits demonstrated that both ATPase and helicase activities can tolerate at least three mutant subunits within the hexamer (Fig. 6) (180). This observation was further confirmed by combining various ratios of subunits containing *cis* and *trans* mutations; the recovery of helicase activity at certain subunit ratios indicates that interactions between two adjacent active sites, but not among all six, are needed for activity. This has led to the proposal that helicase activity in the SsoMcm complex occurs semisequentially, with DNA unwinding requiring only two to three adjacent wild-type subunits within the SsoMcm ring (180).

This semisequential cooperativity requires physical interactions between the N- and C-terminal SsoMcm domains. In contrast with the subunit-mixing experiments that utilized full-length SsoMcm, analogous experiments using SsoMcm truncations that lack the N terminus (but contain the AAA^+ domain) demonstrated no cooperativity, with DNA unwinding being strictly proportional to the number of wild-type subunits (12). The basis for communication between the N- and C-terminal domains appears to be mediated at least in part by a conserved N-terminal loop (allosteric control loop [ACL]) (12), as mutations in this loop within a full-length SsoMcm protein destroy allosteric coupling between ATPase active sites. Evidence suggests that the ACL physically couples the N-terminal hairpin on one subunit with the presensor 1 hairpin

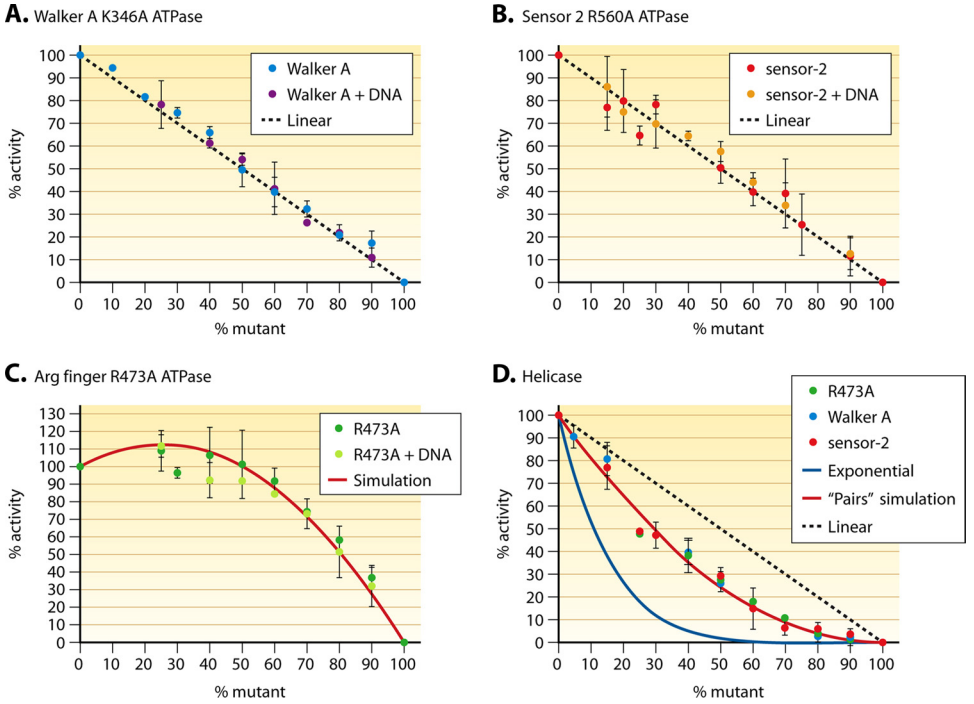

FIG. 6. Involvement of archaeal Mcm structural motifs in ATP hydrolysis and DNA unwinding. (Adapted from reference 180 with permission from Elsevier.) Data from mutant doping studies with Walker A (K346A) (A and D), sensor 2 (R560A) (B and D), and arginine finger-alanine mutants (R473A) (C and D) are shown. The indicated mutants were mixed with wild-type SsoMcm and then assayed for ATPase (A to C) in the presence and absence of DNA or helicase (D) activities; assays were performed between 3 and 10 times, and error bars represent the standard deviations of the results. Red or blue lines represent mathematical simulations (for details, see reference 180). In A and B, the indicated simulation assumes that ATPase activity is linearly proportional to the number of wild-type Mcm active sites present. In C, the simulation is according to the "wild-type–mutant pairs simulation" model with a value of *s* equal to 3. This simulation assumes that although the active sites containing an arginine finger mutation are catalytically inactive, they still bind ATP and stimulate the activity of the adjacent wild-type ATPase active site. In D, the red simulation is the "pairs" model with a *P* value of 2 and follows the assumption that the activity at any wild-type Mcm ATPase active site depends on the presence of an adjacent wild-type active site. The exponential decrease that would be observed if a single mutant subunit within the hexamer blocks helicase activity is shown in blue.

on the adjacent subunit, thus suggesting a mechanism for coupling activity between subunits (12, 30). As the ACL region is reasonably well conserved among the subunits of the eukaryotic Mcm2-7 complex (Fig. 2C) (12), similar allosteric interactions may also extend to Mcm2-7 (see "Mcm10—a bridge to primase?" below).

DNA Binding: a Plethora of Helping "Fingers"

The DNA binding activity of the archaeal Mcm proteins has been extensively analyzed. Structural analysis indicates that unlike the well-studied phage T7 helicase, the central channel of the Mcm complex contains an unusually high concentration of positive charge (76). These charged residues appear to be important for DNA binding, as substitution mutations that neutralize these charges decrease DNA binding (79). With an affinity similar to those of other hexameric helicases (197), MthMcm binds ssDNA substrates in an Mg^{2+}-dependent manner (K_d for ssDNA of ~130 nM [42]), with a slight preference for ssDNA over dsDNA (213). The SsoMcm complex apparently prefers substrates that contain both ssDNA and dsDNA: forked DNA substrates were bound with higher affinity than partial dsDNA probes containing a poly(dT) bubble or a 5′ ssDNA tail (62), but both forks and bubbles were preferred over simple ssDNA probes (204, 209). However, recently reported electron microscopy data suggest that dsDNA may also bind on the outside of the complex rather than in the central channel, an activity possibly reflecting the initial contacts between the Mcm proteins and dsDNA during helicase loading (51). In total, these results suggest that the SsoMcm helicase may productively interact with both ssDNA and dsDNA at the same time.

Further analysis of both DNA binding and the Mcm structure (see below) has led to the discovery that a variety of β-hairpin fingers (Fig. 2 and 7A and see below). To date, the involvement of four such hairpins in ssDNA binding has been described.

N-terminal hairpin. In contrast to typical helicases that bind ssDNA in an ATP-dependent manner (197), the level of MthMcm binding is decreased in the presence of either ATP or the nonhydrolyzable ATP analogue ATPγS. Crystallographic analyses of the N-terminal domain of both the MthMcm (76) and SsoMcm (153) proteins (lacking the AAA^+ domain) demonstrated that the N terminus in isolation can oligomerize into a hexameric ring with an ATP-independent DNA binding activity (76). Individual subunits in each structure contain a positively charged β-hairpin "finger" that resides in the central channel (76, 153). Alanine substitution mutations that elimi-

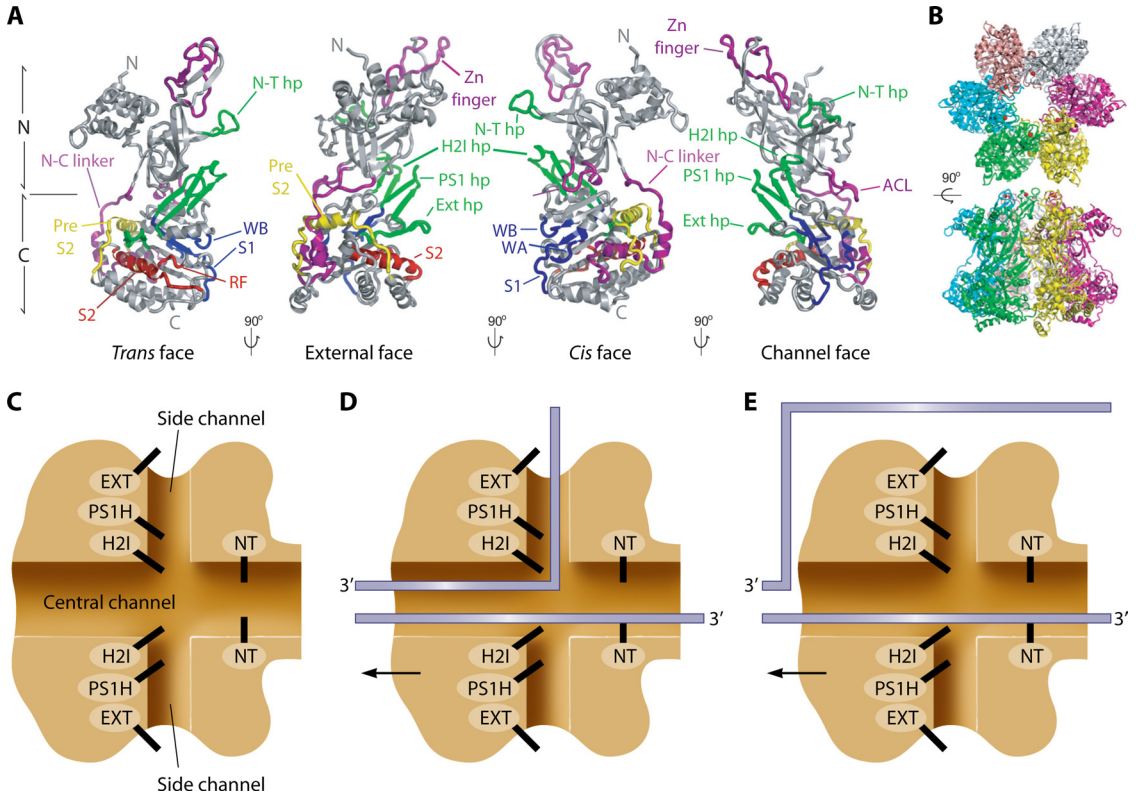

FIG. 7. Archaeal Mcm structural architecture and proposed unwinding modes. (A) Location of Mcm structural motifs within the monomer crystal structure of SsoMcm. The images were made using PYMOL (http://pymol.sourceforge.net/) and Protein Data Bank accession number 3F9V, which includes amino acids 7 to 601 of SsoMcm (30). The coloring and abbreviations are the same as those defined in the legend of Fig. 2A. Each of the four different views are 90° rotations of the monomer crystal structure. The *trans* and *cis* faces show the dimer interface between subunits that contains the *trans*- or *cis*-acting ATPase motifs. The external face views the monomer from the outside of the hexamer looking toward the central channel, while the channel face views the monomer from inside the central channel looking out. (B) Top and side views of the hexamer organization of SsoMcm. (Reprinted from reference 30 with permission of the publisher. Copyright 2008 National Academy of Sciences, U.S.A.) (C) Schematic representation of an SsoMcm hexameric helicase. The four β-hairpins (N-terminal β-hairpin [NT], helix 2 insert β-hairpin [H2I], presensor 1 β-hairpin [PS1], and external β-hairpin [EXT]) are represented by short solid bars; the central channel and side channels are in darker shading. (D) dsDNA pump mode showing ssDNA extrusion from the side channel. (E) Steric exclusion model of a single SsoMcm helicase. DNA is shown as gray lines. Arrows indicate the direction of helicase movement. (Panels B to E reprinted and legends adapted from reference 30 with permission of the publisher. Copyright 2008 National Academy of Sciences, U.S.A.)

nate the positive charge on this hairpin result in a 10-fold loss in ssDNA binding affinity (174). Moreover, the loss of the SsoMcm Zn^{2+} finger motif, a mutation expected to alter the structure of the N-terminal domain (76), results in a complex that binds ssDNA in an ATP-dependent manner (200). This, coupled with the fact that the central channel itself is highly positively charged, argues that DNA binding occurs in the center of the Mcm toroid. The N-terminal hairpin was also confirmed in the nearly full-length SsoMcm crystal structure (30), but sequence homology in this region with the eukaryotic Mcm2-7 proteins is rather poor (Fig. 2C). This raises doubts concerning the involvement of the N-terminal hairpin in Mcm2-7, although mutations that remove three of the positively charged residues in this region of Mcm5 result in defective initiation (148).

