Published in final edited form as:

Nat Struct Mol Biol. 2014 August ; 21(8): 664–670. doi:10.1038/nsmb.2851.

Mechanism of asymmetric polymerase assembly at the eukaryotic replication fork

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Abstract

Eukaryotes use distinct polymerases for leading- and lagging-strand replication, but how they target their respective strands is uncertain. We reconstituted *Saccharomyces cerevisiae* replication forks and found that CMG helicase selects polymerase (Pol) ϵ to the exclusion of Pol δ on the leading strand. Even if Pol δ assembles on the leading strand, Pol ϵ rapidly replaces it. Pol δ -PCNA is distributive with CMG, in contrast to its high stability on primed ssDNA. Hence CMG will not stabilize Pol δ , instead leaving the leading strand accessible for Pol ϵ and stabilizing Pol ϵ . Comparison of Pol ϵ and Pol δ on a lagging-strand model DNA reveals the opposite. Pol δ dominates over excess Pol ϵ on PCNA-primed ssDNA. Thus, PCNA strongly favors Pol δ over Pol ϵ on the lagging strand, but CMG over-rides and flips this balance in favor of Pol ϵ on the leading strand.

Chromosomes are replicated by multiprotein replisome machines that duplicate both strands of DNA in a coordinated fashion^{1–3}. Eukaryotes use different DNA polymerases for the leading (Pol ϵ) and lagging (Pol δ) strands, whereas bacteria use multiple copies of an identical subunit^{4–6}. All cells, in eukaryotes, archaea and bacteria alike, use circular sliding clamps that tether the polymerases to DNA for highly stable synthesis (for example, eukaryotic proliferating cell nuclear antigen (PCNA))^{7,8}. Sliding clamps are opened and closed around DNA by a multiprotein clamp loader (for example, eukaryotic replication factor C (RFC))^{7,9}. Both Pol ϵ and Pol δ function with the PCNA clamp, yet their actions are confined to opposite strands of the replication fork. How this asymmetry is achieved is largely unknown.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

R.E.G., L.L. and M.E.O. conceived the project and designed the experiments. R.E.G., L.L., N.Y.Y. and T.A. executed the experiments and analyzed the results together with M.E.O. L.D.L., O.Y. and J.F. designed the yeast strain constructions, and O.Y. and J.F. carried them out. R.E.G., L.D.L., O.Y. and D.Z. carried out protein purifications. M.E.O. and R.E.G. wrote the manuscript with input from L.D.L.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

At the heart of the eukaryotic replisome is an 11-subunit helicase referred to as the Cdc45, Mcm2–7 and GINS (CMG) complex^{10,11}. The motor of CMG is the Mcm2–7 complex comprising Mcm proteins 2–7, a heterohexamer of AAA+ ATPase subunits. The Mcm2–7 subunits form a ring^{12,13} with demonstrable 3′-5′ helicase activity¹⁴. The helicase is activated upon association of Mcm2–7 with Cdc45 and the four-subunit GINS to form the CMG complex^{15,16}. Assembly of CMG occurs at origins of replication in a highly regulated series of reactions involving several proteins and two S-phase kinases^{1,3,17–23}. Once CMG is formed, the polymerases and other replisome proteins assemble with CMG to form the replisome. The composition of the eukaryotic replisome is uncertain. Use of epitope tags on CMG subunits enables isolation of a replication-progression complex (RPC) from *S. cerevisiae* that consists of CMG in addition to Ctf4, Mcm10, FACT, Tof1, Csm3 and Mrc1, proteins involved in repair, cohesion and nucleo-some remodeling²⁴. DNA polymerases are not present in the RPC and thus are thought to bind it only weakly. However, Pol α -primase has been isolated with the RPC at low ionic strength²⁵. Although Pol ϵ has not been isolated with the RPC, the noncatalytic Dpb2 subunit of Pol ϵ has been demonstrated to bind the Psf1 subunit of GINS, thus indicating that Pol ϵ binds CMG^{26–28}. How Pol δ and RFC are held in the replisome, if they are at all, is presently unknown.

In the current report, we asked how Pol ϵ and Pol δ are targeted to their respective strands. To address this, we purified CMG helicase and reconstituted a minimal leading-strand replisome from CMG, RFC, PCNA, RPA or single-strand-binding protein (SSB) and either Pol δ or Pol ϵ comprising 27 different polypeptides. Prior to isolation of CMG, biochemical studies of SV40 replication forks relied on use of the SV40 T-antigen helicase^{29,30}. However, in the SV40 system Pol δ replicates both the leading and lagging strands, rather than two different polymerases being used asymmetrically to replicate cellular chromosomes^{31,32}. The present study showed that Pol δ is inefficient and distributive on the leading strand with CMG, in contrast to its efficient action with the SV40 T antigen. Pol ϵ appeared much more efficient and rapid than Pol δ in function with CMG. In polymerase mixing experiments, CMG selected Pol ϵ over Pol δ , even when Pol δ was present in excess. We observed the opposite behavior in studies of a lagging-strand model, using PCNA on RPA-coated primed single-stranded DNA (ssDNA). Polymerase mixing experiments demonstrated that Pol δ efficiently outcompeted Pol ϵ for a PCNA-primed ssDNA complex even when Pol ϵ was in 20-fold molar excess over Pol δ . These results suggest that highly stable Pol δ function with PCNA is the default mode, but CMG alters this balance on the leading strand by selecting and stabilizing Pol ϵ over Pol δ .

