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The eukaryotic CMG helicase pumpjack and integration into the replisome

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ABSTRACT

The eukaryotic replisome is α multiprotein machine that contains DNA polymerases, sliding clamps, helicase, and primase along with several factors that participate in cell cycle and checkpoint control. The detailed structure of the 11-subunit CMG helicase (Cdc45/Mcm2-7/GINS) has been solved recently by cryoEM single-particle 3D reconstruction and reveals pumpjack motions that imply an unexpected mechanism of DNA translocation. CMG is also the organizing center of the replisome. Recent *in vitro* reconstitution of leading and lagging strand DNA synthesis has enabled structural analysis of the replisome. By building the replisome in stages from pure proteins, single-particle EM studies have identified the overall architecture of the eukaryotic replisome. Suprisingly leading and lagging strand polymerases bind to opposite faces of the CMG helicase, unlike the long-held view that DNA polymerases are located in back of the helicase to act on the unwound strands.

Introduction

DNA is duplicated in a complex choreography performed by a mechanical interplay of numerous proteins referred to as a replisome.[1,2] The replisome machine unzips the duplex, copies the 2 strands, and communicates to checkpoint and repair systems that preserve the integrity of the genome. Assembly of the replisome occurs at origins of replication in a 2-phase reaction that ensures replication occurs only once per cell cycle.[3,4] In brief, a 6-subunit origin recognition complex (ORC) binds to an origin sequence and 2 Mcm2-7 rings are placed around double-strand (ds) DNA with the help of Cdt1 and Cdc6, forming the PreRC complex (Pre-Replicative Complex).[5-7] Loading of the Mcm2-7 ring around dsDNA involves opening the Mcm2-5 interface, referred to as the Mcm2-5 gate.[8-10] PreRC assembly occurs exclusively in G1 phase and underlies origin "licensing." Upon entry into S phase, cell cycle kinases and other factors assemble Cdc45 and GINS onto Mcm2-7, forming 2 active CMG helicases (Cdc45, Mcm2-7, GINS heterotetramer)[11] for bidirectional replication forks. Recombinant CMG from budding yeast, fly and human all display helicase activity.[12-14]

The core replisome proteins include the CMG helicase, DNA polymerase-primase (Pol α), the leading and lagging strand DNA polymerases ε (Pol ε) and δ (Pol δ), PCNA clamps, the RFC clamp loader, and the RPA single-strand bindingprotein. Pull-outs of CMG from actively replicating budding yeast, followed by mass spectrometry has identified many other proteins that bind CMG, referred to as the RPC (Replisome Progression Complex).[15,16] RPC contains CMG, Pol α, Ctf4, Mcm10, the checkpoint factors Tof1 (hTim), Csm3 (hTippin), Mrc1 (hClaspin) and TopoI.

We have recently purified the eukaryotic replisome proteins and have reconstituted a functional replisome that extends both the leading and lagging strands *in vitro*.[12,17,18] Furthermore, Pol ε was only active on the leading strand, and Pol δ was most active on the lagging strand, properties that are consistent with many cell biology studies in *S. cerevisiae* and *S. pombe* that assign Pol ε and Pol δ to the leading and lagging strands, respectively.[19-24] However, there is one study to suggest that Pol δ functions on the leading strand as well as the lagging strand.[25] This paper summarizes our recent advances on the structure of CMG [26] and

the replisome,[27] and places the findings in the context of previous studies from many other laboratories.

Replicative helicases are hexameric motor rings

Cellular replicative helicases are homohexameric rings that encircle DNA.[28-30] The most intensively characterized homohexameric helicases are *E. coli* DnaB, archaeal MCM, phage T4 gp41, phage T7 gp4, simian virus 40 large T-antigen and bovine papilloma virus (BPV) E1 helicase. In each case the monomeric unit consists of 2 domains, a N-terminal domain (NTD) and C-terminal domain (CTD). Thus the circular hexamers appear as 2 stacked rings, referred to here as the NTD tier and CTD tier. The motor domains are located in the CTD tier. Bacterial and phage helicase CTD motors are based on the RecA fold and travel 5′-3′ on ssDNA, while archeal and viral motors are based on the AAA+ fold and travel 3′-5′ on ssDNA.