Presensor 1 hairpin. Motivated by analyses of other helicases, structure-aided sequence alignments (174) predict that both the archaeal and eukaryotic Mcm complexes contain an additional β-hairpin motif contributed by the presensor 1 insert similar to that found in the SV40 TAg (84, 149) and E1 (1) helicases. Alanine substitution mutations of the presensor 1 hairpin in SsoMcm modestly reduce ssDNA binding affinity (approximately threefold) but eliminate DNA-unwinding activity; combining the presensor 1 mutations with those of the N-terminal hairpin completely abrogates DNA binding (174). The existence of at least two DNA binding regions of SsoMcm was also verified using domain deletion constructs (13, 153, 203).

Helix 2 insert and the external hairpin. The crystal structure of the nearly full-length monomeric SsoMcm reveals two additional β-hairpins, one corresponding to the helix 2 insert that is located within the central channel and the other (external hairpin) (Fig. 7) that is slightly upstream of the Walker A box that is predicted to be located on the outside of the hexameric Mcm structure (30; for additional details, see the section below on archaeal Mcm structural biology). Previous investigations of the helix 2 insert motif in the MthMcm complex demonstrated that it is essential for coupling ATP hydrolysis to helicase activity, and its movement can be affected by residues in both the N- and C-terminal domains (115). The finding that this

TABLE 2. Interactions between archaeal Mcm proteins and other archaeal replication proteins[a]

Organism	Gene	Physical interaction	Effect on Mcm				Mcm phosphorylation	Reference(s)
			ATP hydrolysis	DNA binding		Helicase		
				Origins	Nonorigins			
M. thermoautotrophicum	Cdc6-1	+	ND	ND	ND	−	ND	225
	Cdc6-2	+	ND	ND	ND	−	ND	225
Thermoplasma acidophilum	Cdc6-1	−	ND	ND	ND	ND	ND	99, 100, 225
	Cdc6-2	+	+	ND	ND	+	ND	99, 100, 225
S. solfataricus	Cdc6-1	+	−	−	+	−	ND	61–63, 117
	Cdc6-2	+	ND	+	ND	ND	ND	61–63, 117
	Cdc6-3	+	ND	−	+	ND	ND	61–63, 117
	Gins15/Gins23	+	ND	ND	ND	ND	ND	164
Aeropyrum pernix	Cdc6-1	ND	ND	ND	ND	−	ND	7
	Cdc6-2	ND	ND	ND	ND	−	+	7
P. furiosus	Gins15/Gins23	+	ND	ND	ND	+	ND	272

[a] ND, not determined.

motif is another β-hairpin finger whose conformation depends upon ATP is consistent with these results (30). As the conservation of the helix 2 insert is a defining feature of the Mcm clade of AAA^+ proteins (see the introduction), this hairpin is likely functional in the eukaryotic Mcm2-7 complex as well (Fig. 2C). The external hairpin is similar to the acidic pin of the RuvA helicase (108), and primary sequence homology among the eukaryotic Mcm proteins is good in this region (Fig. 2C), suggesting that its function is also conserved. Mutational analysis indicates that the external hairpin is functionally important, as mutations in this hairpin block helicase activity without affecting subunit oligomerization, ssDNA binding, or ATP hydrolysis (30).

The Archaeal Mcm Proteins Are Robust Helicases

The MthMcm complex has robust and reasonably processive 3′→5′-directed helicase activity (it can unwind up to 500 bp of dsDNA in vitro) (42) that is dependent on ATP and Mg^{2+} (115). This complex demonstrates very little DNA substrate specificity: blunt, singly tailed, and forked substrates are all similarly unwound (227). DNA/RNA hybrids can also be unwound as long as the helicase traverses along the DNA strand (228). The SsoMcm complex displays similar DNA unwinding characteristics: it too is a 3′→5′ helicase (13, 34, 174, 204) whose activity depends upon ATP hydrolysis (ATP>dATP>ATPγS and all other NTPs and deoxynucleoside triphosphates; AMP-PNP, ADP, and the transition-state analogue ADP-AlF_4 fail to support DNA unwinding [13, 34]). Although the complete SsoMcm complex cannot unwind a fully duplex substrate, constructs lacking the N terminus gain this activity, suggesting that this domain normally inhibits duplex-unwinding activity (13).

The relative orientation of the archaeal Mcm complex on a forked DNA substrate has also been studied. Fluorescence resonance energy transfer experiments demonstrate that SsoMcm hexamers bind as predicted for the steric model: they encircle the 3′ tail of forked DNA probes and then slide with 3′→5′ polarity toward the duplex region of the fork (174). SsoMcm loads such that the AAA^+ domain is near the ssDNA/dsDNA junction of the fork, and the N terminus is closer to the end of the 3′ tail. Single-molecule fluorescence resonance energy transfer has confirmed these findings but also indicates that the interaction of the 5′ tail of a fork substrate with the surface of the SsoMcm hexamer increases the stability of the Mcm-DNA interaction (209).

Interactions of the Archaeal Mcm Proteins with Other Replication Factors

The study of physical and functional interactions between the archaeal Mcm proteins and other archaeal replication factors is at an early stage. Much of the current data involves the multiple Cdc6 homologues present in each species. Although most of these factors demonstrate physical interactions with the corresponding Mcm complex, the observed functional interactions are diverse; both the stimulation and inhibition of helicase activity and ATP hydrolysis have been observed, as have changes to the DNA binding specificity of the Mcm complex (Table 2). While many of these differences likely reflect both the diversity of investigators and organisms involved in these experiments, it should be noted that these studies are implicitly based on the supposition that archaeal proteins should fulfill functions similar to those of their biologically better-studied eukaryotic counterparts. However, much less is known of the biology of archaeal DNA replication (although great strides are being made to rectify this situation [159, 258]), so it is currently difficult to interpret the significance of these results.

In addition to the archaeal Cdc6 homologues, interactions with several other putative replication factors have been examined (Table 2). Archaea contain poorly conserved homologues of the eukaryotic GINS heterotetramer (composed of Sld5 and Psf1-3 in *S. cerevisiae*). In both the euryarchaeote *Pyrococcus furiosus* and the crenarchaeote *S. solfataricus*, the GINS complex is composed of two proteins: Gins15, which is homologous to Psf1 and Sld5, and Gins23, which is related to Psf2 and Psf3 (164, 272). In both species, GINS physically interacts with the Mcm helicase (164, 272), but *P. furiosus* Gins ($Gins_{Pf}$) also stimulates *P. furiosus* Mcm (PfuMcm) helicase activity (272). Interestingly, even though the $Gins15_{Pf}$ and $Gins23_{Pf}$ proteins display similarity to all four eukaryotic GINS subunits, they still exist in a 2:2 ratio as a heterotetramer.

Structural Biology of the Archaeal Mcm Proteins

In contrast to Mcm2-7, for which little structural data are available, the SsoMcm and MthMcm complexes have been the subject of detailed structural analyses using transmission electron microscopy (39, 49, 50, 78, 91), cryo-electron microscopy (51), as well as X-ray crystallography (8, 30, 76, 153). Although a high-resolution structure of a full-length hexameric archaeal Mcm complex is not yet available, a low-resolution structure of the complete MthMcm double hexamer has been derived by electron microscopy (51, 91). Additionally, four crystal structures currently exist for discrete pieces of the archaeal complexes: two structures of the N-terminal domains of the proteins in the form of either hexamers (SsoMcm [153]) or double hexamers (MthMcm [76]), structures of a nearly full-length SsoMcm monomer (30) (Fig. 7A), and a structure of a nonfunctional Mcm monomer from *Methanopyrus kandleri* (8). To model the structure of the full hexamer and its corresponding dimer interfaces, the two monomer structures were fitted into a hexameric arrangement using the previously derived hexameric N-terminal structures and then docked into the electron microscopy reconstruction of MthMcm (8, 30) (Fig. 7B). Given the potential limitations of these structures, caution should be exercised in their interpretation; however, they have provided considerable information that facilitates the interpretation of mechanistic studies.

Mcm oligomerization. In contrast to well-studied viral helicases (197), archaeal Mcm oligomerization is independent of exogenous ATP (129) but dependent upon contacts made throughout the length of the protein (30, 76). Presumably, contacts within the N-terminal domains are stronger, as isolated N-terminal domains form both single and double hexamers in solution (76, 153), while the C terminus of SsoMcm is unable to hexamerize in the absence of chemical cross-linking (13).

Despite considerable similarity over their entire lengths (43% identical and 63% similar), SsoMcm and MthMcm demonstrate different oligomeric forms. Gel filtration and glycerol gradient centrifugation studies demonstrate that the SsoMcm (34, 204) and other lesser-studied archaeal Mcm complexes (95, 100) usually form hexamers (although SsoMcm may form double hexamers at high protein concentrations [see reference 13]). In contrast, the MthMcm complex tends to form double hexamers in solution (42, 129, 200, 222) in a head-to-head orientation with juxtaposed N termini (76, 153), a structural organization strongly reminiscent of SV40 TAg (discussed in reference 74). In addition to the double-hexameric form, MthMcm has been observed in a range of additional forms by electron microscopy (closed or gapped hexamers [193], heptamers [277], double hexamers [42], or helical filaments [39, 277]), differences that may be due in part to the unusual sensitivity of MthMcm oligomerization to solution conditions (i.e., the concentration of salt, Mg^{2+}, ATP, and DNA as well as temperature [49, 50, 91]). The double-hexamer form of MthMcm likely represents the in vivo state, as double hexamers have about 10-times-greater in vitro helicase activity than single hexamers (78), and subcomplexes and single subunits lack DNA-unwinding activity altogether (125).

Mcm domain structure. Both MthMcm and SsoMcm contain defined N- and C-terminal domains connected by a long structured linker localized on the outside of the hexamer (Fig. 7A). Electron microscopy and crystallographic studies demonstrate that these domains form two bulky rings stacked on top of one another, which are joined at a thinner "waist" (Fig. 7A and B) (30, 193). These domains can be separated by limited proteolysis (203), and they have been engineered by recombinant means and studied independently of one another (13, 76, 203). Each Mcm domain makes different functional contributions, as discussed below.

(i) N-terminal processivity domain. The N-terminal processivity domain (essentially defined as amino acids 1 to 265 in SsoMcm [30, 203]) contains the N-terminal β-hairpin (see "DNA Binding: a Plethora of Helping 'Fingers'" above) and the zinc finger motifs (Fig. 2A and C and 7A). In isolation, the MthMcm N-terminal domain has the propensity to oligomerize into toroidal double hexamers (76), and both the corresponding MthMcm and SsoMcm domains are capable of binding DNA (76, 203), properties facilitated by the N-terminal β-hairpin (76). The double-hexamer formation of MthMcm is mediated by the Zn^{2+} finger motif (76, 78, 125). It should be noted that the zinc fingers do not mediate oligomerization directly but rather facilitate double-hexamer formation by mediating and stabilizing the proper folding of the N-terminal domain. In contrast to the archaeal Mcm proteins, the significance of the zinc finger motifs in the eukaryotic Mcm2-7 complex is controversial. Although mutations in the *S. cerevisiae* Mcm2 zinc finger motif are lethal (269) and those in Mcm5 result in temperature sensitivity (56), the canonical motif is routinely absent from Mcm3 and not universally conserved among the other Mcm proteins (Fig. 2C). These findings are consistent with the observation that Mcm2-7 double hexamers have not yet been observed (23, 58, 218).

