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When proteins play tag: the dynamic nature of the replisome

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DNA replication, or the copying of DNA, is a fundamental process to all life. The system of proteins that carries out replication, the replisome, encounters many roadblocks on its way. An inability of the replisome to properly overcome these roadblocks will negatively affect genomic integrity which in turn can lead to disease. Over the past decades, efforts by many researchers using a broad array of approaches have revealed roles for many different proteins during the initial response of the replisome upon encountering roadblocks. Here, we revisit what is known about DNA replication and the effect of roadblocks during DNA replication across different organisms. We also address how advances in single-molecule techniques have changed our view of the replisome from a highly stable machine with behavior dictated by deterministic principles to a dynamic system that is controlled by stochastic processes. We propose that these dynamics will play crucial roles in roadblock bypass. Further single-molecule studies of this bypass will, therefore, be essential to facilitate the in-depth investigation of multi-protein complexes that is necessary to understand complicated collisions on the DNA.

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Title Page

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Abstract
Keywords : Single-molecule biophysics, DNA replication, DNA damage, multi-protein complexes, concentration-dependent exchange dynamics.

DNA replication, or the copying of DNA, is a fundamental process to all life. The system of proteins that carries out replication, the replisome, encounters many roadblocks on its way. An inability of the replisome to properly overcome these roadblocks will negatively affect genomic integrity which in turn can lead to disease. Over the past decades efforts by many researchers using a broad array of approaches has revealed roles for many different proteins during the initial response of the replisome upon encountering roadblocks. Here, we revisit what is known about DNA replication and the effect of roadblocks during DNA replication across different organisms. We also address how advances in single-molecule techniques have changed our view of the replisome from a highly stable machine with behavior dictated by deterministic principles to a dynamic system that is controlled by stochastic processes. We propose that these dynamics will play crucial roles in roadblock bypass. Further single-molecule studies of this bypass will, therefore, be essential to facilitate the in-depth investigation of multi-protein complexes that is necessary to understand complicated collisions on the DNA.

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Stefan H. Mueller, Lisanne M. Spenkelink, and Antoine M. van Oijen declare that they have no conflict of interest.

Introduction

DNA replication, or the duplication of genomic DNA prior to cell division, is a fundamental step in the process of reproduction of living organisms. When the double-helix structure of DNA was proposed by Watson and Crick (1953), aided by X-ray crystallographic images obtained by Rosalind Franklin in 1953, a general mechanism for DNA replication became apparent: separating the two strands of the parental DNA and pairing both with the complementary nucleotides to create two identical daughter strands. The initial discoveries of the underlying mechanisms were mainly driven by Arthur Kornberg and co-workers starting from 1956 (Kornberg *et al.* 1956). From this point on, tremendous insight was gained into DNA replication, the involved proteins and their functions, and how similar functions are carried out by different proteins in different organisms.

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Not only is DNA replication accomplished with remarkable speed and at high accuracy, the replisome can overcome numerous barriers without much delay. These barriers arise from both endogenous sources, such as other proteins interacting with and bound to the DNA, as well as DNA damage caused by exogenous sources such as UV exposure. The question how DNA replication copes with damage is as old as the field itself. Already in 1929 a correlation between the lethal effect of ultra-violet radiation on living cells and the absorption maximum of nucleic acid in the ultra-violet spectrum was proposed (Gates 1928). The formation of thymine dimers, one of the most abundant DNA lesions, by UV irradiation was demonstrated in 1958 (Beukers *et al.* 1960). DNA replication and other fundamental DNA-metabolism processes, such as transcription or repair, occur simultaneously. Thus, collisions of the replisome with other DNA-bound protein complexes are inescapable. If the replisome is unable to properly overcome these roadblocks, these encounters will have an effect on genomic integrity and stability. Over the past decades many connections between error-prone or malfunctioning DNA replication on the one hand and diseases like cancer on the other were discovered (Macheret and Halazonetis 2015). Research on the initial response of the replisome to collisions has revealed the roles of many proteins in the cause and resolution of genomic instability. Until recently, mostly traditional biochemical methods, averaging over large numbers of molecules and reactions, have been used to study these processes. To reveal the fundamental molecular mechanisms occurring at roadblocks, however, the use of single-molecule techniques seems almost inevitable. Single-molecule assays allow the visualisation of the dynamics of individual proteins, without the need for ensemble averaging. These techniques have already revealed surprising dynamic behavior of proteins involved in replication in interplay with their surroundings. Very much like children playing tag on the playground, some proteins seem to change their kinetic properties upon "being tagged" by competitors while staying still in the absence of such competitor molecules.