The CMG helicase of eukaryotic cells contains a Mcm2-7 AAA+ hexameric motor ring that travels 3′-5′, but it has 2 important differences from the homohexameric helicases. First, Mcm2-7 is a heterohexamer of 6 homologous, but distinct subunits. Second, Mcm2-7 requires 5 accessory factors, Cdc45 and the GINS heterotetramer, to form the active CMG helicase.[11,13] The Cdc45-GINS accessory factors lack ATP sites, and are proposed to hold the Mcm2-7 hexameric ring into an active conformation for helicase function.[13]

Structure of CMG helicase

Recently, we determined a high resolution cryoEM structure of *S. cerevisiae* CMG helicase to 3.8-4.7 Å.[26] Figure. 1a illustrates our earlier 3D reconstruction of CMG at low resolution, showing the Mcm2-7 central channel (top view) and the NTD and CTD tiers (side view). The high resolution cryoEM single-particle 3D reconstruction of yeast CMG apoenzyme enabled atomic model building of each of the 11 subunits, providing very detailed information on the architecture of the complex.[26] The cryoEM study also revealed 2 different conformers, and their high resolution atomic models are shown in Fig. 1b. The 2 conformers suggested an unexpected mechanism of translocation along DNA, as discussed below. Simultaneous with the high resolution structure of yeast CMG is a subnanometer structure of fly CMG (7–9 Å) that provides information on ligand

interactions and nicely compliments conclusions drawn from the structure of yeast CMG.[31]

The 2 conformers of CMG are shown in Fig. 1b. The major difference between the 2 CMG conformers is a large structural change in the Mcm2-7 CTD tier, while the NTD tier and Cdc45/GINS appear rigid, which we refer to as the "NTD platform." Conformer I has a nearly open interface between the CTDs of the Mcm2-5 subunits (i.e. Mcm2-5 CTD remain connected by 1 domain swapped helix), while in Conformer II the Mcm2-5 interface is completely closed. These conformers likely correspond to the conformers identified by negative stain EM studies of *Drosophila* CMG, suggesting Conformer I represents an ATP free state and Conformer II represents an ATP bound state.[9] The partially open Mcm2-5 interface in Conformer I results in an "extended form" with a vertical displacement of the Mcm 2-6-7 CTD domains that reach as much as 20 Å further from the NTD tier relative to the more "compact" CMG Conformer II having a closed Mcm2-5 interface. Thus the central channel becomes longer or shorter in the extended and compact conformers. The CTD domains of the Mcm3-5-4 subunits undergo less motion, possibly through interaction of GINS with the CTDs of Mcm3-5. Switching back-and-forth between Conformers I and II gives a up-down nodding motion of the Mcm 2-6-7 CTD domains relative to the rigid NTD platform. We have likened these motions to that of an oil field pumpjack that looks like a nodding horse head that moves up and down on a fixed platform.[26]