(ii) C-terminal motor domain. The C-terminal motor domain (defined in SsoMcm as amino acids 304 to 617 [30]) contains all of the ATPase motifs (see the introduction and Fig. 2), a predicted winged-helix domain (that is disordered, preventing determination [30]) on the extreme C terminus, and three β-hairpin "fingers" (Fig. 7A and see "DNA Binding: a Plethora of Helping 'Fingers'" above). The *cis*-acting motifs of the ATPase active site (Walker A and B and sensor 1 motifs) are located on loops that extend into one dimer interface (*cis* face), while the *trans*-acting motifs (arginine finger and sensor 2 motifs) extend into the other dimer interface (*trans* face) (Fig. 7A). The presensor 2 loop, in contrast, localizes on the external face of the subunit and is accessible to interact with potential regulatory proteins. Both the helix 2 insert and presensor 1 β-hairpins project from the channel face of the monomer, while the external hairpin points between the *trans* face and the external face of the monomer.

For SsoMcm, this domain has been biochemically studied in isolation, but the analogous region in MthMcm is insoluble and resists experimental analysis (213). In isolation, this domain is competent to bind DNA in an ATP-independent manner, hydrolyze ATP, and unwind DNA (13, 203, 213). Despite the relatively weak oligomerization of this domain in isolation, it still appears to make important contributions to complex formation, as mutations targeting putative dimer interfaces in the C terminus disrupt the oligomerization of the entire full-length subunits (30).

Interdomain communication. Interestingly, many of the properties of the wild-type archaeal Mcm complex can be reconstituted by mixing together individual N- and C-terminal-domain preparations. Although the C termini in isolation can unwind DNA, processivity is enhanced by the addition of the N terminus (13). In isolation, the C-terminal domain can efficiently unwind blunt substrates and those containing 3′ or 5′ ssDNA tails. However, upon the addition of the N-terminal domain, the unwinding of 3′-tailed substrates is stimulated, and the ability to unwind the 5′-tailed and blunt substrates is inhibited, suggesting a role of the N terminus in substrate discrimination (13).

Although these results may suggest that the N-terminal domain is nothing more than a collar that keeps the motor domain attached to DNA in the proper orientation, additional evidence indicates that the N terminus is able to coordinate and control C-terminal function. Even though the two domains physically interact, the stimulation of processivity afforded by the N-terminal domain requires the N-terminal β-hairpin, suggesting that the N terminus also functionally interacts with the C-terminal domain (13). Further work has traced this functional interaction to a conserved loop in the N-terminal domain that projects into the *cis* face of the monomer structure (ACL) (Fig. 7A) (12). A mutational loss of this loop has little effect on ssDNA binding but almost completely eliminates helicase activity. Interestingly, the helicase defect can be largely suppressed by the additional inactivation of the N-terminal β-hairpin, suggesting that in the absence of the ACL, the N-terminal β-hairpin is in a nonproductive orientation (12). Structural analysis indicates that the ACL of one subunit is in physical contact with the presensor 1 hairpin of the neighboring subunit, suggesting that the ACL helps coordinate activity between subunits (12, 30).

Channels. Both the MthMcm and SsoMcm complexes contain two sets of channels (Fig. 7C): (i) a long positively charged central channel boring through the entire hexamer from the N to the C terminus and (ii) six perpendicular side channels at dimer interfaces evident between the N- and C-terminal domains that connect the central channel to the exterior of the complex in a manner similar to that of SV40 TAg (30, 49, 84, 91, 149, 193). Although the MthMcm central channel is wide enough to encircle dsDNA (76), SsoMcm forms a narrower central channel that can accommodate ssDNA but not dsDNA along its entire length (30, 153). The diameter of the central channel is restricted in the N-terminal domain by the N-terminal β-hairpin fingers and is restricted primarily in the C terminus by the helix 2 insert hairpin (and to a lesser extent by the presensor 1 hairpin) (30, 76). The side channels (11 Å wide) may function as exits for ssDNA that is extruded from the central channel during unwinding (30). This model seems particularly attractive for the SsoMcm complex because (i) it was observed that the C-terminal domain is loaded toward the duplex portion of a DNA fork substrate (174), (ii) the central channel is wide enough at the C terminus to allow the passage of dsDNA (30) but narrows toward the N terminus (due to the N-terminal hairpins) to a diameter that would allow the passage of ssDNA only (30, 153), and (iii) it could involve all four β-hairpin fingers during unwinding. Conversely, the SsoMcm hexamer is also fully compatible with DNA unwinding by the standard steric mechanism (Fig. 5B), although in this case, one predicts a less functional involvement of the β-hairpins (Fig. 7E).

BIOCHEMISTRY OF THE EUKARYOTIC Mcm2-7 HELICASE

Unlike a homohexameric helicase, the eukaryotic Mcm2-7 complex is a heterohexamer containing six distinct and essential subunits. This immediately suggests a puzzle: why are all of the subunits different? The complete evolutionary conservation of this heterohexameric-subunit arrangement among eukaryotes strongly implies a functional importance (see Speculation on Mcm Oligomerization and Evolution below). Considering this, two nonexclusive models come to mind. One, based on the observation that their sequence conservation is most prominent within their AAA^+ domains, would be that each of the six Mcm2-7 active sites are biochemically equivalent, with the functional distinction among them resulting from a differential ability to bind other replication proteins through their divergent N and C termini. Alternatively, the six subunits might be catalytically distinct and contribute differentially to DNA unwinding. Genetic studies indicated that analogous mutations within the ATPase active site of different Mcm2-7 subunits result in different phenotypes (e.g., see references 90 and 218), and biochemical studies (22–24) have identified catalytic differences among the six active sites, suggesting the evolution of active-site specialization. These results will be discussed below and are summarized and compared to data for the archaeal Mcm proteins in Table 1. Although the bulk of these experiments focus on the *S. cerevisiae* Mcm complex, recent work with the *Drosophila* Mcm complex has confirmed and expanded many of the key observations cited below (M. Botchan, submitted for publication).

Subunit Architecture of Mcm2-7 and Evidence of a Gapped Toroidal Structure

The early fractionation of the eukaryotic Mcm proteins from cell extracts yielded a variety of dimeric, trimeric, and tetrameric complexes (that can now be viewed as Mcm2-7 subcomplexes) (2, 58, 109, 110, 121, 137, 144, 160, 185, 201, 217, 218, 223, 241, 267, 275) as well as one form (Mcm2-7) containing all six subunits (2, 58, 110, 137, 144, 160, 201, 218). Mcm2-7 maintains a stable physical association among the six subunits in the absence of exogenous ATP, as demonstrated by both coimmunoprecipitation assays (2) and gel filtration analyses in a variety of systems (2, 58, 144, 218, 270), and represents a heterohexamer-sized (~600 kDa) complex that likely contains each Mcm subunit in a 1:1:1:1:1:1 stoichiometry. Furthermore, electron microscopy indicates that Mcm2-7 forms a ring-shaped complex (2, 23) similar to that of the archaeal Mcm proteins and other AAA^+ proteins.

However, this toroidal complex may contain a discontinuity. While detailed structural data are unavailable for Mcm2-7, several association studies utilizing both the yeast and human Mcm subunits (22, 58, 278) have identified five dimeric subunit pairs. These pairs are named such that the subunit contributing the arginine finger motif comes first: Mcm5/3, Mcm3/7, Mcm7/4, Mcm4/6, and Mcm6/2. Assuming that each subunit contacts only two other subunits, these dimeric pairs suggest

the subunit arrangement for Mcm2-7 depicted in Fig. 9A. Results suggest that the Mcm2/5 interface is relatively labile (22, 58). Although these two subunits are predicted to physically interact to form the toroidal Mcm2-7 structures observed in electron micrographs, a direct interaction between Mcm2 and Mcm5 has never been demonstrated (except by cross-linking [54, 278]); these two subunits in isolation do not stably interact in vitro (58) or following coexpression in insect cells (22). The possible significance of this discontinuity will be discussed below.

The Individual Mcm2-7 ATPase Active Sites Are Functionally Distinct

Both the study of specific Mcm subcomplexes and mutations in conserved active-site motifs indicate that the six Mcm2-7 ATPase active sites contribute differentially to all biochemical activities examined:

Helicase activity: discovery of the Mcm467 subcomplex. By the biological criteria discussed in the introduction, in vivo studies indicate that Mcm2-7 is the replicative helicase. However, this oligomeric form has historically lacked in vitro helicase activity (58, 109, 218, 270). Instead, a specific dimeric heterotrimer (Mcm467) was isolated from a variety of systems that lacked the Mcm2, Mcm3, and Mcm5 subunits but contained two copies each of the Mcm4, Mcm6, and Mcm7 subunits and possessed an ATP-dependent, 3′→5′ DNA-unwinding activity (109, 122, 144, 273). The helicase activity of this complex apparently requires only the Mcm7/4 active site, as Mcm4 and Mcm7 can oligomerize into a hexamer possessing helicase activity in the absence of Mcm6 (121). Furthermore, the addition of Mcm2 or the Mcm5/3 dimer inhibits Mcm467 helicase activity (214). These results strongly suggest that the Mcm2-7 ATPase active sites form two distinct functional groups: those contributed by Mcm4, Mcm6, and Mcm7 are needed for helicase activity, while those contributed by Mcm2, Mcm3, and Mcm5 serve as negative regulators (218).

Unequal role of Mcm2-7 active sites in ATP hydrolysis and ssDNA binding. (i) ATP hydrolysis. A weak ATPase activity was first discovered for the *S. pombe* Mcm2-7 complex (144), followed by a detailed study of the ATPase activity of the *S. cerevisiae* Mcm2-7 complex (218). Unlike either a typical helicase or the Mcm467 subcomplex (19, 109, 143, 274, 275), neither ssDNA nor dsDNA stimulates the ATP hydrolysis of the *S. cerevisiae* Mcm2-7 complex (22, 58, 218). Measurement of ATP hydrolysis as a function of ATP concentration demonstrates three different kinetic states within Mcm2-7 (218). Although similar kinetic results have been observed with homohexameric helicases and explained as negative cooperativity among the active sites (17, 64, 103), the fact that Mcm2-7 contains structurally distinct ATPase active sites suggests an alternative possibility, that the three different kinetic states represent ATP hydrolysis at three physically distinct active sites (218).

Analyses of individual Mcm active-site dimers demonstrate that only three of the five stable dimers hydrolyze ATP to a significant degree: Mcm3/7 has high levels of ATPase activity similar to that of the entire Mcm2-7 complex, Mcm7/4 has intermediate levels of ATPase activity, and Mcm6/2 has low levels of ATPase activity. The Mcm5/3 and Mcm4/6 dimers have negligible activity (22, 58). Further analysis of the Mcm3/7, Mcm7/4, and Mcm6/2 dimers confirms a canonical AAA^+ active-site configuration; dimers containing mutations in conserved ATPase motifs demonstrate that the Walker A and B boxes are donated by one subunit (*cis*) and that the arginine finger motif is contributed by the other (*trans*) (22, 58). It should be noted that each Mcm dimeric subunit combination contains two sets of *cis* motifs and two sets of *trans* motifs; however, only one set of each is juxtaposed to form a complete active site, while the remaining sets form incomplete "half-sites" (Fig. 2B).