RESULTS

Comparison of Pol ϵ and Pol δ function with CMG

To reconstitute leading-strand replication, we purified the 11-subunit CMG from yeast (Supplementary Fig. 1). CMG contains equimolar amounts of each subunit and displays time-dependent unwinding of a forked substrate with a specific activity similar to that of *Drosophila* CMG¹⁵ (Supplementary Fig. 1). For a substrate, we constructed linear duplexes of either 2.8 kb or 3.2 kb, each having the same synthetic forked junction at one end, and annealed a DNA 37-mer primer to the leading strand, leaving 54 nt of ssDNA between the 3′

terminus and the forked junction for CMG to bind. Synthesis of the leading strand can be detected either with [^{32}P]dNTPs or by 5'- ^{32}P end-labeling of the DNA primer. We staged the reaction by loading CMG onto DNA for 10 min, then assembled PCNA on DNA with RFC and either Pol ϵ or Pol δ for 2 min. Two dNTPs (dATP and dGTP) were present to prevent excision of the primed site by the 3'-5' exonuclease activity inherent in the polymerases and to support clamp loading. We initiated synchronous replication by adding the remaining dNTPs along with RPA and 5 mM ATP to fuel CMG helicase unwinding (Fig. 1a). The results using a 3.2-kb fork and [^{32}P]dCTP demonstrate CMG-dependent replication by Pol ϵ on the linear forked DNA (Fig. 1b and Supplementary Fig. 2). The extension products appeared as a wide distribution extending along the full length of the substrate. Scans of leading-strand products at early time points (2 and 4 min), before the replisome reaches full length, showed an average rate of 4.4 ± 0.6 nt/s, with some replisomes faster and others slower (Fig. 1b). This rate, although slower than the *in vivo* rate³³, is in line with replication forks in yeast extracts^{34,35}. Omission of CMG, Pol ϵ or ATP (Fig. 1b, lanes 6, 7 and 9) strongly reduced synthesis. The reaction was halved in the absence of PCNA and RFC (Fig. 1b, lane 5 versus 8, quantitation in Supplementary Fig. 2d), in agreement with yeast Pol ϵ not being completely dependent on PCNA³⁶. RPA stimulated the reaction but was not absolutely required (Fig. 1, lane 10, and Supplementary Fig. 2d), similarly to observations of the effects of SSB on leading-strand synthesis in the *Escherichia coli* system³⁷. The control of Pol ϵ in the absence of CMG showed a low level of full-length product, which we have identified as originating from polymerase-exonuclease idling at the tip of the duplex (Fig. 1b, lane 5 versus 6). This background reaction did not depend on the 37-mer primer and could be eliminated by an end-labeled 5'- ^{32}P primer instead of [α - ^{32}P]dNTPs. We performed the remaining experiments with a 5'- ^{32}P primer (scheme in Fig. 2).

The ^{32}P primer, unlike [^{32}P]dNTPs, enables evaluation of substrate utilization because both replicated and unreplicated products are visualized. The results demonstrate that all the ^{32}P -labeled primers are extended to the forked junction, and about 28% are extended beyond it (Fig. 2a, lanes 1–5). We presume that the products that do not progress past the forked junction do not contain CMG. We used a 17-fold excess of CMG over DNA. Use of an excess of replicative helicase over DNA is common in assays lacking specific helicase-loading factors, and we note that assays of *E. coli* DnaB helicase, without its loader, have typically contained 100-fold molar excess of DnaB over DNA to unwind 30% of the substrate^{38,39}. We also note that a 40-fold molar excess of *Drosophila* CMG was required to unwind 24% of the substrate in a previous study¹⁵. Thus yeast CMG loads onto DNA quite well. During replication, the full-length product was detectable at 4 min and was readily apparent at 8 min, for a rate of about 5.6 nt/s. We note the persistence of products in the range of 0.5–2 kb, possibly because of pause sites in the template (lane scans in Fig. 2a). These products were less apparent with [^{32}P]dNTPs because [^{32}P]dNTPs underestimate short products.

RPA blocks CMG helicase activity if it is added before CMG, probably by preventing CMG loading onto DNA (Supplementary Fig. 3). Consistently with this interpretation, RPA did not inhibit when added after preincubation of CMG with DNA (Supplementary Fig. 3).

Because RPA was present during the replication phase of the assay, we presume that the bulk of observed replication products were the result of a single CMG bound to the same DNA during the 20-min reaction.

When we compared Pol δ function with CMG (Fig. 2b) to the Pol ϵ –CMG reactions (Fig. 2a) the results were strikingly different. Although we observed a time-dependent extension of the ^{32}P primer, the DNA product length was only 494 ± 4 nt in 8 min (1 nt/s), and then it progressed even more slowly, averaging 761 ± 6 nt in 32 min (0.4 nt/s). This length is within the region of the DNA where the Pol ϵ –CMG replisome appeared to progress with difficulty. The percentages of forks that passed the forked junction were 13, 17 and 20% (Fig. 2b, lanes 3, 4 and 5, respectively). Overall, the observations reveal that Pol δ can function with CMG, but the extension rate is 5- to 10-fold slower than that of Pol ϵ –CMG.

Genetic studies have inferred that Pol δ can replicate the leading strand when the polymerase domain of Pol ϵ is deleted^{40,41}, and therefore we expect that other proteins or conditions may increase the efficiency of Pol δ with CMG. The dropout assays (Fig. 2b, lanes 6–9) demonstrate that the Pol δ reaction is dependent on ATP, CMG, Pol δ , PCNA and RFC. The nearly complete dependence of Pol δ on PCNA and RFC is consistent with studies using a primed ssDNA substrate^{42,43} and demonstrates that PCNA was efficiently loaded on the leading-strand primer in these assay conditions.

CMG selects Pol ϵ over Pol δ for leading-strand synthesis

That both Pol ϵ and Pol δ function on the forked DNA with CMG suggests that they may compete when present at the same time. In this event, Pol δ should occupy some of the PCNA on the primed site and inhibit overall synthesis, owing to its slow speed with CMG compared to that of Pol ϵ . We repeated the assay, using a mixture of 10 nM Pol ϵ and increasing amounts of Pol δ (0, 10, 20, 40 and 80 nM) (Fig. 3a), mixing the polymerases before adding them into the reactions. A distinct feature of Pol ϵ –CMG synthesis is the presence of full-length product that was not present in Pol δ –CMG reactions. One may expect that as the ratio of Pol δ to Pol ϵ increases, the full-length product will decrease, owing to the higher Pol δ /Pol ϵ molar ratio, and thus result in greater occupancy by Pol δ on DNA. Interestingly, the result showed very little decrease in the full-length product even at the highest concentration of Pol δ (8:1 Pol δ /Pol ϵ), indicating that Pol ϵ was selectively recruited to CMG–PCNA–DNA over Pol δ in this leading-strand system.