Proposed inchworm pumpjack model for CMG

The pumpjack motion of CMG immediately suggests a simple inchworm translocation mechanism.[26] Directional translocation of a protein along DNA requires at least 2 DNA binding sites that change distance during the ATP cycle, thus driving translocation. The CTD AAA+ domains have conserved loops that bind DNA, as exemplified by RFC and bacterial clamp loaders,[32,33] and by BPV E1.[34] Separately, the NTD of Mcms contain loops that bind ssDNA.[35] The distance between the CTD and NTD tiers changes between Conformers I and II, the proposed ATP free and ATP bound states of CMG, respectively. Thus, the DNA binding sites in the CTD and NTD tiers would fulfill the requirement of ATP driven distance changes
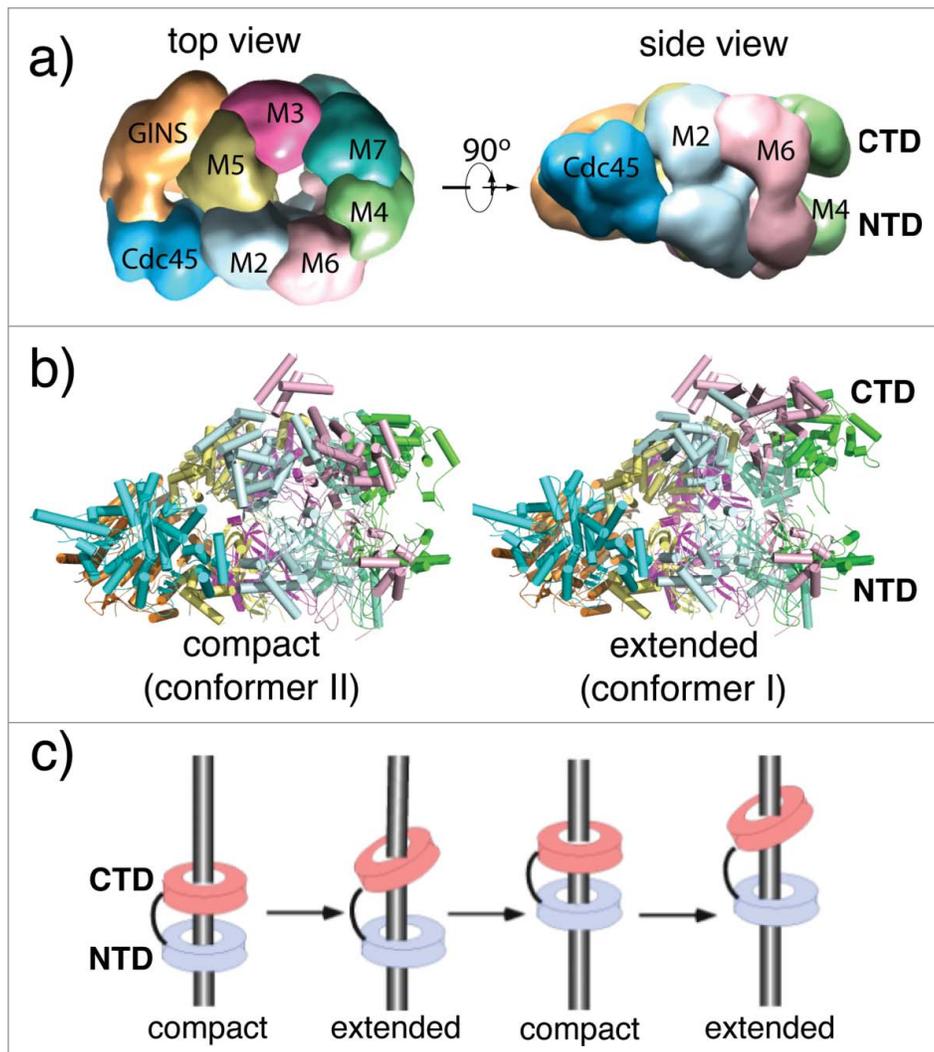
Figure 1. CMG helicase structure and translocation mechanism. (a) Low resolution stucture of yeast CMG obtained by negative stain and single particle 3D EM reconstruction.[27] The top view (left) shows the central channel through Mcm2-7 with the Cdc45/GINS located to one side of the ring, and the side view (right) shows the CTD-NTD tiers of the Mcm2-7 ring. Adapted from Fig. 1b of ref.[27] (b) High resolution cryoEM structure of 2 conformers of yeast CMG, oriented similar to the side view in panel A. The 2 conformers differ in the CTD tier of the Mcm2-7 ring. Conformer II (left): all intersubunit interfaces of the CTD tier are closed, and the CTD and NTD tiers are roughly parallel. Conformer I (right): The Mcm2-5 interface is open, and the CTD tier tilts upward. Adapted from Fig. 2f,g of ref.[26] (c) Proposed inchworm mechanism of translocation. CMG binds DNA at 2 sites (illustrated here as the CTD and NTD tiers). Cycles of ATP hydrolysis cause CMG to alternate between compact (conformer II) and extended (conformer I) forms, driving CMG translocation along DNA like an inchworm. See text for details.

needed for translocation. In this hypothesis, ATP fuels a CMG pumpjack that "inchworms" along DNA (Fig. 1c). Interestingly, the cryoEM study of Drosophila CMG indicated that DNA occupancy in the CTD is higher in the ATP bound state, while DNA occupancy in the NTD is higher in the ATP free state, leading the authors to suggest that DNA may be handed-off from the CTD to the NTD during ATP hydrolysis.[31] This suggestion fits nicely with our proposal of a CMG inchworm.[26] We note that an inchworm process has abundant precedent in the monomeric SF1 and SF2 family helicases.[36,37] Whether CMG truly functions as an inchworm will require further studies.