Interestingly, in at least the case of the Mcm6/2 and Mcm7/4 dimers, the Walker A motifs that form such "half-sites" also appear to contribute to ATP hydrolysis activity at the corresponding complete active site; not only does the loss of the Walker A motif (lysine-to-alanine ["K→A" or "KA"] substitution) at the expected dimer interface eliminate ATPase activity (i.e., Mcm2KA and Mcm4KA mutations), but the loss of the "half-site" Walker A motifs (i.e., Mcm6KA and Mcm7KA mutations) also reduces ATPase activity (22). Although these data do not exclude the possibility that the half-site Walker A motif participates in a complete noncanonical active site involving the other subunit, these results do suggest that it still binds ATP and stimulates the ATP hydrolysis of the adjacent complete canonical active site. This scenario is similar to that of archaeal Mcm subunit doping experiments, results accommodated by the hypothesis that ATP hydrolysis is stimulated by the nucleotide occupancy of an adjacent active site (180).

The unequal contribution of Mcm active sites to ATP hydrolysis also occurs within the context of the intact heterohexamer. Biochemical analysis of Mcm2-7 complexes with mutations in either the Walker B (aspartate/glutamate-to-asparagine/glutamine ["DENQ"] mutations) or arginine finger (arginine-to-alanine ["RA"] mutations) motif of individual Mcm subunits indicates that only the Mcm3/7 and Mcm7/4 active sites contribute substantially to steady-state ATP hydrolysis (22). Note that these results suggest essentially no coordination among active sites for ATP hydrolysis, as individual mutations in four of the six sites are unable to poison the ATP hydrolysis of the remaining active sites.

In sharp contrast to both the analysis of the Mcm2-7 Walker B and arginine finger mutations as well as a similar analysis of the archaeal Mcm proteins (180), the inclusion of a single Walker A mutation in any of the six Mcm subunits poisons the ATPase activity of the entire complex (218). This result indicates that Mcm2-7 ATPase active sites function in an interdependent manner (218). The different results generated by the different ATPase mutations in the same active site likely reflect the distinct roles of these motifs in ATP hydrolysis. In better-studied systems, Walker A mutations often block both ATP binding and hydrolysis, while Walker B and arginine finger mutations block ATP hydrolysis with much less effect on ATP binding (reviewed in reference 96). Assuming that the Mcm2-7 Walker A mutants behave similarly, coordination among the six active sites requires ATP occupancy rather than ATP hydrolysis at each active site.

Analysis of Mcm2-7 complexes with multiple subunits containing a Walker A mutation supports this conclusion (218). Under these conditions, the functional interdependence between active sites is lost, and the observed steady-state ATP hydrolysis corresponds to the remaining wild-type active sites. Remarkably, an Mcm2-7 complex that simultaneously contains

Walker A mutations in Mcm2, Mcm3, and Mcm5 demonstrates wild-type levels of ATP hydrolysis and the same three kinetically defined ATP hydrolysis modes (218). In contrast, double or triple mutations among Mcm4, Mcm6, and/or Mcm7 have even less ATP hydrolysis than any of the single-mutant complexes (218). In combination, these results indicate that the Walker A motifs of Mcm4, Mcm6, and Mcm7 are required for the majority of the ATPase activity in the wild-type Mcm2-7 complex, while the remaining active sites contribute little to bulk hydrolysis.

(ii) ssDNA binding. The relationship between specific ATPase active sites in Mcm2-7 and DNA binding was also studied. Both wild-type Mcm2-7 and Mcm467 complexes from *S. cerevisiae* demonstrate ATP-dependent DNA binding activity with ~100-fold-greater affinity for ssDNA than dsDNA (35 nM for ssDNA versus ~2 to 5 μM for dsDNA [23]). This result is in contrast to data for the archaeal Mcm proteins, which bind ssDNA in a largely ATP-independent manner (42, 174), a fact suggesting a less prominent role for the N-terminal β-hairpins in eukaryotic Mcm ssDNA binding. Only the binding and not the hydrolysis of adenosine triphosphates stimulates the Mcm2-7 association with ssDNA, since it is supported by both dATP and the nonhydrolyzable analogue ATPγS but not non-adenosine triphosphates. In contrast, the ability of ADP to support ssDNA binding appears to be controversial (see reference 23 versus reference 237). Maximal binding requires ssDNA of greater than 35 nucleotides in length (23) and is strongly base composition dependent, with polypyrimidine tracts being about five- to ninefold preferred over polypurines (23), an effect previously noted for the archaeal Mcm complex (129) and for Mcm467 (145, 275). Considerable subunit oligomerization is also required for ssDNA binding, as most Mcm subcomplexes are incapable of significant ssDNA binding, and those that do bind contain an intact Mcm7/4 active site (23).

Biochemical analysis of the effects of Mcm mutations on ssDNA binding is not as advanced as studies of ATP hydrolysis, but existing data support the unique importance of the Mcm3/7 and Mcm7/4 active sites in this activity. Analysis of the single Walker A mutant complexes demonstrated that only the Walker A box of Mcm4 is necessary for ssDNA binding (23). A previous report that the *S. pombe* Mcm4KA mutant blocks the in vivo chromatin association of Mcm2-7 (90) supports this observation. Furthermore, although the Mcm7KA hexamer retains intermediate levels of ssDNA binding, this activity is no longer stimulated by ATP (23). Taken together, these results suggest that ATP binding at both the Mcm3/7 and Mcm7/4 ATPase active sites is required for ATP-dependent ssDNA binding in Mcm2-7, a conclusion generally supported by analyses of Mcm2-7 complexes containing either Walker B or arginine finger mutations (our unpublished data). Given that Mcm467 binds ssDNA as well as Mcm2-7 and by definition lacks the Mcm3/7 active site, it seems likely that the Mcm7/4 site is the more important of these two active sites for ssDNA binding. Although the DNA binding of mutant Mcm2-7 complexes has not been examined extensively in vivo, single and double Walker A mutations in Mcm6 and Mcm7 were examined in vitro using the *Xenopus* replication system; these mutants block elongation but not chromatin associations (270), suggesting that they are able to load into the pre-RC but are unable to unwind DNA.

Reconstitution of Mcm2-7 Helicase Activity: Clues to ATPase Active-Site Function

To both ascertain the function of the Mcm2, Mcm3, and Mcm5 subunits as well as understand why Mcm2-7 lacks DNA-unwinding activity in vitro, a comparative study of the Mcm2-7 and Mcm467 complexes was conducted (23). The results demonstrated several informative differences that provided clues regarding Mcm2, Mcm3, and Mcm5 function that ultimately led to the reconstitution of Mcm2-7 helicase activity (24). Extending these studies to mutant Mcm2-7 complexes, the differences between Mcm2-7 and Mcm467 can be traced to the Mcm2/5 active site, the site of the putative discontinuity in the Mcm2-7 toroid (22, 24).

Mcm2-7 and Mcm467 have ATP-dependent differences in ssDNA binding. Both Mcm2-7 and Mcm467 bind linear ssDNA in an ATP-dependent manner with similar affinities, but they differ in their abilities to bind circular ssDNA. Although experiments conducted with different methodologies detected the binding of Mcm467 to circular ssDNA (121), a direct comparison of the two complexes demonstrates that Mcm467 has an apparent affinity for circular ssDNA that is ~20 times lower than that of Mcm2-7 (24). This difference depends upon the temporal order in which ATP enters the reaction; if Mcm2-7 is mixed with ATP prior to the DNA addition, the complex binds circular ssDNA poorly. In contrast, if Mcm2-7 is mixed with circular ssDNA prior to the ATP addition, considerably higher levels of circular ssDNA binding are observed (24). Mcm467 binds circular ssDNA poorly regardless of the order of addition of ssDNA and ATP.

As both complexes are toroidal, the difference between Mcm2-7 and Mcm467 may reflect their relative abilities to open their ring structure to facilitate ssDNA binding to the central channel. This possibility was examined through an analysis of a gapped Mcm complex (a pentameric complex lacking Mcm6) that was shown to bind linear and circular ssDNA with the same affinity regardless of ATP preincubation (24). These results suggest a simple hypothesis, that Mcm467 hexamers formed in the same cytosol normally form tightly closed toroids that are poorly able to bind ssDNA lacking a free end, while Mcm2-7 has the ATP-dependent ability to transiently open to allow circular ssDNA access to its central channel.

The second difference between the two Mcm complexes is in their association rates with linear ssDNA: Mcm467 binds relatively quickly, while under identical conditions, Mcm2-7 binds more than five times more slowly (23). Similar to the observation with circular ssDNA binding, the preincubation of Mcm2-7 with ATP increased its ssDNA association rate to that observed for Mcm467 (24), indicating that some aspect of the association between Mcm2-7 and ATP is rate limiting. Although the biochemical rationale of the difference is unknown, the full stimulation of the association rate or the inhibition of circular ssDNA binding requires roughly 30 min of ATP preincubation at 30°C, a finding more consistent with a slow ATP-dependent conformational change in Mcm2-7 rather than slow ATP binding per se (23, 24).

Evidence for an Mcm2/5 "gate." Since ATP preincubation dramatically changes the ssDNA binding properties of Mcm2-7, one or more ATPase active sites are likely involved in this effect. Moreover, since ATP preincubation has little effect on Mcm467 ssDNA binding, the basis of these differences

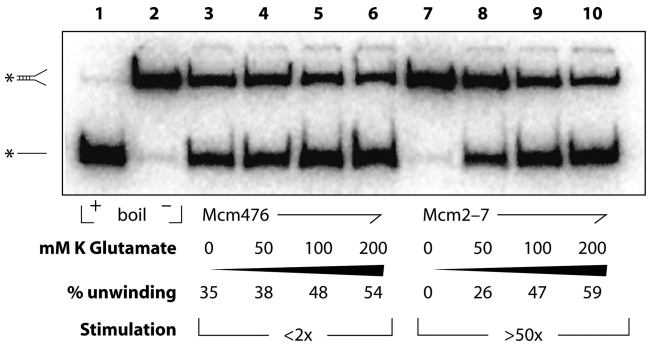

FIG. 8. Mcm2-7 helicase activity is glutamate dependent. Lanes contained either no Mcm (1 and 2), 400 ng Mcm467 (lanes 3 to 6), or 400 ng Mcm2-7 (lanes 7 to 10) and were supplemented with potassium glutamate as indicated. (Figure reprinted and legend adapted from reference 24 with permission from Elsevier.)

likely resides in ATPase active sites found in Mcm2-7 but not Mcm467 (i.e., those involving Mcm2, Mcm3, and/or Mcm5). To identify the responsible active site(s), Mcm2-7 complexes containing specific ATPase mutations were assayed (23), and complexes containing either the Mcm5KA or the Mcm2RA mutation enhanced the ssDNA association rate in the absence of ATP preincubation. Interestingly, these mutations also eliminate the ATP preincubation difference in circular ssDNA binding but in different ways. Heterohexameric complexes containing Mcm5KA bind circular ssDNA well, in a manner similar to that of the gapped pentameric complex, perhaps consistent with a loss of ATP binding at the Mcm2/5 active site. In contrast, complexes containing Mcm2RA bind circular ssDNA poorly, in a manner similar to that of Mcm467, perhaps consistent with ATP binding without hydrolysis at the Mcm2/5 site (23, 24).

As noted above, these two motifs are predicted by subunit association studies to be present in the same active site (Mcm2/5). Combined with the studies that indicate little physical association between Mcm2 and Mcm5, these results suggest that they form a reversible ATP-dependent discontinuity or "gate" in the Mcm2-7 toroid: closed when Mcm2/5 binds ATP and open when the active site is empty.