Pol ϵ takes over a moving Pol δ –CMG replisome

The selective recruitment of Pol ϵ over Pol δ on the leading strand could be explained by Pol ϵ being quicker than Pol δ to assemble with CMG and PCNA or by Pol ϵ being more stable with CMG compared to Pol δ . To distinguish these possibilities—faster or more stable—we first assembled Pol δ (10 nM) on the forked DNA with CMG, PCNA and RFC, then initiated synthesis for 4 min before adding various amounts of Pol ϵ (scheme in Fig. 3b). Strikingly, the result (Fig. 3b, lanes 5–9) showed that even at the lowest concentration of Pol ϵ (10 nM) the full-length product was still synthesized, demonstrating that Pol ϵ takes over the CMG replisome from a moving Pol δ –PCNA–CMG. Given the dominance of Pol ϵ over Pol δ in leading-strand replication with CMG, one may expect Pol δ not to gain entry into a Pol ϵ

leading-strand replisome. We tested this by reversing the order of polymerase addition (Fig. 3b, lanes 1–4). Adding Pol δ to a moving Pol ϵ replisome had little effect on the appearance of full-length product, thus providing further evidence that Pol ϵ was selected over Pol δ for leading-strand synthesis. In sum, even if Pol δ were to gain access to the leading strand, Pol ϵ would quickly take its place during replication.

One mechanism to explain how Pol ϵ takes over the leading strand from Pol δ is that Pol δ may not be stabilized by CMG and may cycle on and off the DNA. Each Pol δ dissociation event would give opportunity for Pol ϵ to bind. This would contrast sharply with the high stability of yeast Pol δ with PCNA on primed ssDNA, on which it can extend a primed terminus 5.4 kb without dissociating⁴⁴. We examined the stability of Pol δ with CMG by varying the concentration of Pol δ while keeping the concentration of DNA and other proteins constant (Fig. 4). A distributive polymerase comes on and off DNA during the reaction and thus samples all the DNA substrates, extending them as a population. The higher the concentration of polymerase, the faster the population of DNA will be extended. In contrast, a polymerase that remains stably bound to the same DNA substrate will, at low polymerase concentration, extend only a fraction of DNA substrate, but the rate will be the same as at high polymerase concentration.

Titration of Pol ϵ gave similar-length products at each concentration tested, thus indicating that Pol ϵ is stable with CMG (Fig. 4a). We then titrated Pol δ into the replication-fork assay while keeping the concentrations of DNA, CMG, PCNA, RFC and RPA constant (Fig. 4b). The result shows that increasing concentrations of Pol δ result in increasing lengths of replication products. Thus, Pol δ is not stabilized by CMG on the leading strand and cycles on and off the template.

Pol δ is dominant over Pol ϵ on a model lagging-strand DNA

Given the dominance of Pol ϵ over Pol δ on the leading strand, it is a mystery how Pol ϵ is excluded from lagging-strand primers. Two large differences from the leading strand are the absence of CMG and the presence of RPA on the lagging strand. We studied the behavior of Pols δ and ϵ with PCNA in a lagging-strand model system, a singly primed 5.4-kb circular ϕ X174 ssDNA coated with either *E. coli* SSB or yeast RPA (scheme, Fig. 5a).

First we compared the rates of synthesis by Pol ϵ and Pol δ with PCNA on *E. coli* SSB-coated primed ϕ X174 ssDNA (Fig. 5b,c). We assembled the polymerase–PCNA clamp on the primed ssDNA in a preincubation that contained PCNA and RFC along with either Pol ϵ or Pol δ and two dNTPs. Then we added the remaining dNTPs to initiate synthesis and removed timed aliquots for analysis in an alkaline gel. The result showed that Pol ϵ extends the primer at a rate of 24 nt/s (Fig. 5b, lanes 4–6, and Supplementary Fig. 4a,c), whereas Pol δ is about three times faster (82 nt/s) (Fig. 5c, lanes 5–8, and Supplementary Fig. 4b,d). We then repeated these reactions with RPA in place of *E. coli* SSB. RPA appears to slow Pol ϵ –PCNA about 37% (15 nt/s) (Fig. 5b, lanes 11–14, and Supplementary Fig. 4a,c) but to slow Pol δ –PCNA by only about 12% (72 nt/s) (Fig. 5c, lanes 14–17, and Supplementary Fig. 4b,d). These rates of synthesis are similar to those in previous studies^{42,45}.

Next we examined the speed at which the polymerase assembles with PCNA. It has been proposed that the rate of Pol δ assembly with PCNA-primed ssDNA may be faster than that of Pol ϵ , thereby explaining how Pol δ achieves dominance over Pol ϵ on the lagging strand⁴⁵. If Pol ϵ is slow in binding PCNA-primed ssDNA, we may expect a lag time in synthesis of a reaction initiated upon addition of Pol ϵ to a preassembled PCNA-primed ssDNA, in comparison to preincubation of Pol ϵ with the PCNA–DNA complex before initiation of synthesis (scheme in Fig. 5a). However, the experimental result showed no detectable advantage of preincubating Pol ϵ with PCNA-primed ssDNA and either SSB or RPA, indicating that Pol ϵ rapidly associated with PCNA-primed ssDNA (Fig. 5b, lanes 1–4 and 9–12). For comparison, we performed these experiments with Pol δ ; the results showed that Pol δ also rapidly assembled with PCNA-primed ssDNA (Fig. 5c, lanes 1–4 and 10–13).