There are many possible DNA binding elements in the Mcm2-7 central channel, and we suggest here another possible source of 2 DNA sites needed for translocation. Specifically, the 2 halves of the CTD tier could harbor 2 distinct DNA binding sites, 1 utilizing the DNA binding loops in the CTD of Mcm2-6-4, and 1 that utilizes the corresponding loops in the CTDs of Mcm5-3-7. As discussed above, the Mcm2-6-4 AAA+ domains in the CTD tier move relative to the Mcm5-

3-7 AAA+ domains in the 2 CMG conformers. In this hypothesis, the 2 halves of the CTD tier walk along DNA, and the NTD tier could serve as a processivity ring.

Comparison to other replicative helicases

The current view of homohexameric helicase action proposes that the DNA binding site in each subunit walks along DNA, taking turns as ATP is hydrolyzed in sequential fashion around the ring (reviewed in ref[28]). Crystal structures of BPV E1-ssDNA [34] and *E. coli* DnaB-ssDNA [38] reveal an open lockwasher arrangement of the CTD tier, similar to CMG Conformer I that has an open Mcm2-5 interface. But instead of an open/close cycle as in CMG, homohexameric helicases are proposed to hydrolyse ATP to open an adjacent subunit interface at the same time as closing another, which drives the DNA binding site of the "bottom" subunit in the lockwasher to leapfrog to the "top" of the lockwasher and thus translocate along DNA. In this "staircasing" mechanism, an open interface is ever present, and is rotationally translated around the ring.

Rotary models predict that each ATP site is equivalent and the 6 sites fire in sequence around the ring. Unfortunately, the homooligomeric status hinders mutational tests because single subunits of homohexameric helicases can not be individually mutated. However, individual subunits can be mutated in the Mcm2-7 heterohexamer, and mutagenesis of specific Mcm2-7 ATP sites demonstrate that the sites are not equivalent.[39,40] Studies of Drosophila CMG demonstrate that mutation of either the Mcm3 or Mcm5 ATP site eliminate helicase function, while the other ATP sites only contribute 2-fold or less to helicase activity.[13] ATP sites in AAA+ oligomers are at subunit interfaces, and interestingly, the Mcm5 site is at the Mcm2-5 interface that opens and closes in Conformers I and II. The fact that not all ATP sites are required for helicase activity is consistent with a non-rotary model for CMG, but further studies are required to understand the mechanisim of hexameric helicases, and whether they operate in different ways.

Integration of CMG into the replisome

We have recently determined the organization of a minimal eukaryotic replisome.[27] Replisome structure studies were facilitated by our finding that Pol ε binds to CMG, forming a "CMGE" leading strand complex.[18] Pol ε consists of 4 subunits, the Pol2 polymerase, and the Dpb2, Dpb3, Dpb4 accessory subunits.[41] The single-particle 3D reconstruction of CMGE showed that Pol ε binds on top of the accessory factors, to one side of Mcm2-7 (Fig. 2a-c).[27] Only about 70% of the expected density for the mass of Pol ε was observed in the 3D reconstruction, suggesting conformational flexibility. Interestingly, the POL2 gene encodes 2 DNA polymerases; the N-half is the active polymerase while the C-half is an inactive polymerase.[42] Concievably, the 2 polymerases form separate domains with a flexible link. Indeed, our cross-linking/mass spectrometry study revealed only 1 cross-link between the 2 halves of Pol2, indicating the 2 halves may be separated in space.[27] The N- and C-halves of Pol2 must be close to one another, as the cross-linking/mass spectrometry results demonstrate that both halves of Pol2 cross-link to positions on the C-surface of CMG.[26] Since, the C-half of Pol2 interacts with the Dpb2-3-4 subunits, and the Dpb2 subunit binds directly to GINS,[43,44] the C-half Pol2-Dpb2-3-4 complex is probably present in the visible density in CMGE. We speculate that the Pol2 NTD might be connected to the Pol2 CTD by a flexible hinge (e.g illustrated in Fig. S6 b of ref.[27]).

