Review

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Special Focus: DNA

New insights into replisome fluidity during chromosome replication

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Several paradigm shifting advances have recently been made on the composition and function of the chromosomal DNA replication machinery. Replisomes appear to be more fluid and dynamic than ever imagined, enabling rapid and efficient bypass of roadblocks and template lesions while faithfully replicating chromosomal DNA. This fluidity is determined by many layers of regulation, which reach beyond the role of replisome components themselves. In fact, recent studies show that additional polymerases, post-transcriptional modifications, and chromatin structure are required for complete chromosome duplication. Many of these factors are involved with the more complex events that take place during lagging-strand synthesis. These, and other recent discoveries, are the focus of this review.

Conservation of the replisome stops at the fork

The numerous proteins required to advance a replication fork act together as a machine referred to as the replisome [1]. The basic enzymatic activities of cellular replisomes are common to all domains of life and include the DNA polymerases, proofreading 3′-5′ exonucleases, a hexameric helicase, primase, and a heteropentameric clamp loader that assembles ring-shaped sliding clamps onto primed sites to tether polymerases to DNA for high processivity [1–3]. The leading strand is synthesized in the direction of DNA unwinding, but the antiparallel structure of duplex DNA requires the lagging strand to be extended in the opposite direction of fork movement, as a series of Okazaki fragments. As leading strand synthesis progresses, the lagging-strand template accumulates single-strand (ss) DNA, which is tightly bound by ssDNA binding proteins. Connection between the leading and lagging-strand polymerases results in a growing replication loop during Okazaki fragment extension [4]. After the Okazaki fragment is finished, the loop dismantles, the RNA primer is processed and individual fragments are sealed together by ligase.

Although the conservation of these replisome components is substantial, this is where the similarity between eukaryotic and prokaryotic replisomes stops. This review outlines recent advances that change our view of the prokaryotic replisome structure, and reveal a more dynamic machine than previously thought possible. Several new findings in the eukaryotic replication field reveal a replisome that is very different from the prokaryotic counterpart, and is highly regulated by post-translational modifications. Eukaryotes package the DNA into chromatin, with nucleosomes as the most basic unit. Recent findings reveal a role of the nucleosome in eukaryotic lagging-strand synthesis in a fascinating mechanism that controls Okazaki fragment length and regulates removal of the RNA/DNA hybrid primer made by the low fidelity Pol α/primase.

Prokaryotic replisomes

Bacterial replisomes contain three DNA polymerases

An illustration of the *Escherichia coli* replisome is shown in Figure 1a. The *E. coli* DnaB hexameric helicase encircles

Glossary

Nanoscale DNA biopointers: nanoscale DNA biopointers consist of short (<200 bp) DNA duplexes labeled with biotin. They bind the multivalent streptavidin molecule, which can also bind a biotinylated protein, thereby coupling the DNA molecule to the protein, through the streptavidin bridge. Binding of biopointers to proteins is used to map the localization of proteins in EM. Streptavidin-labeled biopointers have a high specificity for their target and are small enough not to obscure the target, yet are large enough to visualize by EM.

SOS DNA damage response: bacterial cells undergo a response to DNA damage (SOS DNA damage response) that helps them to survive. The major trigger for the SOS response is accumulation of ssDNA, which is recognized and bound by RecA to form filamentous structures. RecA (recombinase A) filament formation activates RecA to function as a co-protease for cleavage of the transcriptional repressor, LexA (locus for X-ray sensitivity A). This results in dissociation of the LexA repressor from DNA and expression of more than 40 genes involved in the cellular response to damaged DNA. These proteins include enzymes required for nucleotide excision repair, base excision repair, DNA recombination, and cell division.

Stroboscopic illumination: a frequent challenge for fluorescence microscopy that applies high intensity illumination is production of reactive oxygen species that are toxic to cells (phototoxicity) and also bleaching of fluorophores during the course of extended or repeated measurements (photobleaching). Use of a high power light-emitting diode (LED), which can emit short pulses of light (0.5–2 ms) (stroboscopic illumination) to excite fluorophores, maximizes signal intensities and minimizes illumination time, thus reducing phototoxicity and photobleaching.