Mcm2-7 helicase activity is anion dependent. Oddly, ATP appears to both promote and inhibit the Mcm2-7/ssDNA association rate effect; ATP preincubation (suggesting a positive role for ATP) and mutations that ablate the Mcm2/5 active site (suggesting a negative role for ATP) have similar effects. These data suggest that the role of ATP in this phenomenon may be indirect. A specific possibility is that an unknown copurifying inhibitor is lodged in the Mcm2/5 active site; this inhibitor can be displaced either by competition with exogenous ATP or by the mutational ablation of its binding site.

Attempts to identify a potential inhibitor revealed that ssDNA binding by Mcm2-7 is very sensitive to the nature of the salt present in the binding buffer; although potassium chloride supports ssDNA binding, the potassium salts of bromide and iodide do not (Bochman, unpublished). Conversely, larger anions (e.g., glutamate or acetate) actually stimulate the Mcm2-7 association rate with ssDNA and inhibit circular ssDNA binding to nearly the same extent as ATP preincubation (24).

Mcm2-7 helicase activity is also very sensitive to specific anions; the replacement of chloride with either glutamate or acetate in the helicase assay reconstitutes the Mcm2-7 DNA-unwinding activity (Fig. 8) (24). Since the binding constant of Mcm2-7 with ssDNA is the same in either 100 mM potassium chloride or glutamate, the stimulatory effect of glutamate is not caused by an increased affinity for ssDNA. Moreover, because the effect is anion specific, it is unlikely to be occurring through alterations of the DNA (a polyanion) but rather must function by causing an alteration of the Mcm2-7 complex itself. Limited proteolysis of Mcm2-7 also implicates a conformational change in the presence of glutamate (Bochman, unpublished). Finally, an analysis of helicase activity in the presence of Mcm subunit-specific neutralizing antibodies confirms that glutamate does not stimulate DNA unwinding by physically liberating Mcm2, Mcm3, and Mcm5 from the complex to generate active Mcm467 (24).

Mcm2-7 ATPase Active Sites Contribute Unequally to Helicase Activity

In addition to ATP hydrolysis and ssDNA binding, the helicase activity of Mcm2-7 also depends upon a subset of ATPase active sites. Analysis of mutant Mcm2-7 complexes demonstrates that those containing Walker A (K→A) mutations in either Mcm4, Mcm5, Mcm6, or Mcm7 ablate helicase activity, while complexes containing Walker A mutations in Mcm2 or Mcm3 retain either partial or complete levels of DNA unwinding, respectively (24). Although the Mcm2KA mutation did not completely abolish activity, the Mcm2RA mutation did, demonstrating the physical requirement for the Mcm2 subunit in Mcm2-7 DNA unwinding. In addition to further substantiating the claim that Mcm2-7 helicase activity was not due to the presence of fortuitous Mcm467, these results indicate that ATPase active sites contribute unequally to helicase activity.

Previous studies of the Mcm Walker A mutants demonstrate that the inclusion of a single-mutant subunit into Mcm2-7 largely poisons the ability of the entire complex to hydrolyze ATP (see above). To better ascertain the contributions of individual active sites, mutant complexes containing subunits with either single Walker B or arginine finger mutations or multiple Walker A mutations were assayed for helicase activity. Two complementary results emerged from this analysis. Mcm2-7 complexes that contain individual Walker B or arginine finger mutations within the Mcm7/4 and Mcm4/6 sites are essentially devoid of helicase activity, while mutations in the other active sites have considerably less effect on DNA unwinding (our unpublished results). Also, while double or triple Walker A mutations among the Mcm2, Mcm3, and/or Mcm5 subunits suppress the steady-state ATPase defect of complexes containing a single KA mutant subunit (218), these combinations cannot suppress any corresponding DNA-unwinding defects (24). Although Mcm2-7 complexes containing a single Walker A mutation in either Mcm2 or Mcm3 retain substantial helicase activity, analogous complexes containing this mutation in both subunits have little to no helicase activity (24). These results demonstrate that the Mcm5/3 and Mcm6/2 active sites have partially redundant but essential roles in coupling ATP hydrolysis to DNA unwinding.

In summary, analysis of a variety of Mcm2-7 biochemical

activities—ATP hydrolysis, ssDNA binding, and helicase activity—clearly demonstrates that the individual Mcm ATPase active sites make unequal contributions. Although the specifics are complicated, the results in aggregate confirm that active sites contributed by Mcm4, Mcm6, and Mcm7 are largely required for ATP hydrolysis, ssDNA binding, and helicase activity, while Mcm2, Mcm3, and Mcm5 play a secondary role possibly involving the coordination of ATP hydrolysis with DNA unwinding. Numerous observations suggest that the Mcm2/5 active site makes unique regulatory contributions to various biochemical activities, possibly involving the formation of an ATP-dependent discontinuity in the toroidal Mcm2-7 complex. The significance of these observations will be discussed below.

INVOLVEMENT OF Mcm2-7 IN PRE-RC FORMATION

In vivo results derived largely from chromatin immunoprecipitation studies determined that Orc1-6, Cdc6, and Cdt1 are necessary to target and load Mcm2-7 at origins of replication to form the pre-RC (reviewed in reference 14). Despite the superficial simplicity of these interactions, they have proven very difficult to reconstitute in vitro using purified recombinant components. Although some work has been done with the corresponding *Xenopus* proteins (89), the currently best-characterized system uses partially purified G_1- and S-phase *S. cerevisiae* extracts and origin DNA coupled to magnetic beads (221). With this system, it was shown that origin-bound Orc1-6 (that is also bound to ATP) is competent to recruit Cdc6 (205). When Cdc6 binds ATP, it recruits Cdt1 bound to Mcm2-7. ATP hydrolysis by Cdc6 leads to the loading of Mcm2-7 at origins and the release of Cdt1 (that hydrolysis event also destabilizes the association of Cdc6). ATP hydrolysis by the Orc1 and Orc4 subunits completes the Mcm2-7 loading reaction and allows for further rounds of reiterative helicase loading (26). While Orc1-5 is sufficient to bind origin DNA, Orc6 is necessary for interacting with Cdt1 and loading the Mcm2-7 helicase (37). The nature of the DNA bound by Mcm2-7 within the pre-RC (e.g., single stranded versus double stranded) and any possible contribution that Mcm2-7 ring topology (i.e., Mcm2/5 gate open or closed) makes to pre-RC formation remains to be addressed.

MODEL OF Mcm2-7 FUNCTION

In combination, in vitro studies of the ATPase active sites of both the archaeal and eukaryotic complexes are beginning to provide a coherent picture of how the complex functions. There are two particular pieces of evidence, as discussed below.

ATPase Active-Site Specialization: DNA Unwinding versus "Gate"

As discussed in detail above, ATP hydrolysis at only a subset of Mcm2-7 active sites (Mcm7/4, Mcm4/6, and possibly Mcm3/7) is uniquely involved in DNA unwinding (24; our unpublished data). Analogous experiments with the archaeal Mcm complex suggest that ATP hydrolysis at half of the active sites or less is sufficient for DNA unwinding (180). It should be noted, however, that while ATP hydrolysis of the nonhelicase eukaryotic active sites is not required for in vitro helicase activity, most of the corresponding alleles are lethal in vivo (22). Thus, ATP binding and/or hydrolysis at these sites still performs an essential function, possibly to regulate the loading, activation, or unloading of Mcm2-7 during the corresponding phases of DNA replication.

These observations can most economically be explained by postulating that Mcm2/5 represents an ATP-dependent discontinuity in the Mcm2-7 toroidal structure. As the evidence for this discontinuity is indirect (i.e., subunit association and ability to bind circular ssDNA), the Mcm2/5 active site might alternatively be viewed as an allosteric regulatory site in Mcm2-7. To simplify the following discussion, Mcm2/5 will be assumed to form or regulate a "gate" within the toroidal Mcm2-7 structure, but many of the same arguments would also apply if Mcm2/5 were simply an allosteric regulatory site with no direct involvement in gate activity.

Although the notion that only a subset of the active sites are involved in DNA unwinding may appear heretical to those who study homohexameric helicases, the natural abundance of dimeric helicases indicates that DNA unwinding does not mechanistically require six interdependent active sites. Although many AAA^+ ATPases are homooligomeric, some are heterooligomeric and commonly contain ATPase active sites that are either functionally degenerate (i.e., Orc4 [26]) or inactive (processivity clamp loader [116]). In addition, an ATP-mediated subunit association, as suggested for Mcm2/5, is commonly observed for various viral helicases (i.e., SV40 TAg [259]), and gapped structures have been observed for other toroidal hexamers (i.e., SV40 TAg [21, 175, 176], RecA [15, 240], and the Rho transcription terminator [234]).

Evidence for Functional Coupling between Gate Closure and Helicase Activity

In contrast to mutations in either the Walker B or arginine finger motif, the inclusion of just a single Walker A mutant subunit strongly inhibits ATP hydrolysis by the entire Mcm2-7 complex, suggesting that even active sites that demonstrate little or no ATP hydrolysis still act as allosteric activators of ATPase activity. This feature appears to be Mcm2-7 specific, since the analogous mutation in the archaeal complex eliminates ATP hydrolysis at the affected site without poisoning the ATP hydrolysis or helicase activity of the entire hexamer (i.e., see Fig. 6 and reference 180).

There are several additional pieces of data to suggest why all of the Mcm2-7 active sites need to bind ATP. Mcm2-7 complexes with mutations that perturb gate function (Mcm2RA and Mcm5KA) lack helicase activity; this suggests that either the inability to close the gate (Mcm5KA mutation) or possibly a defect in the ability to communicate the state of gate closure to the rest of the complex (Mcm2RA) blocks helicase activity (Fig. 9A and B). In addition, the two active sites flanking Mcm2/5 (i.e., Mcm5/3 and Mcm6/2) appear to be involved in coupling ATP hydrolysis to productive DNA unwinding (24), as Mcm2-7 complexes that contain Walker A mutations in both sites demonstrate high levels of ATP hydrolysis but little DNA unwinding.