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In this review we will revisit what is known about the effect of DNA-based roadblocks during DNA replication across different organisms. We also address advances in single-molecule techniques that will facilitate the in-depth investigation of multi-protein complexes that is necessary to understand these complicated processes. ~~We will use recent findings to discuss how our understanding of this fundamental process has changed and will continue to change in future.~~

The Replisome

The replisome is the protein complex responsible for the duplication of genomic DNA. Over the past decades a variety of different organisms has been studied. The fundamental mechanism of DNA replication is surprisingly similar from the most primitive viral systems up to the most complex organisms like humans. In the following section we will provide an overview of the basic principles of DNA replication on the basis of the replisomes from bacteriophage T7, the prokaryotic *Escherichia coli* and eukaryotic *Saccharomyces cerevisiae*, as these are extensively studied and represent model organisms for the major domains of life. Life evolved molecular mechanisms to ensure robust and highly regulated DNA replication. That robustness means that every gene is copied once and only once for every cycle of cell division. Furthermore, for correct genome duplication, replication has to start and end at well-defined positions. In one of the simplest of all studied systems, the

bacteriophage T7, while technically not a living organism, this process of initiation is rather simple. Only two proteins are involved in initiating replication at a specific site: DNA polymerase or gene product 5 (gp5) and T7 RNA polymerase (Richardson 1983). The bacterium *E.coli* has a more complex mechanism, yet much simpler than the eukaryotic initiation system. Briefly, an initiator protein, DnaA binds to a unique sequence, the origin of replication or oriC. This origin sequence consists of multiple binding sites for DnaA and AT-rich segments that melt upon binding of DnaA. This process, called origin unwinding, is driven by DnaA hydrolysing ATP. Subsequently, the replication proteins are recruited to the origin and replication is initiated.

Eukaryotic cells follow a cycle to highly regulate and control replication cell division. Both events occur only once per cycle. Due to the size of eukaryotic genomes each chromosome contains multiple origins of replication. The origins are prepared (licenced) during late M phase and G1 phase (Li and Jin 2010; Truong and Wu 2011). Licensing involves the assembly of pre-replication complexes (pre-RCs) on the origins. The core component that first binds the origins is the hexameric origin-recognition complex (ORC), followed by cell division cycle proteins Cdc6/Cdc18 and Cdt1. See Tognetti *et al.* (2015) for a detailed review on replication initiation. ORC then recruits the mini-chromosome-maintenance-proteins 2–7 (Mcm2–7) to the origin. This complex has been shown to be stable at the origin for a long time, until replication is initiated by post-translational modifications. These modifications trigger the recruitment of additional replication proteins and ultimately the start of replication from a specific site on the chromosome (origin firing). For a more detailed review on origin licencing see Méchali (2010).

Often referred to as the core of the replisome is the helicase, responsible for unwinding of the double-stranded parental DNA. Interestingly, all replicative helicases seem to be hexamers that translocate along single-stranded DNA (ssDNA) to unwind the double helix. While T7 gene 4 product (gp4) (Kolodner and Richardson 1977) and *E.coli* DnaB helicases form homo-hexamers that translocate in a 5′–3′ direction (on the lagging strand; see figure 1a). The eukaryotic CMG helicase is an assembly of three major complexes: The core helicase complex, a circular hetero hexamer of the before mentioned Mcm2–7, Cell-division-cycle-protein 45 (Cdc45), and the GINS complex (Sld5, Psf1, Psf2, Psf3). In contrast to the bacterial helicase, this assembly translocates on ssDNA in a 3′–5′ direction and excludes the lagging strand (Takayama *et al.* 2003; Bochman and Schwacha 2008; Fu *et al.* 2012; Costa *et al.* 2014; Sun *et al.* 2015b).