Pol ϵ and Pol δ differ in the way in which they attach to a PCNA-primed site⁴⁵. Pol ϵ has very low affinity for PCNA but binds to the ssDNA junction with double-stranded DNA quite well, even in the presence of RPA⁴⁵. Pol δ , however, does not bind well to DNA but binds tightly to PCNA even without DNA^{43,45}. To determine which polymerase dominates in use of PCNA-primed ssDNA, we performed a competition experiment containing a four-fold molar excess of Pol ϵ (20 nM) over Pol δ (5 nM) in the same reaction (Fig. 6). We preincubated the mixture of polymerases with PCNA, RFC and primed ssDNA (1.5 nM) coated with RPA. Each polymerase was in molar excess over the DNA, and therefore the polymerases would compete with one another for the limiting DNA. After allowing time for proteins to assemble on DNA, we initiated replication and analyzed timed aliquots in an alkaline agarose gel. We chose time points such that Pol δ –PCNA would complete the 5.4-kb DNA, whereas Pol ϵ –PCNA would form only incomplete DNA products (Fig. 6a). Therefore, if the two polymerases were to bind PCNA-primed ssDNA equally well, the Pol ϵ , at its four-fold-higher amount, should compete with the Pol δ and reduce the amount of full-length product in comparison to reactions with Pol δ alone. However, the same amount of 5.4-kb product was formed by the Pol ϵ –Pol δ mixture compared to the Pol δ control in the absence of Pol ϵ (Fig. 6b, lanes 7–12 versus 13–18, and Fig. 6c). This result indicates that Pol δ binds PCNA-primed DNA much more tightly than does Pol ϵ . In a further demonstration of the dominance of Pol δ over Pol ϵ , we first assembled 100 nM Pol ϵ with PCNA-primed DNA and allowed it to replicate for 10 s before adding 5 nM Pol δ (Supplementary Fig. 5). The result showed that Pol δ still recruited the PCNA-primed ssDNA from a moving Pol ϵ with no change in full-length product. This result is also consistent with those from an earlier study that allowed Pol ϵ to extend DNA for 90 s before addition of Pol δ (ref. 42). Hence, even in the face of a 20-fold excess of Pol ϵ under moving conditions, Pol δ was dominant on the lagging-strand model DNA.

DISCUSSION

Mechanism of asymmetric polymerase selection at the fork

The current report studies the behavior of Pol ϵ and Pol δ with CMG and PCNA to clarify how Pol ϵ and Pol δ are specifically targeted to their respective leading and lagging strands. Prior to purification of CMG, eukaryotic replisome studies used SV40 T-antigen helicase^{29,30}. However, SV40 replication uses Pol δ on both strands, whereas Pol ϵ is not

required^{31,32}. Hence, studies using T antigen did not reveal how Pol ϵ and Pol δ are targeted to their respective strands. In contrast, studies using CMG helicase revealed the asymmetric and mutually exclusive function of Pol ϵ over Pol δ on the leading strand. Pol ϵ not only was faster with CMG than Pol δ but also was selected by CMG for leading-strand synthesis even when Pol δ was present in eight-fold excess over Pol ϵ . Moreover, Pol ϵ could take over the leading strand when it was added after Pol δ had time to assemble on the DNA with PCNA and CMG.

CMG appeared to select Pol ϵ over Pol δ by two processes (Fig. 7a,b). First, Pol δ -PCNA lacked highly stable action on the leading strand with CMG, in contrast to its highly stable action with PCNA on primed ssDNA in the absence of CMG. Hence, CMG appears unable to stabilize Pol δ on the leading strand. Second, the selection process is probably facilitated by direct protein-protein interaction. Previous immunoaffinity assays have detected a weak Pol ϵ -GIN5 interaction²⁶, and recent studies have shown that the Dpb2 subunit of Pol ϵ binds to the Psf1 subunit of GIN5, thus suggesting at least one point of interaction between CMG and Pol ϵ (ref. 27). We propose that this interaction contributes to CMG selection of Pol ϵ over Pol δ (illustrated in Fig. 7c). Indeed, this may be the main function of Dpb2; *in vitro* studies have shown that Dpb2 is not required for Pol ϵ to function with PCNA⁴⁶. In contrast, Pol δ may not bind directly to CMG, although evidence exists for a weak interaction between Pol δ and GIN5²⁶.

This report also demonstrates that Pol δ is far superior to Pol ϵ on a lagging-strand model DNA, the opposite of results on the leading strand. Polymerase mixing experiments showed that Pol δ was dominant over Pol ϵ in assembly with PCNA on RPA-coated primed ssDNA (Fig. 6). Pol δ was also able to replace a moving Pol ϵ on the model lagging-strand DNA (Supplementary Fig. 5). Hence, even if Pol ϵ were to bind PCNA on the lagging strand, it would quickly be replaced by Pol δ . The current report demonstrates that both Pol ϵ and Pol δ associated rapidly with PCNA-primed ssDNA (Fig. 5). The observed greater affinity of Pol δ to PCNA probably accounts for the observed dominance of Pol δ over Pol ϵ (ref. 45). It is also interesting that Pol ϵ was not as fast with RPA as with SSB; this was also indicated in a previous study⁴⁷. Perhaps RPA has evolved to impede Pol ϵ and facilitate its exclusion from the lagging strand.

In overview, this study indicates that two protein-protein contacts control asymmetric positioning of Pol ϵ and Pol δ at the replication fork (Fig. 7a,b). PCNA selects Pol δ over Pol ϵ on the lagging strand, whereas CMG alters this balance on the leading strand and selects Pol ϵ while being unable to stabilize Pol δ with PCNA on the leading strand.

We presume that these studies in yeast generalize to humans. However, we note that a study of human Pol ϵ (in the presence of RFC and PCNA) without CMG produced >10 kb of DNA by strand-displacement synthesis of Pol ϵ alone¹⁶. Further, human Pol ϵ appeared to be highly processive in strand displacement because only about 1% of the substrate was extended. In contrast, yeast Pol ϵ did not strand-displace past a forked junction in the absence of CMG (Fig. 2). Human CMG stimulated Pol ϵ only five-fold, thus suggesting that human Pol ϵ may not be tightly coupled to CMG, in comparison to the tightly coupled yeast CMG-Pol ϵ . The human system was inhibited by RPA, even when RPA was added after

initiation of synthesis; this further suggests the presence of a different mechanism compared to that of the yeast system. It seems unlikely that the two systems would have evolved along such different lines, but clarification of this issue will require more-detailed studies.

