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Establishing the human rolling circle reaction

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In eukaryotes, the complex comprised of Mcm2–7, Cdc45 and GINS (CMG) is essential for DNA replication. Several lines of evidence indicate that the Mcm2–7 complex is the motor of the replicative helicase (reviewed in ref. 1), which is activated by its association with Cdc45 and GINS.[2] Recently, we described the isolation and characterization of the human (h) CMG complex.[3] In HeLa cells, this complex was formed only on chromatin and, following its isolation from cells, exhibited DNA helicase activity. Purified from Sf9 cells, hCMG possesses 3'→5' DNA helicase activity, indicating that it moves ahead of the leading-strand DNA polymerase (pol). In contrast, the prokaryotic helicase DnaB, which unwinds DNA in the 5'→3' direction, moves on the lagging strand. Detailed information about the progression of the prokaryotic replication fork was obtained using the rolling-circle method (ref. 4 and references therein). These studies permitted a detailed characterization of the joint action of the replicative pol and replicative helicase. In the rolling-circle reaction, the pol extends the 3' end of a primer annealed to a minicircle that is then unwound simultaneously by the helicase (for a possible arrangement of proteins at the replication fork, see **Fig. 1**). The emerging single-stranded 5'-tail provides the template for lagging-strand synthesis. In most experiments, minicircles were engineered to contain only three nucleotides, allowing the distinction between leading- and lagging-strand nucleotide incorporation.

We initiated experiments to develop a eukaryotic replication fork in order to investigate whether the hCMG helicase activity could be coupled with the replicative pols.[3] We set up rolling circle reactions using a 200-nt minicircle, the putative leading strand pol ε[5] and hCMG and showed that DNA chains longer than 10 kb were produced (representing > 50 turns of the circle). The putative lagging strand pol δ,[6] however, did not replace hpol ε in this reaction, though both pols extended primers on single-stranded M13 to full-length products (about 7 kb). It is tempting to speculate that an interaction between hCMG and hpol ε, but not hpol δ, contributes to their different activities. Specific interaction between GINS, a component of the CMG complex, and hpol ε has been demonstrated.[7] However, it is presently unclear whether this contributes to the observed preferential role of pol ε and thus requires further examination.

The processivity of the CMG complex alone was about 500 bp, which was stimulated to about 1 kbp by the addition of a single-strand DNA binding protein, either *E. coli* SSB or hRPA. The rolling circle reaction is also dependent on *E. coli* SSB, presumably to sequester the emerging single-stranded 5' tail. Surprisingly, hRPA did not replace *E. coli* SSB in the rolling circle reaction. This was attributed to its inhibitory effects on pol ε activity in vitro. The influence of hRPA on eukaryotic fork progression is presently unclear. In the in vitro SV40 viral DNA replication system, hRPA is essential for DNA synthesis and cannot be replaced by *E. coli* SSB (reviewed in ref. 8). In this system, the SV40 large T-antigen acts as the replicative helicase, and hRPA is essential for its interaction with the hpolα/primase complex, which positions primase to initiate RNA chains. In the SV40 replication reaction, hpol δ synthesizes both leading and lagging strands. Surprisingly, while prokaryotic pols (and their processivity factors) can replace hpol δ and its auxiliary proteins in the in vitro SV40 elongation reaction, hpol ε does not play a role,[8] suggesting that, in this system, the action of hpol ε is preferentially excluded. Importantly, no rolling circle synthesis was detected when hpol δ was used in lieu of hpol ε.[3] Whether a similar mechanism leading to the exclusion of hpol δ from leading-strand synthesis is operational with the CMG helicase remains to be investigated. Using an archaeal system consisting of Pol B, RFC, PCNA, the 3'→5' DNA helicase Mcm and the DNA primase, we have performed both leading- and lagging-strand synthesis on a rolling circle substrate.[9] Currently, our efforts are focused on the synthesis of the lagging-strand with human proteins.

In cells, the replication machinery duplicates chromatinized DNA. Thus, it is likely that chromatin remodeling factors and nucleosome chaperones play roles in the progression of the replication fork. In support of this notion, FACT was identified as a component of the yeast replisome progression complex.[10] Various other proteins associate with the replication fork, such as Mcm10, Ctf4, Tim-Tipin and Claspin. The effects of these proteins on the in vitro replication reaction in eukaryotes remain to be examined.

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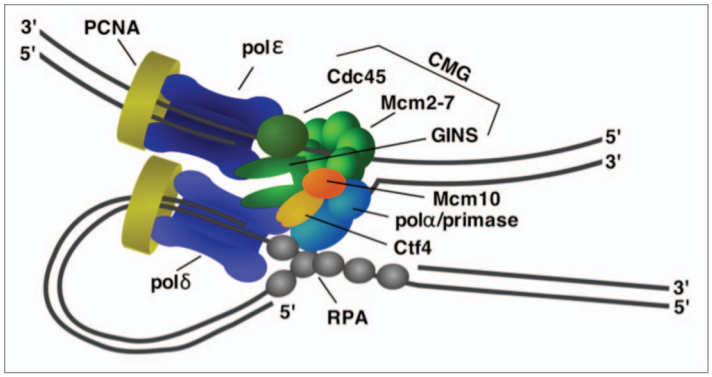

Figure 1. Model of the human replication fork. The CMG complex unwinds DNA in the 3′→5′ direction. Polα/primase synthesizes primers to initiate leading- and lagging-strand synthesis. Polε and polδ are assigned as leading- and lagging-strand polymerases based on evidence in yeast.[5,6] Both pols require the processivity factor PCNA. RPA binds to single-stranded DNA. Additional proteins are required for DNA replication, of which only Ctf4 and Mcm10 are shown for simplicity.

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