After initial unwinding a short RNA primer is synthesized to provide a binding substrate for DNA polymerases and to allow the DNA polymerase to initiate DNA synthesis. While such a primer is crucial to initiation of DNA replication among all organisms, it varies in length and composition. The T7-phage priming activity is carried out by gp4, therefore called a helicase–primase, and occurs at specific primer recognition sites (Kusakabe and Richardson 1997). The synthesized primer consist of 2–4 ribonucleotides (Tabor and Richardson 1981). In *E.coli* a separate protein, DnaG, synthesizes an RNA primer of 26–29 bp (Bouché *et al.* 1978). In eukaryotes, DNA polymerase α (Pol α) synthesizes a primer of about 9–11 ribonucleotides and directly extends it by circa 20 deoxyribonucleotides (Nethanel and Kaufmann 1990; Santocanale *et al*. 1993). The result is the creation of a mixed RNA-DNA primer.

The primers are extended by a group of proteins called DNA polymerases. ~~Again, higher organisms evolved more specialized DNA polymerases for different purposes.~~ While the T7 phage possesses only the gp5 DNA polymerase, in *E.coli* polymerases I through V are known. Of these, Polymerase III (Pol III) is the major replicative polymerase (Kornberg and Gefter 1972) while the others are important for a variety of other processes, mainly associated to DNA repair, as reviewed in more detail by Fijalkowska, Schaaper, and Jonczyk (2012) In contrast to the phage T7 DNA polymerase, Pol III is a complex of three subunits. The α subunit exerts the actual DNA polymerase activity, the ε subunit has 3′– 5′ exonuclease activity, and the θ subunit, the function of which is not well understood. Two to three of these core complexes are thought to form a complex at replication forks in the presence of auxiliary factors and form the Pol III holoenzyme (Maki *et al.* 1988; Kim *et al.* 1996; Reyes-Lamothe *et al.* 2010). Due to its exonuclease activity, the holoenzyme possesses an inherent proofreading mechanism, leading to error rates as low as 10^{-7}per synthesized base pair (Fijalkowska, Schaaper, and Jonczyk 2012). Eukaryotes have three replicative polymerases. Polymerase α (Pol α), discovered first in 1986, has the primase activity, but has been shown to be capable of replicating DNA in absence of other

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polymerases (Campbell 1986; Tsurimoto *et al.* 1990). The other replicative polymerases are Polymerase δ (Pol δ) and Polymerase ε (Pol ε). How these three enzymes interact at replication forks has been subject to controversy. Currently, most evidence points towards a model in which Pol ε is physically tethered to the CMG helicase and exclusively replicates the leading strand. Pol δ synthesizes Okazaki fragments and also removes parts of the primers created by pol α through its strand-displacement synthesis activity (Byrnes *et al.* 1976; Budd *et al.* 1989; Higuchi *et al.* 2003; Pursell *et al.* 2007; Nick McElhinny *et al.* 2008). Similar to the *E.coli* Pol III, Pol ε and Pol δ are capable of both DNA-synthesis and exonuclease activity. Unlike the *E. coli* polymerase which has dedicated subunits for synthesis and exonuclease activity, in Pol ε and Pol δ these activities are supported by different domains of the same subunit. The three additional accessory subunits to Pol ε and Pol δ partially facilitate interactions with other components of the replisome and have unknown or not fully understood functions (Pursell and Kunkel 2008). Interestingly all polymerases rely on additional coenzymes to achieve processive DNA replication. Processivity is defined as the number of nucleotides synthesized before dissociation of the protein. T7 bacteriophages utilize the thioredoxin protein from their bacterial host for this purpose. The processivity of the T7 DNA polymerase is increased about 80 fold upon binding of thioredoxin (Bedford *et al.* 1997). Cellular organisms possess a class of proteins called DNA-sliding clamps to achieve this processivity and to play an organisational role in the replisome. In *E. coli* this clamp is called the β clamp and it is a part of the Pol III holoenzyme (McHenry and Kornberg 1977; Stukenberg *et al.* 1991). The eukaryotic version is the Proliferating Cell Nuclear Antigen (PCNA) (Tan *et al.* 1986). These clamps encircle DNA and freely slide along it. By binding DNA polymerases, the net affinity of the polymerases to DNA is increased.