Why use distinct leading- and lagging-strand polymerases?

Bacteria and phages use multiple copies of an identical subunit for replication of both strands of DNA². Hence, this raises the question of why eukaryotes evolved a replication strategy using different polymerases for the leading and lagging strands. The most obvious explanation is that leading- and lagging-strand operations are very different, and the two polymerases have distinct properties suited to each strand. An expected property of a leading-strand DNA polymerase is to maintain stable continuous synthesis without dissociating from the fork, as observed here for Pol ϵ -CMG. Interestingly, Pol ϵ -CMG is not fully dependent on RFC and PCNA, unlike Pol δ (Fig. 1). This is consistent with studies showing that Pol ϵ binds only weakly to PCNA compared to Pol δ (ref. 45) and that multiple PCNA clamps are loaded onto DNA during replication of a long primed ssDNA by Pol ϵ , probably owing to Pol ϵ cycling on and off PCNA-DNA during the reaction and thus providing RFC periodic access to the 3' terminus for additional PCNA loading⁴⁵. We propose that at the fork, Pol ϵ cycles on and off PCNA-DNA but holds onto CMG for stable leading-strand synthesis. This on-off action would periodically provide RFC access for repeated assembly of new PCNA clamps on the leading strand. PCNA is required to direct mismatch repair and to assemble nucleosomes on daughter strands^{30,31,48}. Hence, the benefit of a weak Pol ϵ -PCNA interaction that enables repeated PCNA loading during extension would be to populate the leading strand with PCNA clamps that direct mismatch repair and nucleosome assembly. On the lagging strand, a plentiful supply of PCNA is provided by use of a new PCNA for each Okazaki fragment.

An important property of a lagging-strand DNA polymerase is strand-displacement activity, required to remove the low-fidelity RNA and DNA primers synthesized by Pol α -primase^{49,50}. Previous studies have demonstrated that Pol δ is efficient at limited strand-displacement synthesis and functions with FEN1 nuclease to maintain a ligatable nick⁵⁰. In contrast, Pol ϵ was nearly inactive in strand-displacement synthesis and did not function with FEN1 to maintain a ligatable nick⁵⁰. Another property expected of a lagging-strand enzyme is distributive behavior because Okazaki fragments are only 100–200 bp. Accordingly, Pol δ dissociates from PCNA shortly after converting ssDNA to dsDNA⁴⁴.

Bacterial and phage replisomes maintain a connection between the leading- and lagging-strand polymerases for coordinated leading- and lagging-strand synthesis. It is not yet clear whether Pol ϵ and Pol δ are physically coupled in the eukaryotic replisome. Pols ϵ and δ do not copurify with RPCs, but DNA is removed by DNase treatment before RPC isolation¹⁸. Thus it remains possible that when the replisome is bound to DNA, the polymerases form tighter connections with other replisome proteins. These and other questions must await future studies on the architecture of a complete eukaryotic replisome that performs both leading- and lagging-strand synthesis.

ONLINE METHODS

Reagents and proteins

Radioactive nucleotides were from PerkinElmer. Unlabeled nucleotides were from GE Healthcare. DNA-modification enzymes were from New England BioLabs. DNA oligonucleotides were from Integrated DNA Technologies. Protein concentrations were determined with the Bio-Rad Bradford Protein stain and bovine serum albumin as a standard. Buffer A is 20 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.1 mM EDTA and 4% glycerol. Buffer B is the same as buffer A except 20 mM Tris-acetate, pH 7.5, was used in place of 20 mM Tris-HCl. Buffer C is 25 mM Tris-Cl, pH 7.9, 10% glycerol, 1 mM DTT, 1 mM MgCl₂, 5 mM imidazole, 20 mM KOAc and 350 mM KCl. Buffer H is 20 mM HEPES, pH 7.5, 10% glycerol, 1 mM EDTA, 2 mM DTT, 350 mM KCl, 1 mM ATP and 4 mM MgCl₂. Buffer D is 25 mM Tris-OAc, pH 7.6, 40 mM KOAc, 40 mM K glutamate, 2 mM MgOAc₂, 1 mM DTT, 20% glycerol and 0.25 mM EDTA. Stop buffer is 1% SDS, 40 mM EDTA.

Expression and purification of CMG

The 11 subunits of CMG were coexpressed in yeast. Genes were integrated into the chromosome of strain OY001 (*ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 bar1 MATa pep4::KANMX6*), a strain constructed from W303 (a gift from A. Tackett and B. Chait, Rockefeller University). The order of integration was as follows. Genes encoding Psf1 and Psf2 (cloned into pRS403/GAL), genes encoding Sld5 and Psf3 (cloned into pRS402/GAL), genes encoding Mcm3 and Mcm4 (cloned into pRS404), genes encoding Mcm6 and Mcm7 (cloned into pRS405), a gene encoding C-terminally tagged Cdc45-Flag (C-terminal 3× Flag tag) and finally genes encoding Mcm2 and N-terminal His₆-kinase-tagged Mcm5 (cloned into pRS406/GAL; sequence of tag as in ref. 51). Prior to integration of the Mcm2 and Mcm5 genes, Cdc45-Flag was cloned into pIS375, and after integration, the Ura marker was removed by back selection with 5-FOA⁵². A second version of CMG was as described above except a 3× Flag tag was exchanged for the histidine tag on Mcm5, an N-terminal GST tag was placed on Sld5, and Cdc45 was untagged. The final change in genotypes of these strains relative to strain OY001, respectively, are: OY157 (*HIS3::GAL1, 10 – PSF1, PSF2 in pRS403 ADE2::GAL1, 10 – PSF3, SLD5 in pRS402 LEU2::GAL1, 10 – MCM6, MCM7 in pRS405 TRP1::GAL1, 10 – MCM3, MCM4 in pRS404 met15::GAL1, 10 – CDC45-FLAG in pIS375 URA3::GAL1, 10 – MCM2, HK/P-MCM5 in pRS406*) and OY163 (*HIS3::GAL1, 10 – PSF1, PSF2 in pRS403 ADE2::GAL1, 10 – PSF3, GST/P-SLD5 in pRS402 LEU2::GAL1, 10 – MCM6, MCM7 in pRS405 TRP1::GAL1, 10 – MCM3, MCM4 in pRS404 met15::GAL1, 10 – CDC45 in pIS375 URA3::GAL1, 10 – MCM2, FLAG-MCM5 in pRS406*).