These observations can be accommodated by postulating that the closure of the Mcm2/5 gate is transmitted by ATP-

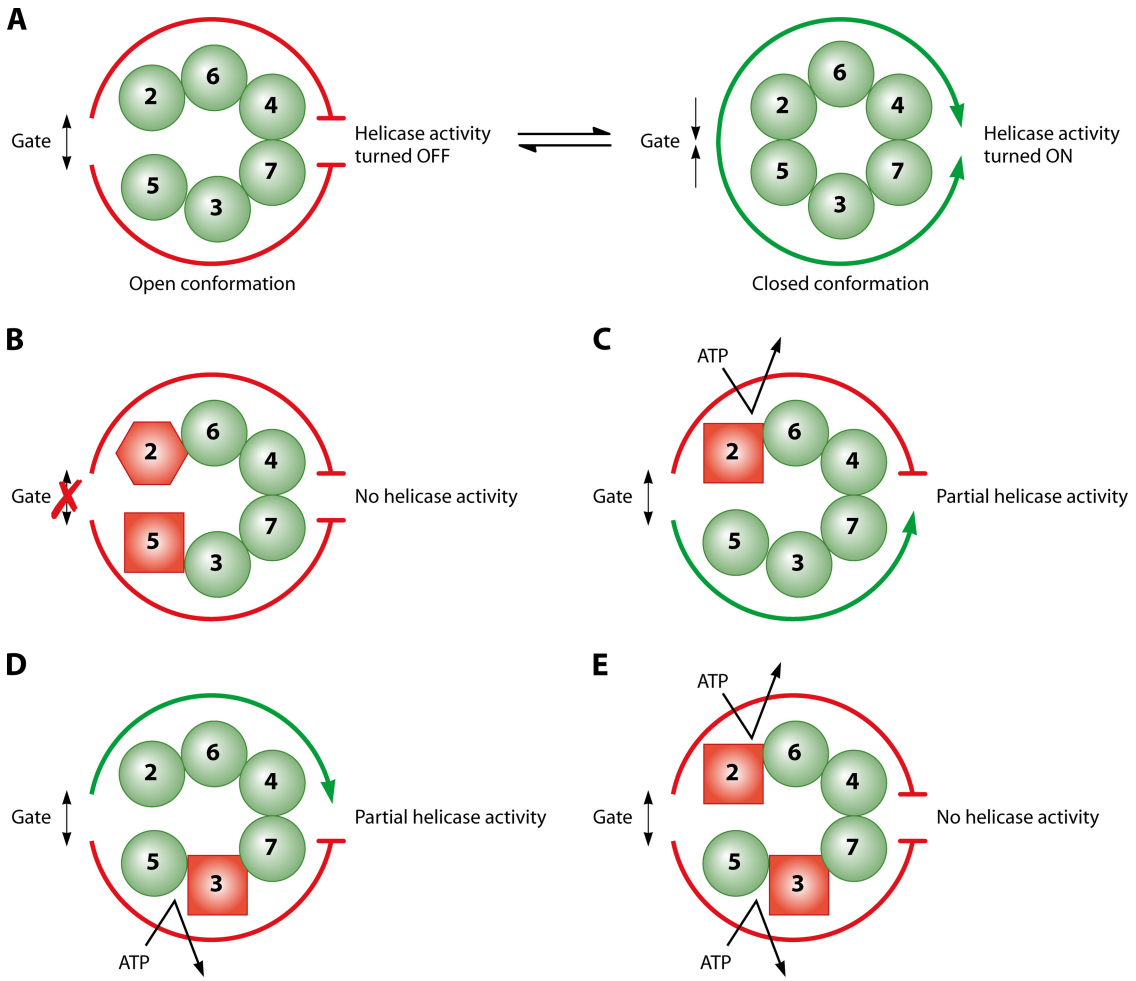

FIG. 9. Speculative model for coupling Mcm2/5 gate activity with DNA unwinding. (A) Wild-type Mcm2-7. The Mcm2/5 gate alternates between open and closed conformations. In the open conformation, the helicase activity is turned off by conformational changes propagated through the complex; in the closed conformation, a different set of conformational changes is propagated through the complex that activates helicase activity. (B) Mutations that destroy normal gate activity (i.e., Mcm5KA and Mcm2RA) prevent propagation of the activating conformation. (C and D) Mcm2KA (C) or Mcm3KA (D) mutations cause a partial reduction in helicase activity by a loss of ATP binding at the affected active site (indicated) that partially blocks the activating conformation from reaching the Mcm7/4 site. (E) The double-mutant complex has little or no ATPase activity since no activation signal reaches the Mcm7/4 site. Green circles represent wild-type subunits, red hexagons represent arginine finger R→A mutations, and red squares represent Walker A K→A mutations.

dependent conformational changes in both directions around the toroid to the sites responsible for helicase activity; the inability to transmit this information (i.e., the loss of both Mcm2/5 flanking sites) blocks helicase activity (Fig. 9C to E). The Mcm2/Mcm3 double Walker A mutant complex demonstrates no defect in toroid closure (i.e., normal circular ssDNA binding [Bochman, unpublished]), suggesting that the inhibition of helicase activity is not due to abnormal gate closure but rather to the inability to transmit this information to the helicase active sites. Since Walker A mutations frequently block both ATP binding as well as hydrolysis, this at least suggests that the ATP occupancy of the Mcm5/3 and Mcm6/2 active sites is required to activate the helicase.

The above-described model assumes that the Mcm5/3 and Mcm6/2 active sites transmit a positive signal upon gate closure that activates helicase activity. The opposite may also be true, that these sites transmit a negative signal when the gate is open that blocks helicase activity. The addition of Mcm2, Mcm3, or the Mcm5/3 dimer in any combination to the Mcm467 subcomplex blocks helicase activity (144, 267; Bochman, unpublished). One reasonable explanation would be that these treatments break apart the Mcm467 toroidal structure. This indeed seems to be the case with the addition of the Mcm5/3 dimer to the Mcm467 subcomplex, but it is known that the mammalian Mcm2467 tetramer is still toroidal (267). Interestingly, in the case of the Mcm2 addition, the inhibition of Mcm467 helicase activity requires an intact Walker A motif on Mcm2; a wild-type Mcm2 preparation inhibits helicase activity, whereas an Mcm2KA mutant preparation does not (237). This result does not occur through a trivial loss of associations between the mutant Mcm2 subunit and Mcm467, since both wild-type and mutant Mcm2 associate robustly with Mcm467. Assuming that Mcm2-7 functions in a similar manner, these data combined with the results discussed above

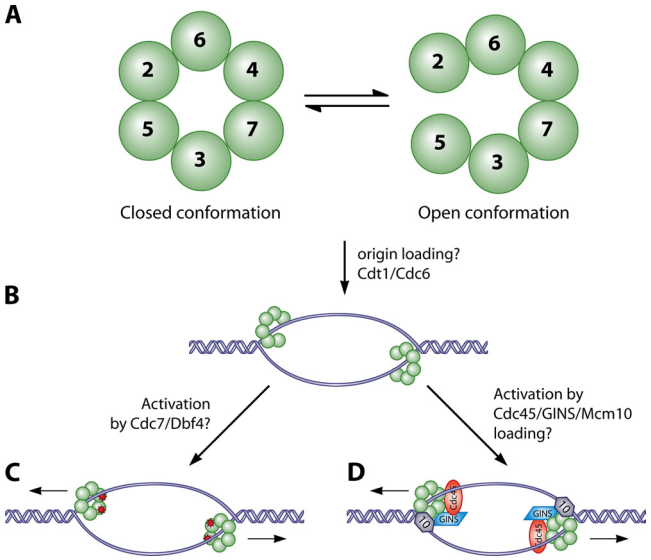

FIG. 10. Speculative model for Mcm2/5 gate involvement during DNA replication. (A) In vitro, the Mcm2-7 complex exists in equilibrium between open and closed states, but its topology in vivo is unknown. (B) The open state may facilitate DNA loading. Cdc6 and Cdt1 recruit the Mcm2-7 complex to origins of replication (marked by Orc1-6 [not shown]). An open Mcm conformation may be required to load DNA into the central channel. In late G_1/early S phase, the Mcm2-7 complex is activated for DNA unwinding. The closure of the Mcm ring and helicase activation may occur by several means. (C) Cdc7/Dbf4 phosphorylation may close the Mcm2/5 gate and activate DNA unwinding. Small red circles indicate phosphorylation. (D) Alternatively, the loading of Mcm10, the GINS complex, and Cdc45 may either (i) activate the helicase, (ii) act as processivity factors by stabilizing the closure of the Mcm2/5 gate to prevent dissociation from the DNA (depicted), or (iii) regulate the activity of individual Mcm active sites in some unknown fashion. Purple hexagons, Mcm10; blue parallelograms, GINS heterotetramer; red ovals, Cdc45.

suggest that both stimulatory and inhibitory conformational changes likely require the nucleotide occupancy of the Mcm6/2 active site.

Taken together, these data suggest a simple model for Mcm2-7 activity (Fig. 9A). Unwinding by the Mcm3/7, Mcm7/4, and Mcm4/6 sites is regulated by the state of the Mcm2/5 gate: when the Mcm2/5 active site is "open" (no nucleotide bound), the helicase is actively turned off; when it is closed (nucleotide bound), the helicase is actively turned on. The possible regulatory significance of this scenario is discussed below.

AVENUES FOR Mcm2-7 REGULATION

It is attractive to imagine that the Mcm2/5 gate has regulatory significance. Although in vivo ionic conditions would be expected to favor the closure of the Mcm2/5 gate (glutamate levels are high [see reference 147 and references therein]), and (chloride levels are low [~150 μM] [52]), the addition of neither glutamate nor ATP is enough to force Mcm2/5 dimerization from isolated subunits in vitro (our unpublished data). These data suggest that in vivo, the Mcm2/5 interface is still relatively labile (Fig. 10A). This property could be leveraged in a variety of ways during DNA replication. For instance, to load Mcm2-7 onto DNA prior to the start of unwinding or unload it from DNA at the end of S phase, the Mcm2-7 complex must be at least transiently opened to allow DNA access to its central channel, and the natural weakness in the Mcm2/5 interface suggests its potential involvement in this process (Fig. 10B). Alternatively, closing the Mcm2/5 gate might be the regulated event that activates DNA-unwinding activity from the dormant pre-RC upon passage through the G_1/S-phase transition (Fig. 10C and D).

Role of Pre-RC Components in Mcm2-7 Loading and Activation

In vivo ATP levels are high and relatively constant (~3 mM [252]), suggesting that the Mcm2/5 ATPase active site may normally be filled with ATP. If the Mcm2/5 site functions as the access point in chromosome loading during pre-RC formation, it suggests that at least one possible role of the putative Mcm2-7 loading factors (Cdc6 and Cdt1) would be to regulate either ATP turnover or ATP exchange at this site, essentially biasing the gate from a closed to an open conformation (more below). Moreover, since Mcm2-7 is functionally inert within the pre-RC, some component of the pre-RC might be necessary to maintain the open and inactive conformation of the Mcm2-7 complex until passage through the G_1/S transition. In bacteria, there is a precedent for this type of regulation, as interactions between DnaC (helicase loader) and DnaB (replicative helicase) inhibit DnaB ATPase and helicase activity (57).

This prediction is supported by at least some of the results from the archaeal DNA replication systems that show that specific Cdc6 homologues inhibit either the ATPase activity or helicase activity of their cognate archaeal Mcm complex (Table 2) (7, 61–63, 99, 100, 117, 225, 226). Although data from previous work with Walker A alleles of Mcm6 and Mcm7 suggested that Mcm2-7 ATP hydrolysis was needed only for DNA unwinding rather than pre-RC formation (270), this interpretation holds only if all six Mcm2-7 active sites participate directly in DNA unwinding. Examination of pre-RC formation using ATPase alleles of the nonhelicase subunits might identify defects in origin association rather than elongation.

Possible Role of Replication Factors during Mcm2-7 Activation and Elongation

Kinases: Cdc7/Dbf4 (DDK), CDKs, and ATR. Although the mechanism by which DDK activates DNA replication is unknown, Mcm2-7 is a likely functional target. A specific *S. cerevisiae* mutation in *MCM5* (*mcm5-bob1*, P83L) obviates the need for DDK (97), and various Mcm subunits (Mcm2, Mcm4, and Mcm6) serve as both in vivo and in vitro substrates for this kinase (82, 146, 167, 168, 219, 224). Although the DDK phosphorylation of Mcm4 has been shown to assist Cdc45 loading (224), a role for Mcm2 and Mcm4 phosphorylation remains unknown.

Analysis of the *mcm5-bob1* mutant indicates that it facilitates a conformational change in the Mcm complex. Since structural work with the eukaryotic Mcm2-7 complex has thus far proved intractable, the *bob-1* mutation has been studied by X-ray crystallography following incorporation into the archaeal MthMcm complex. This mutation causes a modest conformational change in the MthMcm N-terminal domain (39, 76), a result that led to the "domain-push" hypothesis, which predicts

that Mcm2-7 helicase activation occurs through a DDK-dependent conformational change (76, 220). It should be noted, however, that the in vivo ability of this *M. thermoautotrophicus* "*bob-1*-like" mutation to bypass the need for DDK activation cannot be tested, since this organism lacks a DDK homologue. Moreover, since the MthMcm complex is homohexameric, the resulting structure contains the mutation in all six subunits, as opposed to only a single subunit change in Mcm2-7. Paradoxically, although the *bob-1* mutation is thought to activate eukaryotic Mcm2-7 helicase activity, MthMcm complexes containing this allele have decreased in vitro helicase activity with little effect on ssDNA binding or ATP hydrolysis (77). While the effect of the *bob-1* mutation in Mcm2-7 awaits structural studies, it is tempting to speculate that DDK activation may involve conformational changes at the Mcm2/5 interface directly or indirectly through the recruitment of additional DNA replication factors (Fig. 10).