Interestingly, cells remain viable upon deleting the N- half of Pol2, but cell growth is slow and S phase progression is severely compromised.[45,46] Presumably, another polymerase takes over for Pol ε when Pol2 is missing. Polymerase replacement is established in *E. coli*. Cells with a deletion of *dnaE* encoding the replicative Pol III are viable, but grow slowly because Pol I takes over replication.[47] In vitro studies confirm that the *E. coli* replisome can function with other DNA polymerases in place of Pol III.[48] Interestingly, yeast cells with point mutations in the active center of Pol ε are not viable, indicating that Pol ε is dominant negative over other polymerases.[45] Concievably, the point mutant still binds CMG helicase at the correct position with wild type enzyme affinity, preventing alternative polymerases from substituting for Pol ε. Surprisingly, the inactive C-half polymerase of Pol2 is essential to cell viability,[45] possibly to tether the essential Dpb2 into the replisome.

Building the eukaryotic replisome in stages, and examination in the EM revealed the organization of proteins in the core replisome.[27] Fig. 2d shows 2D class averages of CMG-Ctf4 which reveal that Ctf4 binds CMG on the opposite side from Pol ε (compare
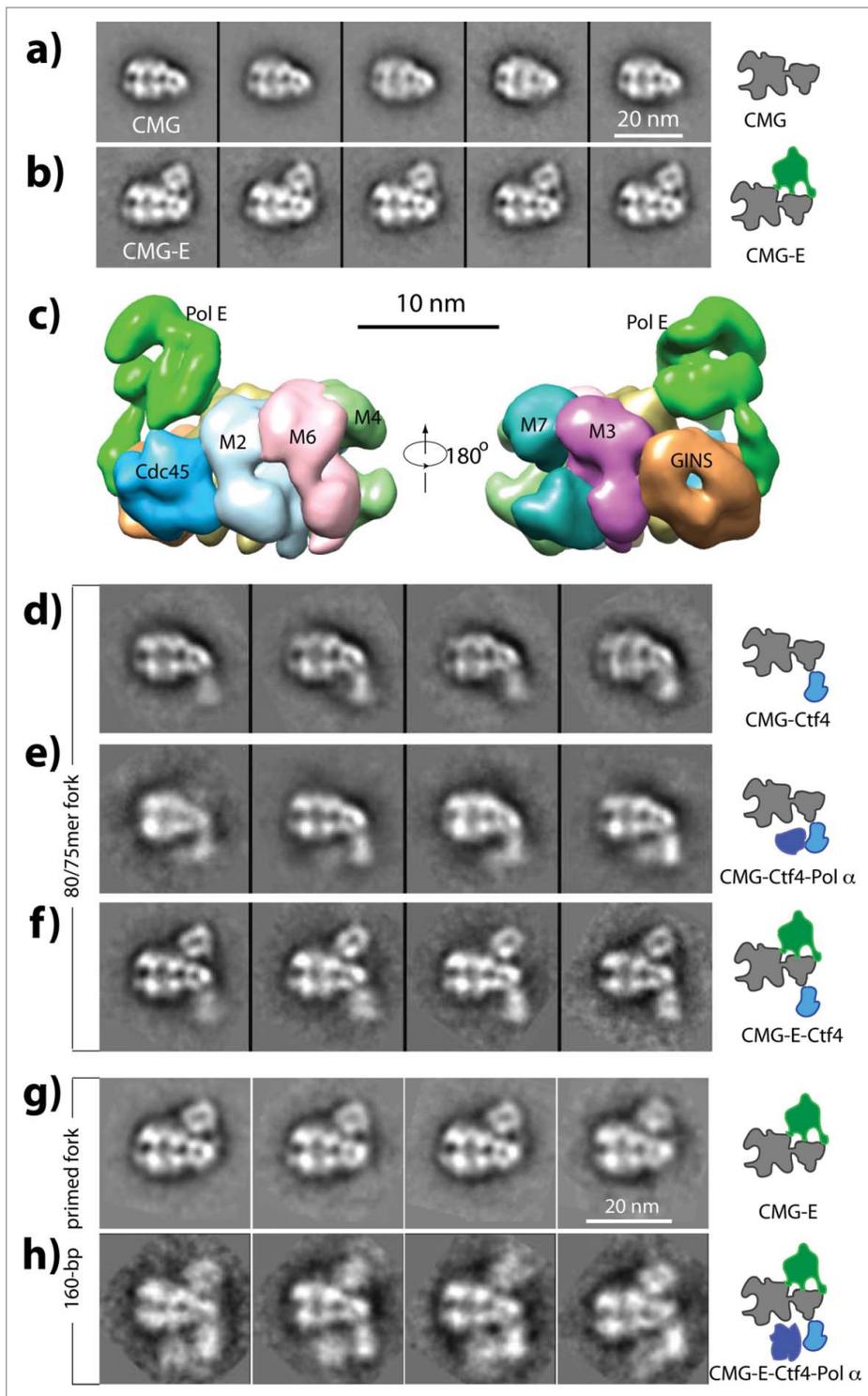
Figure 2. Structure of CMGE and architecture of the replisome. Class average side view images of: (a) CMG, and (b) CMGE. Panel (c) is the 3D single-particle EM reconstruction of CMGE. Panels (d-h) are class average side-view images of: (d) CMG-Ctf4, (e) CMG-Ctf4-Pol α, (f) CMGE-Ctf4 in the presence of forked DNA. Panels (g) and (h) are CMGE and CMGE-Ctf4-Pol α, respectively, in the presence of a primed fork DNA. Illustrations to the right of the class averages are the interpretation of proteins in the images. Adapted from Figs. 2,5 of ref. [27]