Cells were grown under selection at 30 °C in SC glucose, then split into 12L YP-glycerol and grown to OD₆₀₀ of 0.7 at 30 °C before induction for 6 h upon addition of 20 g of galactose/L. After 6 h, cells were harvested by centrifugation, resuspended in a minimal volume of 20 mM HEPES, pH 7.6, 1.2% polyvinyl-pyrrolidone, and protease inhibitors and frozen by dripping into liquid nitrogen. Purification of CMG was performed by lysis of 6 L of frozen cells with a SPEX cryogenic grinding mill (6970 EFM). Ground cells were thawed

and debris removed by centrifugation (19,000 r.p.m. in a SS-34 rotor for 1 h at 4 °C); then the supernatant was applied to a 1-ml anti-Flag M2 affinity column (Sigma) equilibrated in buffer H. Before elution, the column was equilibrated in buffer C. Elution was in buffer C containing 0.15 mg/ml 3× Flag peptide (EZBiolab, Carmel, Indiana, USA). Peak fractions were loaded onto a 1-ml HisTrap HP column (GE Healthcare) and washed with buffer C. Elution was with a 7.5-ml linear gradient of 5–750 mM imidazole in buffer C. Fractions containing CMG are shown in Supplementary Figure 1. We also purified CMG with a GST tag instead of the histidine tag. This preparation was obtained as described above through the Flag column step, then loaded onto 0.3 ml glutathione Sepharose 4B (GE Healthcare), washed with Buffer H with 300 mM KCl but without ATP or MgCl₂ and eluted with 5 × 300-μl pulses of Buffer H with 100 mM KCl supplemented with 40 mM reduced - glutathione. Elution buffer also contained 0.2 mg/ml human insulin (Sigma) as carrier protein. Each pulse of elution buffer was incubated on the column for 20 min before flow was resumed. The GST tag was removed by cleavage with Prescission protease for 4 h in a cold room, and products were applied to a 0.1-ml Mono Q column to remove protease and GST-cleavage product. Both forms of CMG were equally active in the linear forked DNA assay, and therefore these tags on the N terminus of Mcm5 or Sld5 do not affect this activity. Fractions containing CMG (0.3 mg) were pooled and dialyzed against buffer D, and then CMG was divided into aliquots and stored frozen at –80 °C.

Other proteins

Proteins were purified as previously described: RPA⁵³, *E. coli* SSB⁵⁴, PCNA⁵⁵ and RFC⁵⁶. Pol ε was expressed in yeast similarly to methods previously described⁵⁷, except a 3× Flag tag was placed on the N terminus of Pol2 in pRS425/GAL and transformed into yeast along with pJL6 expressing genes encoding Dpb2, Dpb3 and Dpb4 (ref. 57). Pol ε was routinely purified from 6 L induced cells by the same methods described above for CMG, through the Flag column and then a Mono S column (Supplementary Fig. 6). Approximately 2 mg Pol ε was obtained from 6 L cells. Protein was divided into aliquots and stored frozen at –80 °C. For Pol δ, a C-terminal Flag tag was placed on Pol3, and the gene was placed under control of the Gal1/10 promoter in the pRS405 vector; this was followed by integration into the yeast genome. The Pol31/32 subunits were expressed in *E. coli* as described⁵⁸, except the Pol31/32 subunits were untagged. 6-L cultures of induced yeast and *E. coli* cells were cocrushed in a cryogenic mill, and Pol δ was purified as described above for CMG, through the Flag column, then a Mono S column (Supplementary Fig. 6). Typical yields of Pol δ were 1.0–1.5 mg. Protein was divided into aliquots and stored frozen at –80 °C.

Oligonucleotides

DNA oligonucleotides used in this study are listed in Supplementary Table 1. To make NB160, 10 nmol of NB160a was mixed with 10 nmol NB160b and 25 nmol NB160bridge in hybridization buffer containing 10 mM Tris, pH 8.5, 300 mM NaCl and 30 mM sodium citrate in a total volume of 690 μl. The mixture was placed in a boiling water bath and cooled slowly to room temperature. After annealing, 80 μl of 10× T4 DNA ligase buffer and 30 μl (12,000 units) of T4 DNA ligase (New England BioLabs) were added, and the ligation reaction was incubated at 16 °C overnight. Ligation was stopped by addition of 40 μl 0.5 M EDTA, and ligase was heat inactivated at 75 °C for 30 min. The reaction was split in two,

and each half (420 μ l) was precipitated with 42 μ l 3 M sodium acetate, pH 5.3, and 1.05 ml 100% ethanol, incubated at -80°C for 1 h, and recovered by centrifugation in a cold room. Liquid was aspirated, and the pellet was air dried overnight. The dried pellet was resuspended in 200 μ l gel-loading buffer with formamide and separated on a 7% UREA/PAGE gel. The band corresponding to the ligated product, NB160, was cut out of the gel and the DNA eluted from the crushed gel slice into 12 ml $1\times$ TE overnight at room temperature. The supernatant from the DNA elution was transferred to an Amicon Ultra 15 Centrifugal Filter Unit (MWCO 10 kDa) (Millipore) and spun at 3,500 r.p.m. ($\sim 3,500g$) in a Sorvall RC3B until the retentate volume was $<500\ \mu\text{l}$.