Closely associated with these sliding clamps are the proteins required to support their initial loading onto the DNA. These ATP-dependent enzymes are called clamp-loader complexes. The *E. coli* clamp loader complex (Clc) composed of five distinct peptides ($\tau_{(n)}\gamma_{(3-n)}\delta\delta'\chi\psi$) (Maki and Kornberg 1988) is fully integrated into the pol III holoenzyme and therefore travels with the replisome (Naktinis *et al.* 1995; Lewis *et al.* 2016). The eukaryotic clamp loader, Replication Factor C (RFC), is a heteropentamer. It is currently unknown whether RFC forms a stable complex with the replisome and is therefore not depicted in figure 1c (Yao and O'Donnell 2012). Another component crucial for faithful DNA replication in all organisms is a protein that protects the stretches of ssDNA that are inevitably created by unwinding. This protein is the so called single-stranded DNA-binding protein (SSB). It is called gene product 2.5 (gp2.5) for T7, simply SSB in *E.coli* and replication factor A (RPA) in yeast. Besides protecting the ssDNA, SSBs play important regulatory roles by serving as an interaction site for many binding partners within the replisome (Shereda *et al.* 2008; Hernandez and Richardson 2019). Even though there are more essential proteins involved in eukaryotic replication, we will not describe these in greater detail here, as their functions are less well known. We will come back to these accessory proteins later as they are thought to be important for certain responses of the replisome to roadblocks.

Roadblocks on DNA

In the context of DNA replication a roadblock can be any obstacle that the replisome has to overcome in order to successfully duplicate the whole genome. Here, we discuss two major types of roadblocks: DNA lesions and protein roadblocks.

Lesions

DNA lesions are sites of damage or defects in the structure or base paring of DNA. Lesions on the genome can be caused by many different factors. A prime cause of DNA lesions is UV radiation, which results in two major defects of DNA: cis-syn-cyclobutane pyrimidine dimers (CPDs), which are most abundant (67–83%), and pyrimidine (6-4) pyrimidone photoproducts (PPs) (Beukers, Eker, and Lohman 2008). Crystal structures of DNA containing lesions show that not only the position of the bases forming dimers is changed, the conformation of the backbone is altered as well. PPs have been reported to be effectively repaired in human cell lines, while repair of CPDs is much slower (Hedglin and Benkovic 2017). The high-fidelity replicative polymerases are very inefficient in incorporating nucleotides across or bypassing such lesions. Trans-lesion synthesis polymerases, however, are able to synthesize across lesions at the cost of higher error rates (O'Day *et al.* 1992; McCulloch *et al.* 2004). A third type of lesion is caused by oxidative stress and results in a chemical

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alteration of the nucleosides. A prominent example is the incorporation of oxidized guanosine opposite of an adenine. This type of lesions induces mutations rather than challenge the process of DNA replication itself and is thought to play a role in carcinogenesis (Cadet and Wagner 2013) .

Protein Roadblocks

Besides lesions, which are mainly induced by external impact on the genome, cells also have to coordinate replication with all other processes taking place within the nucleus (e.g. transcription, translation, repair). Due to the sheer number of proteins interacting with DNA little is known about the exact coordination of all these processes. One major process that could potentially interfere with DNA replication is the transcription of DNA by RNA polymerases. This process is regulated by various transcription factors. A recent review states that around 1600 different transcription factors are known in humans (Lambert *et al*. 2018). Even though the exact coordination of transcription and replication is not understood in detail, various replication barriers have been identified. One such barrier was already identified in 1988 in yeast, when replication forks were observed to stall at the 3′-end of ribosomal RNA genes. This study revealed that transcription and replication are coordinated in these highly transcribed genes by a polar replication block facilitated by the fork blocking protein 1 (Fob1). The activity of this protein ensures that RNA polymerase and replication complexes move through these regions in a coordinated fashion, avoiding collisions (Linskens and Huberman 1988; Brewer and Fangman 1988; Kobayashi and Horiuchi 1996). Another roadblock for DNA replication that is subject to current research are nucleosomes. Nucleosomes are complexes of histones, DNA-binding proteins with DNA wrapped around them. Eukaryotic DNA is organized in such nucleosomes to form chromatin structure that compacts genomic DNA in way that tightly regulates physical access to the DNA to ensure control of gene expression. For replication to take place on chromatinized DNA these nucleosomes have to be remodelled by so called chromatin remodellers. MacAlpine and Almouzni (2013) provide a more detailed review on DNA replication on chromatinized DNA. How this process is orchestrated around DNA replication remains unclear.