DDK preferentially acts on Mcm2-7 complexes that have been "loaded" into the pre-RC, suggesting the involvement of other pre-RC components in this process (82). Interestingly, the DDK phosphorylation of Mcm2-7 requires a prior phosphorylation event (activation) (82) postulated to cause a conformational change in Mcm2-7 that exposes a DDK docking site and/or a target peptide for DDK phosphorylation. While the identity of the activating kinase remains unknown, CDKs and ATM/ATR are two obvious candidates. Alternatively, an additional possibility is suggested by reports of a cryptic kinase activity associated with both the archaeal and eukaryotic Cdc6 proteins (7, 93, 99).

In contrast to DDK, the functional role of CDK and ATM/ATR phosphorylation of Mcm2-7 is controversial and may vary among model organisms. In vivo, it is clear that Mcm2-7 is phosphorylated by both CDK (reviewed in reference 14) and the ATM/ATR kinases (47, 112), and at least in *S. cerevisiae*, CDK phosphorylation promotes the nuclear exit of the Mcm proteins following DNA replication (188). However, the demonstration of an essential role for these putative phosphorylation events has been elusive for several reasons: (i) the only essential targets of CDK phosphorylation in *S. cerevisiae* are the replication factors Sld2 and Sld3, as the appropriate genetic manipulation of these factors obviates the normal dependence of DNA replication on CDK phosphorylation (247, 279); (ii) treatment of purified Mcm2-7 with either λ phosphatase (to completely dephosphorylate the Mcm proteins) or Cdc28/Clb5 (the yeast CDK1 homologue) has no obvious effect on the in vitro ability of the complex to bind DNA (Bochman, unpublished); and (iii) the ablation of all consensus or near-consensus CDK and ATR phosphorylation sites (~60 total, alanine substitution mutations of the critical serine/threonine) from the six *S. cerevisiae MCM* genes demonstrates that, with the exception of a site within the Walker A motif of all six subunits, none of these phosphorylation sites on any one Mcm subunit are essential for viability (M. Patel, J Mitchell, D. Leigley, R. Elbakri, and A. Schwacha, unpublished observations). These results can be interpreted in one of two ways: that CDK and ATR phosphorylation of Mcm2-7 has little or no importance in DNA replication or, conversely, that it is so important that there exists a high level of functional redundancy between phosphorylation sites that has so far masked current analyses, a possibility that has been demonstrated in a different context (189).

These experiments have yet to be duplicated in metazoan systems, but the current data indicate a contrasting role for the CDK phosphorylation of the Mcm proteins in higher organisms. CDK phosphorylation of mammalian Mcm3 on Ser-112 (a residue conserved from yeast to humans) is crucial for the incorporation of Mcm3 into the Mcm2-7 heterohexamer (151). Mcm4 phosphorylation on Ser-3, -32, -54, and -88 and Thr-7, -19, and -110 by CDK1 and CDK2 varies with the cell cycle (133) (in contrast to the constant CDK phosphorylation of Mcm2 [179]) and suggests that these modifications may have distinct and site-specific roles (133). In vitro, CDK phosphorylation of human Mcm4 Thr-19 and -110 decreases Mcm467 helicase activity (111). Interestingly, the phosphorylation of these Mcm4 residues by the Epstein-Barr virus protein kinase also decreases Mcm467 helicase activity in vitro and results in growth arrest in HeLa cells in vivo (138).

It should be noted, however, that computational methods used to compare the conservation and evolution of CDK consensus sites among pre-RC components suggest that the position and number of CDK sites within a given protein are not necessarily important (181). Rather, it is simply the necessity of CDKs for the regulation of replication that is conserved and not necessarily the specific proteins or phosphorylation sites. Thus, the mechanism(s) of this regulation may have been free to evolve, a possibility that may account for the above-described discrepancies.

Cdc45 and the GINS complex: helicase accessory subunits? In addition to Mcm2-7, both Cdc45 and the GINS complex are essential replication factors required for in vivo fork progression (reviewed in reference 5). Cdc45 was isolated as a cell division cycle mutation with numerous genetic interactions with the *MCM* genes and a demonstrable DNA replication defect under nonpermissive conditions (102). The four essential GINS complex proteins (Sld5 and Psf1 to Psf3) were isolated largely as intergenic suppressors of other replication mutants (245). Both Cdc45 and the GINS complex demonstrate an origin association (3, 245) that relocalizes to replication forks during S phase (32), and similar to the Mcm proteins, conditional mutations in either Cdc45 or the GINS complex quickly block replication fork progression when inactivated (85, 249).

Recently, it was found that both Cdc45 and the GINS complex mediate physical interactions between the Mcm proteins and the rest of the replisome. The GINS complex physically interacts with DNA polymerase α (60), and several higher-order complexes containing Mcm2-7, GINS, and Cdc45 were recently isolated following cellular fractionation (85, 184, 192). Although the GINS complex binds DNA (25; our unpublished observations), the association of the GINS complex with Mcm2-7 appears to be specifically through Mcm4 (Botchan, submitted) rather than through DNA (85). The GINS complex mediates the physical interactions between Cdc45 and the Mcm proteins in vitro (Botchan, submitted) and is needed for the association of Cdc45 with pre-RCs in vivo (85, 245). Although no structural work has yet been done with Cdc45, the GINS complex has been crystallized and subjected to high-resolution analysis by several laboratories, who found that the

complex forms either rings (35, 40) or lockwasher-like structures (25) that may potentially encircle DNA.

Mcm10: a bridge to primase? In addition, Mcm10 is also involved in fork progression. In most systems, the replicative helicase is physically and functionally linked to primase (which is localized on the lagging strand to facilitate Okazaki fragment formation). In certain viruses (including a *Bacillus cereus* prophage encoding an Mcm-primase chimera [173]), this situation is structurally formalized, as the replicative helicase and primase are encoded by the same protein (reviewed in reference 46). Since the Mcm proteins are 3′→5′ helicases, they apparently translocate along the leading strand, suggesting the need for a special linkage to connect them to the primase located on the lagging strand. Mcm10 appears to fulfill this role and physically links Mcm2-7 to DNA polymerase α/primase (207). The *S. pombe* Mcm10 protein has even been shown to contain primase activity (75), although this property may be species specific, as no such activity has been observed for the *Xenopus* protein (208). Consistent with a suggestion that Mcm10 is involved in origin unwinding (68), Mcm10 binds both ssDNA and dsDNA (75, 208), and recent structural analysis implicates a specific protein binding surface (oligonucleotide/oligosaccharide binding fold and an adjacent zinc finger) in this interaction (261).

Moreover, genetic studies implicate regulatory interactions between Mcm10 and Mcm2-7 (44, 105, 194). Even though conditional alleles of *MCM10* do not cause a catastrophic loss of fork progression as seen in Cdc45 or GINS mutants, they do cause lethality with associated replication fork defects; forks pause when they reach adjacent unfired origins of replication (177). The reason for fork stalling is unclear, but second-site suppressors in *MCM2* that not only suppress the inviability of the *mcm10-1* allele under nonpermissive conditions but also alleviate the apparent fork-pausing defect have been isolated (B. Tye, I. Liachko, and C. Lee, personal communication). Interestingly, these suppressors map to two different sites within Mcm2: one set maps to residues predicted to be homologous with the archaeal ACL (see "Structural Biology of the Archaeal Mcm Proteins"); the second set maps adjacent to the presensor 1 β-hairpin that is believed to physically interact with the ACL. Mcm10 has also been copurified as part of a higher-order complex with Mcm2-7, although the functional significance of this interaction is currently unknown (85).

Replication checkpoint factors Mrc1, Tof1, and Csm3. When replication forks encounter a DNA lesion, their progression is blocked, and the protein-DNA associations within the fork are stabilized while the damage is repaired (156, 198). As mentioned previously, a complex of Mrc1, Tof1, and Csm3 fulfills this function. These proteins not only physically associate with the Mcm proteins (32, 85, 134, 186) but also associate with the leading-strand DNA polymerase ε (134, 157). This physical coupling provides a way to explain how a block to polymerase function can be communicated to the Mcm2-7 helicase.

Although the Mrc1/Tof1/Csm3 complex was commonly thought only to be involved in this checkpoint function, more recent experiments indicate that it is part of normal DNA replication forks under conditions free of exogenously induced DNA damage (32, 85, 186, 242). However, the exact function(s) of Mrc1, Tof1, and Csm3 at replication forks in vivo and their effect(s) on Mcm2-7 biochemistry in vitro have yet to be elucidated. While these three proteins are often considered to participate together in a common function, some evidence suggests that this may not be true. For instance, the *S. pombe* Tof1 homologue Swi1 was shown to differentially regulate the outcome of the replication machinery colliding with replication fork barriers (202).

Interestingly, the central coiled-coil region of Mrc1 was recently found to directly bind to the unique (among the Mcm2-7 subunits) C-terminal tail of Mcm6 in *S. cerevisiae* (134). The disruption of this interaction via mutations of Mcm6 (I973A and L974A) results in a severe deficiency in DNA replication checkpoint activation in response to methyl methanesulfonate, but not hydroxyurea, treatment; C-terminally fusing Mrc1 to mutant Mcm6 suppresses this phenotype. This suggests that the Mcm2-7 helicase directly senses methyl methanesulfonate-induced (i.e., alkylated) DNA damage by physically interacting with Mrc1. It should be noted that the association of Tof1 and Csm3 with Mcm2-7 is not disrupted in the *mcm6* mutant strain (134). It is therefore likely that Tof1 and Csm3 directly interact with other Mcm subunits, possibly relaying signals from other types of DNA lesions to Mcm2-7. Also of note is that fact that various other putative negative regulators of Mcm2-7 activity (Rad17 [254], ATRIP [47], and the retinoblastoma protein [238]) directly interact with the C terminus of another Mcm subunit, Mcm7. It would appear that while the central AAA^+ domains of the Mcm2-7 proteins are conserved for helicase activity, their divergent N and C termini have evolved as docking points for various other replication factors to control replisome progression.

Potential Role for Mcm Regulation during Replication Termination

In many systems, the Mcm proteins are transported out of the nucleus upon the completion of DNA replication, presumably as part of the fail-safe controls that ensure that only a single round of DNA replication occurs during S phase (189). However, how the Mcm proteins dissociate from DNA during the termination of replication is unknown. If cellular ATP levels bias Mcm2-7 toward a closed conformation, there is a formal possibility that the Mcm proteins may need assistance to dissociate from chromosomes after replication is completed. As essentially all other known aspects of the Mcm proteins are tightly regulated, the disassembly of the replication machinery and the removal of the Mcm proteins during termination deserve future scrutiny.

Possible Mechanisms for Mcm2-7 Regulation

In vivo, replicative helicases need to unwind thousands of base pairs of DNA before dissociation. Although prokaryotes have a backup system to reload the replicative helicase into collapsed forks that have lost physical associations with replication factors (replication restart) (reviewed in reference 101), eukaryotes appear to lack this ability and are unable to reform pre-RCs during S phase after elongation has begun. Although it is likely that cells contain a sufficient number of usually inactive pre-RCs that can be mobilized to mitigate a collapsed replication fork (107, 264), the pathological occurrence of

paused or collapsed replication forks is believed to be an important condition leading to various types of deleterious genomic instability (reviewed in reference 28).