Helicase assays

Ten pmol of oligonucleotide 1T was labeled at the 5' end with 0.05 mCi [γ - ^{32}P]ATP with T4 polynucleotide kinase (New England BioLabs) in a 25- μ l reaction for 30 min at 37°C . The kinase was heat inactivated for 20 min at 80°C . Radiolabeled oligo 1T (10 pmol) was mixed with 15 pmol oligo B100, heated to 90°C and cooled slowly to room temperature. Total reaction volumes, amounts of added CMG helicase and incubation conditions are indicated in the legends of supplementary figures. Reactions contained 0.5 nM radiolabeled DNA substrate and were incubated at 30°C in buffer containing 20 mM Tris-acetate, pH 7.6, 5 mM DTT, 0.1 mM EDTA, 10 mM MgSO_4 , 50 mM sodium glutamate, 40 $\mu\text{g/ml}$ BSA (as carrier) and ATP as indicated in the figure legends. Where noted, CMG was incubated with the DNA before addition of ATP (in a volume equal to one-tenth of the total reaction). Reactions were stopped by addition of SDS/EDTA/loading dye and were flash frozen in liquid nitrogen. Reaction products were thawed quickly in room-temperature water and separated on 10% native PAGE minigels. Gels were washed in distilled water, mounted on Whatman 3MM paper, wrapped in plastic and exposed to a storage phosphor screen that was scanned on a Typhoon 9400 laser imager (GE Healthcare). Scanned gels were analyzed with ImageQuant TL v2005 software.

Linear forked DNA substrates

To make the pUC19 2.8-kb linear fork DNA substrate, pUC19 plasmid was linearized with BsaI-HF (New England BioLabs). BsaI cuts outside its recognition sequence, leaving a 5'-CGGT overhang at one end and a 5'-ACCG overhang at the other end in the case of pUC19. To assemble the fork, a 60-fold molar excess of NB1T was annealed to a ten-fold molar excess of NB160, was mixed with BsaI-digested pUC19 and was then ligated overnight at 15°C with T4 DNA ligase. Annealing of NB160 and NB1T leaves a 5'-phosphorylated ACCG overhang adjacent to the shared duplex region that ligates to one end of the linearized plasmid but not the other. Excess unligated fork was removed by gel filtration over a 20-ml bed volume of Sepharose 4B (GE Healthcare) equilibrated in 10 mM Tris-acetate, pH 7.5, 1 mM EDTA and 100 mM sodium acetate. Peak fractions containing the linear fork DNA were pooled and stored at -20°C . To serve as a primer for replication reactions, 5' radiolabeled oligo C2 was annealed to the linear pUC19 fork DNA in a 1:1 ratio. The substrate in Figure 1 was made by the same procedure, with NB160 and NB1T oligonucleotides, except a 3.2-kb synthetic sequence that contains no G residues on the leading-strand template was used in place of linearized pUC19, and the primer C2 was not labeled.

Leading-strand replication assays

Replication assays contained 1.5 nM ^{32}P -primed replication-fork substrate, 24 nM CMG, 200 nM RPA (as heterotrimer), 20 nM PCNA, 6 nM RFC, Pol ϵ and Pol δ (as indicated in the figure legends) in 25 mM Tris-acetate, pH 7.5, 10 mM Mg-acetate, 0.1 mM AMP-PNP, 60 μM dATP, 60 μM dCTP, 50 mM potassium glutamate, 5 mM DTT, 0.1 mM EDTA and 40 $\mu\text{g/ml}$ BSA. CMG was preincubated with the DNA for 10 min at 30 °C. Then RFC, PCNA and Pol ϵ and/or Pol δ were added, followed by an additional 2-min incubation, and synthesis was initiated upon addition of RPA, 5 mM ATP, 60 μM dGTP and 60 μM dTTP. The addition of proteins to the assay contributes less than 20 mM added salt to the reaction. Although we use glutamate and acetate as an ion in assays, we know of no report that has defined the ion in yeast or the ionic concentration within the yeast cell. Any exceptions to this protocol are noted in the figure legends. Timed aliquots were removed and quenched upon addition of SDS and EDTA to final concentrations of 0.5% and 40 mM, respectively. Quenched reactions were analyzed in 0.7% alkaline agarose gels and imaged in a Typhoon 9400 PhosphorImager (GE/Molecular Dynamics).

Lagging-strand model of primed ssDNA reactions

Reactions on primed circular ssDNA were performed as follows. Reactions contained 20 nM PCNA (as trimer), 10 nM RFC (as pentamer), 1.5 nM ϕX174 circular ssDNA (as circles) primed with a DNA 30-mer, and either 420 nM RPA (as heterotrimer) or 420 nM *E. coli* SSB (as tetramer) in 20 mM Tris-Cl, pH 7.5, 50 mM potassium glutamate, 5 mM DTT, 0.1 mM EDTA, 40 $\mu\text{g/ml}$ BSA, 8 mM MgOAc, 0.5 mM ATP, 5% glycerol, and 60 μM each of dGTP and dCTP. Reactions were preincubated for 5 min at 30 °C. Pol δ (20 nM) or Pol ϵ (20 nM) was either included in the pre-incubation or added upon initiation of replication. DNA synthesis was initiated by addition of 15 μl of 60 μM dATP, 20 μM dTTP and 15 μCi of [α - ^{32}P]dTTP, and reactions were incubated at 30 °C. At the indicated times, 25- μl aliquots were removed and quenched by addition of an equal volume of 1% SDS and 40 mM EDTA. Products were analyzed in 0.7% alkaline agarose gels. Gels were dried, exposed to PhosphorImager screens and imaged with a Typhoon 9400 PhosphorImager (GE/Molecular Dynamics). Reactions that examine which polymerase dominates in a mixture of Pol δ and Pol ϵ were performed similarly to reactions described above with the following differences: reactions were scaled up 1.5-fold, Pol ϵ was at 20 nM, Pol δ was at 5 nM, polymerases were included in the preincubation, RPA was at 400 nM and six timed aliquots were removed instead of four. Control reactions contained only Pol ϵ or Pol δ . A separate set of reactions used 100 nM Pol ϵ in the preincubation, and 5 nM Pol δ was added after DNA synthesis had been initiated with dNTPs.

Original images of gels, autoradiographs and blots used in this study can be found in Supplementary Figure 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for support from the US National Institutes of Health (GM38839) and Howard Hughes Medical Institute to M.E.O. The authors thank A. Tackett and B. Chait (Rockefeller University) for yeast strain OY001.