Single-molecule techniques reveal unexpected plasticity

The replisome carries out one of the most vital cellular processes and therefore must work in an extraordinarily reliable way. It was, therefore, long assumed that the composition of the replisome is very stable. This robustness was, among others, demonstrated by Debyser, Tabor, and Richardson (1994). They demonstrated that the T7 replisome is resistant to dilution or other challenges. Therefore, they concluded that a single polymerase is efficiently recycled for the synthesis of many Okazaki fragments. This view of the replisome, virtually as a machine carrying out its function in a fully deterministic manner, was further confirmed by Kadyrov and Drake (2001) and Kim *et al.* (1996). They demonstrated the same remarkable stability and orchestration of leading- and lagging-strand synthesis for the T4 and the *E. coli* replisome, respectively. However, these studies utilized classical biochemical assays with radiolabelled nucleotides to detect newly synthesized DNA. Although these assays provided valuable information, they were not able to report on transient reaction intermediates, the exact composition of the replisome at a given time, and the conformational changes involved. Our understanding of DNA replication began to change in the last two decades as single-molecule techniques were developed. Such techniques allow us to follow the kinetics of single replisomes in real time revealing unexpected dynamic behaviors. Single-molecule studies have had a drastic impact on our understanding of how DNA replication works and, so we anticipate, will continue to do so. We will discuss key experiments that have contributed to our changing view of multi-protein complexes.

Already in the 1990s, the T7 replisome was known to synthesize DNA at rates of up to several 100 bp/s. Debyser, Tabor, and Richardson (1994) carried out replication experiments using a circular, 6.4-kb DNA template based on the bacteriophage M13 genome that was converted into dsDNA with a ssDNA overhang. The process of replication on this circular template (rolling-circle DNA replication) was highly efficient and resulted in replication products greater than 40 kb in length. Even after assembled complexes of helicase and polymerase were diluted to low nM concentrations, long replication products were detected. However, if replisomes where assembled ~~Yet the~~ *de novo* ~~assembly of replisomes~~ at such low concentrations ~~prevented~~ replication was inhibited. This suggested that a single set of enzymes is capable of synthesizing thousands of basepairs and

many Okazaki fragments once the reaction was initiated. Combined with the observation that the leading-strand synthesis rate was affected by the presence or absence of lagging-strand synthesis, this suggested a spatial coupling of the polymerases on both strands to the replication fork, even though Okazaki fragments are synthesized in opposite direction to the movement of the fork. A model for this coupling had already been proposed by Alberts *et al.* (1975) in which the lagging-strand template is looped, to allow the lagging strand to be spatially coupled to the helicase. In order to demonstrate the existence of such replication loops, Van Oijen and co-workers developed a technique to visualize DNA replication on the single-molecule level. They utilized forked λ- phage DNA that was tethered to the surface of a microscope coverslip on one side and a bead, visible in low-magnification wide-field microscopy, on the other side. Replication was initiated in the presence of a hydrodynamic flow. As replication loops were formed on the lagging strand, the bead moved against the flow direction, as the DNA template was effectively shortened by the loop formation. On release of the loop, the bead abruptly moved with the flow, returning the DNA to its full length. By changing the concentration and nature of ribonucleotides present in solution, the coordination between primer synthesis and loop formation was examined. Their observations suggested that either completion of Okazaki fragments or initiation of primer synthesis can trigger loop release. Between the release of one loop and the formation of the next one, exponentially distributed lag-times were observed and attributed to primer-recognition, synthesis and hand-over to the polymerase (Hamdan *et al.* 2009) (see figure 2a).