panels b and d). Ctf4 is a homotrimer, and the fuzzy appearance of Ctf4 in these 2D class averages is consistent with studies that demonstrate mobility of the N-half of Ctf4 relative to the C-half of Ctf4.[49] Ctf4

is known to bind Pol α,[49] and a complex of CMG-Ctf4-Pol α shows additional density of Pol α on the Ctf4 side of the CMG ring (panel e). A complex of CMG-Pol ε-Ctf4-Pol α shows the 2 DNA polymerases

are located on opposite sides of CMG helicase (panels f and g). Chemical cross-linking with mass spectrometry readout assigned Pol ε to the CTD side of CMG and Pol α to the NTD side.[27] A replisome with polymerases on either side of the helicase was unanticipated. All previous models and illustrations of the replisome, from bacteria to eukaryotes, drew the polymerases on the same side of the helicase where they could act on the separated strands.

DNA threading through the replisome

Which polymerase rides ahead of the helicase, and which trails behind? To answer this question one must determine whether the CMG NTD tier (Pol α side) travels ahead of the CTD tier (Pol ε side), or the reverse. An EM study of Drosophila CMG bound to a 20 bp dsDNA with a 40 nucleotide 3′ ssDNA (i.e., a primed template) concluded that the leading strand enters the CTD side of CMG.[50] Therefore the CTD travels ahead of the NTD as CMG moves along DNA. A moving CMG with the CTD ahead and NTD trailing behind is also consistent with FRET studies of an archaeal Mcm.[51] In contrast, crystal structure analysis of BPV E1-ssDNA reveals that the E1 NTD travels ahead of the CTD.[34] While BPV E1 and Mcms are both AAA+ proteins, they are grouped into distinct helicase superfamilies (SF3 and SF6, respectively[52]). This distinction may explain their different orientation on DNA. However, it remains possible that a

forked DNA may bind the helicases differently from use of ssDNA or a primed template structure.