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Figure 1.

Reconstitution of a leading-strand replisome with CMG, Pol ε, RFC, PCNA and RPA. **(a)** Scheme of the assay. The 3.2-kb forked DNA substrate was constructed by ligation of a synthetic primed replication fork to a 3.2-kb duplex created with nucleotide bias to enable specific labeling of the leading strand with [³²P]dCTP. CMG was assembled on the DNA with AMP-PNP in a 10-min preincubation; then Pol ε, PCNA and RFC were added for 2 min along with cold dATP and dGTP before replication was initiated upon addition of RPA, ATP, dTTP and [³²P]dCTP. Experimental details and protein concentrations are in Online Methods. **(b)** Alkaline agarose gel of reaction products. Lanes 1–5, time course of the full reaction; lanes 6–9, reactions with indicated components omitted. Arrows indicate the peak lengths of DNA product at 2 and 4 min. Quantitation of the results is in Supplementary Figure 2. MW, molecular weight.

Figure 2.

Comparison of Pol ε and Pol δ in leading-strand replication with CMG. Time courses of leading-strand replication with either Pol ε (**a**) or Pol δ (**b**). Reactions were staged as illustrated in the reaction scheme (Fig. 1a), except the leading-strand primer was 5'-end-labeled with ^{32}P instead of with ^{32}P dNTPs, and linearized 2.8-kb pUC19 was used as the duplex region. RFC/PCNA denotes addition of both components. The right lanes of each gel (lanes 6–8 in **a** and 6–9 in **b**) show the results of omitting individual components as indicated. The letters a–c at right of gel in **a** mark the full-length DNA position, the primer-extension product to the forked junction and the unextended ^{32}P primer, respectively. The graphs to the right are ImageQuant analysis (in arbitrary units) of lanes 1–5 (**a**) and 3–5 (**b**), with time (min) indicated, and the peaks of the fits correspond to the average length of DNA. Scans were analyzed by fitting to a double (**a**) or single (**b**) Gaussian distribution (black lines). The uncropped gel images are shown in Supplementary Figure 7.

Figure 3. CMG selects Pol ε from a mixture of Pol ε and Pol δ. **(a)** Top, scheme of the assay. Reactions were performed as in Figure 2, except a mixture of Pol ε and Pol δ were added along with PCNA and RFC for 2 min before initiation of DNA synthesis. Reactions were quenched after 6 and 20 min ('). Bottom, alkaline agarose gel of the reaction products. Concentrations of each polymerase are indicated above the gels. **(b)** Top, scheme of the assay. CMG was assembled on the ³²P-primed forked DNA; then either 10 nM Pol ε or 10 nM Pol δ was added along with RFC and PCNA. Replication was initiated and continued 4 min before addition of the second DNA polymerase—either Pol δ or Pol ε as indicated. Replication was quenched after a further 16 min. Bottom, alkaline agarose gel of products. The order of polymerase addition and the concentration of the second polymerase are indicated above each lane. The numbers at the bottom of each gel represent relative percentage of full-length product in each series of reactions (top row) and total percentage of substrate extended past the forked junction (bottom row).

Figure 4.

Pol ε functions stably with CMG, whereas Pol δ does not. **(a,b)** Leading-strand (1d) replication reactions as described in Figure 2 but with different amounts of either Pol ε **(a)** or Pol δ **(b)**. For each reaction, 5-min and 20-min time points were analyzed. The last two lanes of **a** show the effect of omitting RFC and PCNA. In **b**, arrows indicate the products at the 20-min time point; the small ^{32}P primers were run off the end of the gel. The graphs to the right of each panel are ImageQuant stacked lane scans corresponding to 20-min reaction time at different polymerase concentrations. Scans were fit to single Gaussian distributions (black lines). The error to the fit is noted. The uncropped gel images are in Supplementary Figure 7.

Figure 5.

Pol ϵ and Pol δ rapidly associate with a PCNA-primed template. **(a)** Scheme of the assay and the expected products. The primed ϕ X174 ssDNA is coated with a SSB protein and preincubated with RFC and PCNA to load the clamp on the primed site. Polymerase (Pol ϵ or δ) is either included in the preincubation (top path) or is added after initiating synthesis (bottom path). Time courses of reactions with either Pol ϵ **(b)** or Pol δ **(c)** and either *E. coli* SSB (eSSB) or RPA are as indicated above the alkaline gels. Preinc, preincubation; std, standard. Quantitation of the results is in Supplementary Figure 4, and the uncropped gel image is in Supplementary Figure 7.

Figure 6.

Pol δ is dominant over Pol ϵ on a lagging-strand model template. **(a)** Scheme of the assay and expected products for reactions with a mixture of polymerases. Polymerases were incubated together or alone with DNA along with RFC and PCNA, and then replication was initiated. Expected products, full length or incomplete, are illustrated at right. Reactions were performed as described in Online Methods. **(b)** Alkaline gel of DNA products from reactions with either 20 nM Pol ϵ alone (lanes 1–6), 5 nM Pol δ alone (lanes 7–12) or a mixture of 5 nM Pol δ and 20 nM Pol ϵ (lanes 13–18). Pol ϵ and Pol δ were premixed and incubated together with the DNA substrate before initiation of replication. **(c)** Lane scans of reaction products at 5 min. The inset shows the amount of DNA synthesis at the indicated times as determined by the area underneath the lane scans determined by multipeak (Gaussian distribution) analysis. The error bars represent the standard error of the fit.

Figure 7.

Scheme of asymmetric polymerase assembly at a replication fork. **(a)** CMG recruits Pol ϵ to the leading strand and stabilizes it on DNA. If Pol δ associates with the leading strand, CMG does not stabilize it, and Pol δ dissociates. **(b)** PCNA strongly favors association of Pol δ over Pol ϵ on the lagging strand. **(c)** Replication fork with asymmetric distribution of DNA polymerases. Pol ϵ may bind CMG via contact between the Dpb2 subunit of Pol ϵ and the Psf1 subunit of CMG.