In vitro, the Mcm complexes appear to be poorly suited to become a "juggernaut" able to traverse long stretches of chromatin in a single DNA binding event. The best data suggest that the archaeal Mcm proteins have a processivity of only ~500 bp (129) and have difficulty disrupting very stable protein-DNA interactions (165, 229). Although experiments are at a preliminary stage, the *S. cerevisiae* Mcm2-7 complex is able to unwind only duplex regions of ~200 bp in vitro (our unpublished observations). These data contrast with the several thousand base pairs of in vitro processivity demonstrated by SV40 TAg (263, 274). Moreover, the eukaryotic Mcm proteins have a marked binding preference for polypyrimidines (5- to 10-fold better than mixed sequences [23, 227, 263, 274]), raising the possibility that they might stall while unwinding such regions. Although the Mcm proteins associate with chromatin remodeling factors that might be anticipated to assist in removing nucleosomes (246, 257), many other tightly bound proteins are expected to be present as well.

The ability of the helicase to unwind long stretches of DNA without dissociation, termed processivity, depends upon two factors: the length of time that it associates with DNA and the number of base pairs unwound per second per association. Mcm accessory factors might increase processivity in at least three ways. First, to counteract the potential lability of the Mcm2/5 gate, these proteins may physically interact with Mcm2-7 to prevent its dissociation from DNA. Indeed, recent structural work with both the GINS complex and Mcm10 indicates that these molecules are either toroidal (35, 137, 191) or nearly toroidal (25, 40, 119) in nature. Although there is some controversy regarding whether the central channel of the GINS complex is wide enough to encircle ssDNA (or if the channel size is regulated [35]), both GINS and Mcm10 might function by encircling DNA; simultaneous binding to Mcm2-7 would help prevent the spontaneous dissociation of the replicative helicase during elongation. Additionally, the activity of some of these factors (i.e., DDK phosphorylation) may act to cause a conformational change in the Mcm2-7 complex that results in the closure of the Mcm2/5 gate.

Finally, these factors might serve to regulate the occupancy or hydrolysis of ATP at specific Mcm2-7 active sites. Depending upon the site stimulated or inhibited, this might serve to increase either the rate of DNA unwinding (e.g., the Mcm7/4 site) or the stability of the complex on DNA (e.g., the Mcm2/5 site). By analogy to a multitude of other ATP hydrolases such as Hsp70, it is well established that the activity of these proteins is regulated by factors that control either the nucleotide occupancy of the active site (exchange factors) or its catalytic turnover (reviewed in reference 87). Although little is yet known about this possibility with the Mcm proteins, a recent study demonstrating that the archaeal GINS complex stimulates the corresponding ATPase activity of the Mcm helicase supports this conjecture (272).

Recent evidence suggests that the GINS complex and/or Cdc45 may regulate the processivity as well as alter the ATP hydrolysis cycle of Mcm2-7. Although *Drosophila* Mcm2-7 has a weak helicase activity (M. Botchan, submitted for publication), this activity is greatly enhanced in the CMG complex, a higher-order protein complex formed from Cdc45, Mcm2-7, and GINS (184). A biochemical comparison of these two complexes reveals that the CMG complex has an approximately 10-fold-higher affinity for forked DNA substrates than Mcm2-7, potentially consistent with an increase in processivity (Botchan, unpublished). Moreover, although the *Drosophila* Mcm2-7 complex demonstrates the same three kinetic states of ATP hydrolysis as the *S. cerevisiae* Mcm2-7 complex (218), one of these states in the CMG complex has a considerably altered V_{max}, consistent with a possible role of GINS or Cdc45 in the regulation of Mcm2-7 ATP hydrolysis (Botchan, submitted).

SPECULATION ON Mcm OLIGOMERIZATION AND EVOLUTION

One unsettling aspect of the eukaryotic Mcm2-7 complex is that Mcm subunits can assume a variety of oligomeric states. Although many of these assemblies can be viewed as subcomplexes resulting from Mcm2-7 dissociation, others are more difficult to explain. Mcm oligomerization is not a random and stochastic process (e.g., only five sets of stable heterodimers have been isolated [22, 58]), yet many of the individual Mcm subunits appear to be capable of dimerizing or forming higher-order complexes in isolation (22, 54, 58, 121, 218, 278). More puzzling yet is the fact that at least two complexes, Mcm7/4 and Mcm467, form hexameric complexes with helicase activity under in vitro conditions (109, 121).

Although there is a compelling body of genetic, cell biological, and biochemical evidence to support the claim that Mcm2-7 is the bona fide replicative helicase, how can these alternative and potentially functional Mcm oligomerization states be rationalized? It should be noted that only Mcm2 and Mcm3 contain (partial) nuclear localization signals, and as such, only intact heterohexamers (or, minimally, subcomplexes containing Mcm2 and Mcm3) can enter the nucleus (110, 131, 139, 150, 188, 196). In the absence of cryptic nuclear localization signals on other Mcm subunits, this scheme limits the ability of Mcm7/4 and Mcm467 hexamers to access the nucleus.

These alternative oligomerization states may represent evolutionary artifacts. As discussed above, the six eukaryotic Mcm proteins are partially homologous with one another, suggesting that the putative duplication and diversification of eukaryotic *MCM* genes formed extremely early at the split between the archaea and eukaryotes. Two features of their phylogenetic relationship (Fig. 1) are particularly noteworthy. First, these six families represent one of the most conserved features of DNA replication initiation in eukaryotes (114, 155), with all sequenced eukaryotic genomes to date encoding all six proteins. The extreme conservation of the unusual heterohexameric subunit arrangement strongly suggests that it serves an important functional purpose. Moreover, the sequence relatedness between subunits correlates largely with the accepted subunit organization within the Mcm2-7 complex (Fig. 10A); the most closely related eukaryotic Mcm proteins are those that form physical contacts within the heterohexamer (22, 58).

It is worth considering how the eukaryotic Mcm proteins might have evolved from a primordial Mcm precursor. Typical homohexameric helicases would have considerable difficulty evolving into a heterohexameric helicase, because the tight coupling between active sites would likely restrict the func-

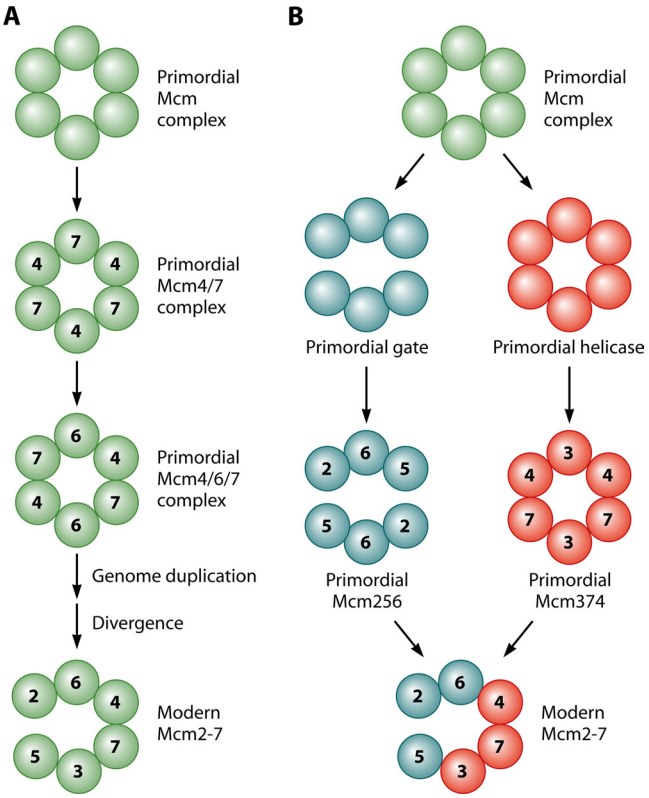

FIG. 11. Models of Mcm2-7 evolution. (A) Divergent evolution of Mcm2-7. (B) Convergent evolution of Mcm2-7. See the text for details.

tional divergence of a duplicated gene. However, the archaeal Mcm proteins appear to be free of this constraint; since only three of the six SsoMcm ATPase active sites are required for helicase activity (180), the complex can tolerate active sites of diminished or altered activity.

Based upon this predication, at earlier evolutionary stages, there should have existed eukaryotic Mcm hexamers containing more than a single type of subunit but fewer than the current six subunits. Despite this likelihood, there are no known cases of any current eukaryote having less than six distinct Mcm2-7 subunits (Bochman, unpublished). Moreover, although various archaeal genomes contain multiple *MCM* genes, some of these extra *MCM* genes represent pseudogenes (8), and none of these genes have increased homology to any of the eukaryotic *MCM2-7* genes (Bochman, unpublished).

Although intermediate stages of Mcm divergence are not evident among modern eukaryotes, several plausible scenarios for their evolution could be considered. A major constraint on Mcm evolution would be the need to maintain helicase activity. Since the eukaryotic Mcm7/4 site is most strongly associated with DNA unwinding and in isolation can form a hexamer with DNA-unwinding activity (121), Mcm4 or Mcm7 likely corresponds to the most primordial of the eukaryotic Mcm subunits and is most closely related to the archaeal Mcm proteins, as supported in published phylogenetic studies of the Mcm proteins (42, 129) (however, see the Fig. 1 legend).

The remaining Mcm subunits might have evolved in at least one of several ways. After the evolution of Mcm4 and Mcm7, an additional gene duplication event might have led to Mcm6, resulting in a primordial Mcm467 complex (Fig. 11A). To maintain a symmetric hexamer (one cannot form such a complex from four or five distinct subunits), the remaining Mcm subunits might have been acquired by genome duplication followed by divergence. Alternatively, both helicase function and gate function could have evolved separately and then at a later time evolved into a heterohexameric Mcm2-7 complex (Fig. 11B). The phylogeny in Fig. 1 suggests that Mcm3, Mcm4, and Mcm7 form one related group, while Mcm2, Mcm5, and Mcm6 form another. Moreover, the degree of sequence divergence among all six *MCM* genes is roughly equal, an observation that is more supportive of the coevolution of the subunits rather than sequential evolution.

What might have driven Mcm differentiation? Eukaryotes have considerable complexities in cell cycle regulation that archaea likely lack; six distinct active sites would allow at least six distinct ways in which to regulate the complex. A reasonable corollary of this supposition would be that factors that would later regulate the Mcm2-7 complex would have coevolved with it. As previously noted (255), although the archaea contain many of the replication factors found in eukaryotes, they appear to lack Cdc7/Dbf4, Cdc45, Mcm10, and the CDKs (255; Bochman, unpublished), eukaryotic replication factors with intimate yet largely unknown functional interactions with Mcm2-7.

CONCLUSION

We are beginning a new stage in the study of eukaryotic DNA replication. With the demonstration of in vitro helicase activity from the Mcm2-7 complex, the potential for reconstituting a eukaryotic replication fork in vitro to elucidate mechanistic aspects is rapidly becoming a reality. The biochemical advances made with the Mcm proteins and the issues raised in this review—the functional divergence of ATPase active sites, processivity, and regulation by other replication factors—provide a framework for much additional experimentation. This will elevate our analysis of DNA replication from a study of factor loading as a function of the cell cycle to a mechanistic understanding of how and why specific factors contribute to DNA replication through the use of biochemically defined mutants. Given both the tractable nature of the archaeal Mcm complex and the ability to generate precise mutations within the eukaryotic Mcm2-7 complex, future structural and biochemical advances are well poised to finally provide definitive evidence for how a hexameric helicase actually unwinds DNA.

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