In Fig. 3, we illustrate the consequence of DNA threading either CTD-to-NTD, or NTD-to-CTD. Provided leading strand ssDNA threads CTD-to-NTD,[50] Pol ε is positioned at the top of CMG and Pol α is below (Fig. 3a). A possible advantage of Pol ε at the prow of the replisome is that it would be first to encounter parental nucleosomes. Pol ε is reported to bind histones, and mutations in Pol ε show it is essential to heterochromatin maintenance.[53,54] In the replisome model of Fig. 3a, unwound leading ssDNA that exits the NTD tier of Mcm2-7 would need to make a U-turn and loop back up to the Pol ε active site. This requires at least 40 nucleotides of ssDNA. Some support for a long leading ssDNA footprint comes from studies in *Xenopus* extracts that identify a 20–40 nucleotide footprint of ssDNA on the leading strand of a replication fork.[55] The *Xenopus* studies also indicate that CMG encircles 1 strand and excludes the other, which appears general to hexameric helicases.[55] Thus, the DNA splits before entering CMG, and in the CTD-to-NTD model the lagging ssDNA must traverse the outside surface of CMG to reach Pol α at the bottom.

Fig. 3b considers the opposite direction of DNA threading, NTD-to-CTD. This model places Pol ε directly behind CMG, where Pol ε was originally expected, but places Pol α in the unexpected "top" position at the fork. If DNA splits at the top of CMG,
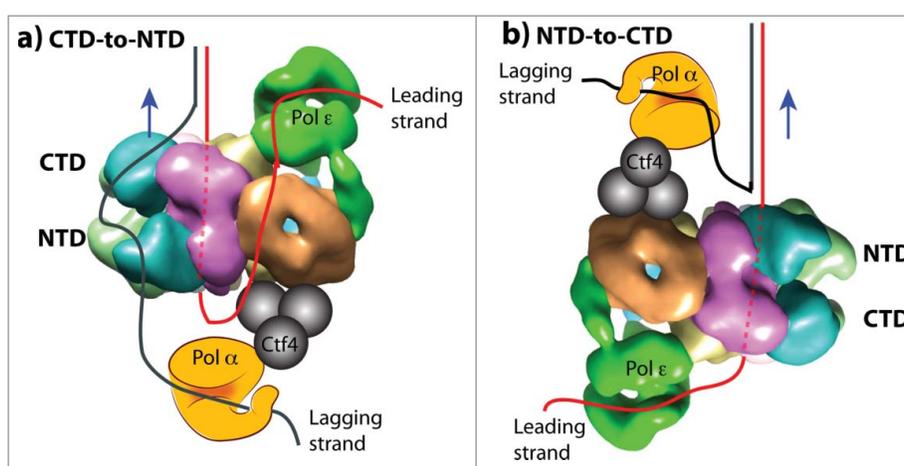
Figure 3. DNA threading through the replisome. (a) CTD-to-NTD model. Studies of Drosophila CMG[50] and archaeal Mcm[51] indicate the leading strand ssDNA enters the CTD tier of CMG. In this model, Pol ε is on "top" of CMG. When the unwound leading strand (red) exits the bottom of Mcm2-7 NTD, it must make a U turn to loop back to Pol ε at the top. The lagging strand (blue) must traverse the outside perimeter of CMG to reach Pol α at the bottom of CMG. Adapted from Fig. 6 of ref.[27] (b) NTD-to-CTD model. If the leading strand enters the NTD tier of Mcm2-7, Pol ε will be positioned to immediately accept the unwound leading strand as it exits CMG. Pol α could act at the top of CMG to prime the unwound lagging strand.

Pol α would be well positioned to act immediately upon the lagging ssDNA for primer formation. Therefore the threading model of Fig. 3b would minimize the amount of ssDNA on both strands. Additionally, the NTD of Mcm2 binds a H3-H4 tetramer,[56,57] and the NTD-to-CTD model would position the histone binding domain of Mcm2 for encounter with parental nucleosomes.

Further studies are needed to firm up the direction of DNA threading through CMG, and thus distinguish between the models in Fig. 3. Regardless of which model is correct, there are still many pressing questions that remain about the organization of the core replisome. For example, what is the connection of Pol δ to the replisome? Are there DNA loops on the lagging strand? Does the RFC clamp loader bind into the replisome, and if so, where is it located? Additionally, there are many other factors that travel with the replisome and remain to be studied biochemically and structurally. Furthermore, the replisome may exhibit alternative arrangements depending on circumstances. For example, activation of the DNA damage checkpoint may induce changes in replisome structure. Thus, despite the recent advances, many questions about this important machine await future studies.

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