T4 system: The replication system of the T4 phage serves as a relatively simple system for studying DNA replication *in vitro*. Reconstituted from purified components it recapitulates formation and propagation of DNA replication *in vitro*. The eight essential components include a DNA polymerase harboring both nucleotide incorporation and 3′-5′ proofreading exonuclease activities (gp43), a ring-shaped homotrimeric processivity factor (gp45), loaded on a primed template by the clamp loader (gp44/62). The polymerase–clamp complex is processive and displaces ssDNA binding protein (gp32) from DNA. A helicase (gp41) unwinds the duplex DNA and primase (gp61) binds the helicase to initiate primer synthesis. A helicase accessory factor (gp59) assists helicase loading.

Total internal reflection fluorescence (TIRF) microscopy: based on the principle that a thin, exponentially decaying, evanescent field of excitation can be generated at the interface of two media of different refractive index (RI) (e.g., the glass coverslip and the biological specimen). This enables the selective excitation of fluorophores in a thin optical plane above the light beam (<200 nm), thereby preventing fluorescence emission from molecules that are not in the primary focal plane (e.g., molecules that are not bound to DNA that is restricted to the focal plane).

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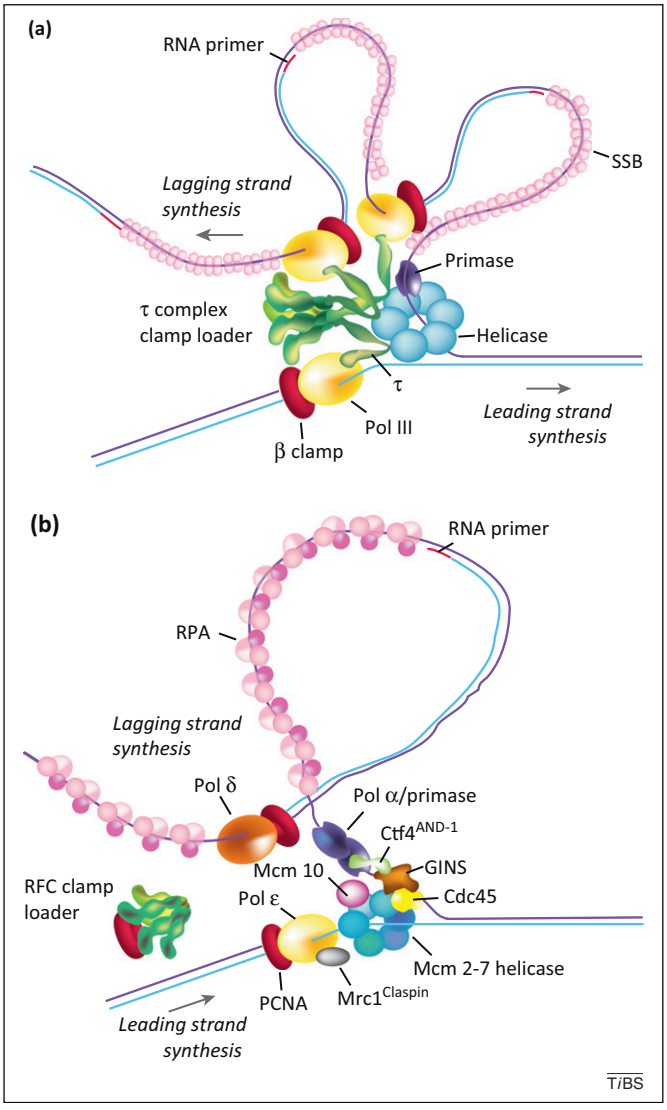