The same research group carried out a study in 2011 using a linear DNA template with *in vitro* reconstituted T7 replisomes. In contrast to earlier work, single DNA molecules tethered to a coverslip surface were directly visualized using fluorescence microscopy. Omitting ribonucleotides from the reaction allowed leading-strand synthesis only, while the lagging strand was converted into ssDNA behind the replisome. With the applied hydrodynamic forces ssDNA adopted a compact random-coil conformation while dsDNA is stretched close to its crystallographic contour length. Replication kinetics were observed by tracking the shortening of DNA for single molecules in real time. When mutant polymerases known to synthesize DNA at a slower rate were used, slower replication kinetics were identified in the single-molecule assay. Therefore, Loparo *et al.* were able to identify which polymerase species was present at a moving replisome at any given time, based on the observed kinetics. Surprisingly, after mixing the two polymerase species, kinetics signatures of both polymerases were observed within trajectories of individual replisomes. Contradictory to the processive nature of the replisome demonstrated before, this showed that polymerases at the fork are dynamically exchanged with competitor molecules from solution (Loparo *et al.* 2011). In 2014 Geertsema *et al.* visualized rolling-circle replication with single-molecule resolution. A circular DNA template based on the phage M13 genome, with a biotinylated ssDNA overhang was tethered to a glass surface and hydrodynamic flow was applied as before. Repeated cycles of replication of the circular template lead to extension of the tethered end of the DNA, which is stretched by the flow. In this study fluorescently labelled polymerases were employed. The fluorescence intensity measured at an elongating fork was proportional to the amount of polymerases present within a diffraction-limited spot. Quantitative intensity measurements gave an estimate of the polymerase stoichiometry at individual replication forks. By labeling polymerases in multiple different colors the authors created an additional readout for exchange kinetics. Using two-color fluorescence microscopy it was demonstrated that polymerases are exchanged with molecules from solution on the lagging strand. The exchange kinetics were shown to be similar to the kinetics of primer synthesis for new Okazaki fragments. The conclusion was that individual polymerases reside at the fork for the synthesis of only a few Okazaki fragments (Geertsema *et al.* 2014). Not only did these results question the mechanism of coupling leading- and lagging-strand synthesis, but they also challenged the general view of the replisome as a highly processive and fully deterministic machine. Hamdan *et al.* (2009) argued that the existence of two distinct triggers for loop release, either initiation of primer synthesis or completion of the previous Okazaki fragment, provides a mechanism needed to overcome the stochastic nature of primer synthesis itself. However, it now became apparent that DNA replication as a whole might occur in a more stochastic manner than expected. Research on DNA replication in model organisms other than T7 tells very similar stories. Polymerase exchange has now been demonstrated in a number of studies, for the bacteriophage T4 replisome (Yang *et al.* 2004) and the *E.coli* replisome *in vitro* as well as *in vivo* (Lia *et al.* 2012; Beattie *et al.* 2017; Lewis *et al.* 2017). More recently, a combination of the previously described single-molecule rolling-circle replication assay with fluorescence-recovery-after-photobleaching (FRAP) studies was used to observe the kinetics of SSB at replication forks and revealed recycling of SSB for multiple Okazaki

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fragments, dependent on the concentration of SSB in solution. For this experiment fluorophores in a small localized area are irreversibly bleached using a high-intensity laser pulse. Subsequent recovery of fluorescence can be attributed to the exchange of bleached molecules with unbleached ones from solution. These measurements revealed that dependent on the concentration of SSB in solution, it can either by rec ycled from a previous Okazaki fragment, which results in no recovery of fluorescence, or recruited from solution, producing increasing fluorescence signal after bleaching (Spenkelink *et al.* 2019) (see figure 2b).

In general the vast literature on DNA replication seems to consist of contradictory findings. Many biochemical studies demonstrated stable, highly processive replisome, yet single-molecule studies revealed stochastic behavior. This apparent paradox of stability in isolation but plasticity in the presence of competitor molecules has been explained by the presence of multiple weak binding sites linking single components of the replisome. This scenario results in dissociation of a protein from the complex in a step-wise process. The process can be imagined as follows: assume a protein that is bound to a complex via two weak binding sites. Dissociation suddenly becomes a two-step process, as it requires unbinding of binding site one followed by the unbinding of binding site two. Complete dissociation therefore becomes less likely (figure 3 a,b). However, if competitors are present in solution, one site might get occupied by a competitor, which leads to exchange (figure 3 c). The kinetics of the exchange depend on the concentration of the competitor in solution. Such a competitor molecule can be a different protein binding the same site or another identical protein (figure 3). Åberg, Duderstadt, and van Oijen (2016) provide a mathematical formalism to describe protein complexes with any number of binding sites and demonstrate that the T7 replisome assembly can be quantitatively described by this method. Qualitatively the multi-site exchange mechanism provides a general solution to the apparent paradox of plasticity and stability in DNA replication under various conditions. This mechanism is not unique to DNA replication and has also been described for other multi-protein complexes, such as the bacterial flagellar motor. For this complex an individual subunit has been shown to rapidly exchange with a pool of membrane-bound inactive proteins in solution (Leake *et al.* 2006; Thormann and Paulick 2010). A second example is given by the facilitated dissociation of transcription factors from DNA, as described by Marko and co-workers (Graham *et al.* 2011; Kamar *et al.* 2017)