Figure 1. Organization of proteins at a moving replication fork. **(a)** Bacterial replication fork. The parental duplex is unwound by the DnaB helicase (blue) that encircles the lagging strand and unwinds DNA ahead of Pol III (yellow). Primase (purple) synthesizes short RNA primers to initiate Okazaki-fragment synthesis on the lagging strand. The lagging-strand single-stranded (ss)DNA is coated by ssDNA binding protein SSB (pink). Three Pol III enzymes are coupled through the three τ subunits of the clamp loader (green), which uses ATP to assemble β processivity clamps (red) onto primed sites. The simultaneous action of two polymerases is a speculative scenario and the frequency with which all three polymerases are used simultaneously is not certain. **(b)** Eukaryotic replication fork. The Mcm2-7 helicase (blue) is assembled on the leading-strand ssDNA. The active form of the helicase is the CMG complex that includes Mcm2-7, the GINS tetramer (brown), and Cdc45 (yellow). Ctf4 (green) interacts with Polα–primase (purple) and GINS. Mcm10 (purple) binds to Mcm2-7. Mrc1 binds the leading strand Pol ε (yellow). Pol ε and the lagging strand Pol δ (chestnut) both make tight contacts to the proliferating cell nuclear antigen (PCNA) processivity clamps (red). The unwound lagging-strand ssDNA is coated by replication protein A (RPA, pink). The replication factor C (RFC) clamp loader (green) uses ATP to assemble PCNA clamps onto DNA, but a connection of RFC to the replisome is not known.

and tracks along the lagging strand to unwind the parental duplex. DnaG primase transiently interacts with DnaB helicase to initiate primer synthesis, thereby localizing the synthesis of lagging-strand RNA primers to the forked junction [1]. Circular β processivity clamps are then loaded onto newly synthesized RNA primers by a multiprotein complex clamp loader [τ-complex ($\tau_3\delta\delta'\psi\chi$)] [2]. In prokaryotes, the τ subunit of the clamp loader interacts with the catalytic α subunit of the DNA polymerase III (Pol) core (α, ε, θ), coupling the polymerases that operate simultaneously on the separated strands of the parental duplex. The τ complex also connects to the helicase, which greatly stimulates the rate of duplex unwinding when the leading strand Pol III is active [5].

It is thought that replication forks may function in the context of a replication factory, in which two interconnected replisomes remain in a fixed position of the cell while the DNA moves through them [6–8]. Recent data using fluorescently labeled proteins and *in vivo* imaging have challenged this view. *E. coli* has a single origin from which two replication forks proceed in a bidirectional fashion around the entire 4.4-Mb genome. Use of high-resolution fluorescence microscopy to visualize replisomes in the cell shows two separate replication forks at quarter and three-quarter positions in a dividing cell, indicating that the two replication forks are distinct entities, and do not remain coupled in a replication factory [9,10]. Recent single molecule microscopy studies on replicating λ DNA in *Xenopus laevis* nuclear extracts suggest that replication forks are uncoupled in eukaryotes as well [11].

Besides a different view on the organization of individual replication forks within the cell, a surprising observation of the organization within the replisome itself has recently come to light. DNA polymerases have long been thought to act in pairs, where one polymerase synthesizes the leading strand and the second polymerase replicates the lagging strand. This view has recently changed. Reconstitution of the *E. coli* DNA polymerase III holoenzyme from purified proteins results in a particle that contains three active Pol III cores, each connected to one of the three identical τ subunits within the clamp loader [12]. Furthermore, a study in the T4 replication system (see Glossary) using electron microscopy (EM) and nanoscale DNA biopointers revealed the presence of three polymerases in about 6% of phage T4 replisomes [13]. Until recently however, it was not known if this tri-polymerase architecture indeed represents the functional unit at the replication fork *in vivo*. This has now been addressed by the recent development of powerful single molecule high-resolution microscopy methods that have enabled the determination of protein stoichiometries in the cell and observations of dynamic processes *in vivo* (Box 1). Using fluorescently labeled proteins and photobleaching techniques, a new study has revealed the presence of three Pol III cores and three τ subunits within the *E. coli* replisome *in vivo* [14] (Box 1), confirming the tri-polymerase replisome structure in the cell. Subsequent studies, both *in vitro* and *in vivo*, have concluded that all three Pol III cores are active during replisome function [10,15]. But why should the replisome contain three polymerases when there are only two DNA strands to replicate? Single-molecule total internal reflection fluorescence (TIRF) microscopy studies of the *E. coli* replisome *in vitro* have shown that the additional polymerase functions on the lagging strand, enhances replisome processivity, and supports more complete Okazaki fragment synthesis [9]. In other words, with numerous RNA primers to extend, two polymerases devoted to the lagging strand are better than one.

In overview, the recent developments of highly sensitive *in vivo* live cell imaging techniques and *in vitro* single