Plasticity as a mechanism to bypass roadblocks

At first sight, the apparent stochastic nature of DNA replication seems to represent a disadvantage. How can a process be of stochastic nature and yet happen in an extremely tightly regulated way to ensure faithful replication of every gene once, and only once per cell-division cycle? On the other hand a dynamic picture maintained through many weak protein–protein interactions as oppose to a few very tight interactions, provides a possible mechanism to deal with unforeseen circumstances. Multi-site exchange could even provide a universal mechanism through which the replisome can achieve efficient bypass of replication roadblocks.

Given the universal presence of roadblocks on DNA even the simplest organisms need some mechanism to overcome such roadblocks. Sun *et al.* (2015) found evidence that the T7 replisome is capable of bypassing CPD lesions without any accessory proteins, even though the gp5 polymerase alone was unable to extend primers across CPD lesions. They show that such bypassing would be dependent on the interaction between the helicase and the polymerase and that the polymerase–DNA interactions become weaker upon encountering lesions. Zou *et al.* (2018) characterize the binding affinities between helicase, polymerase, and DNA utilizing surface plasmon resonance (SPR). They indeed observe a change in the binding affinities upon encountering 8-oxoG or O6-MeG lesions, another type of physiologically relevant DNA damage. Such weakening of binding affinity to the DNA, while the interaction to the helicase stays constant should, according to the theoretical model of van Oijen and co-workers, facilitate either polymerase exchange or transient unbinding. However, how replication could be restarted on the leading strand without concurrent primer synthesis past the lesion was not examined in this study.

In general it can be advantageous for living organisms to bypass damaged DNA without repairing it and even accept the risk of mutations in order to sustain replication, cell division and ultimately survival. Therefore, a number of pathways exist to rescue stalled or damaged replication forks (see Yeeles *et al.* (2013) for a detailed

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review). Little is known about how these pathways are regulated in detail but most of these mechanisms can hardly be imagined without dynamic interactions of the involved proteins. Especially the eukaryotic replisome, which is far more complex than its prokaryotic counterpart, has many components of largely unknown functions. One example is the complex of Mrc1, Tof1 and Csm3 (MTC). It has been shown to be required for maximal rate of replication on one hand, and for stalling in the presence of replicative stress or RFBs on the other hand (Katou *et al.* 2003; Calzada *et al.* 2005; Yeeles *et al.* 2017). In other words, MTC might be required to associate with the replisome when needed and unbind in the other situations. Lewis *et al.* (2017) provided proof for a dynamic interaction of MTC with a fully reconstituted leading-strand *S. cerevisiae* replisome in a single-molecule tethered-bead assay.

Another example where dynamic interactions of a protein with the eukaryotic replisome likely play an important role to promote bypassing of roadblocks is Mcm10. The CMG-helicase stands out among other helicases as its unwinding point seems to take place inside the central pore, meaning that both strands enter the core of the complex to some extent (Georgescu *et al*. 2017). This explains why unwinding of templates containing a bulky roadblock on the non-tracking strand (the lagging strand) is not readily observed in absence of accessory proteins. In presence of the essential protein Mcm10, which is known to play a role in replication initiation (Wohlschlegel *et al*. 2002), however, unwinding rates of CMG are enhanced and lagging-strand blocks are bypassed. This is hypothesized to happen due to a conformational change in CMG induced by Mcm10 (Langston *et al*. 2017; Lõoke *et al.* 2017). Apart from its association with CMG, Mcm10 is a DNA-binding protein (Robertson *et al*. 2008; Warren *et al*. 2008) and physically interacts with Pol α *(Fien et al.* 2004; Ricke and Bielinsky 2004; Zhu *et al.* 2007) and PCNA (Das-Bradoo *et al.* 2006). These multiple interaction sites could allow Mcm10 to dynamically associate with the replisome and facilitate exchange as necessary.

Another mechanism that provides an explanation on how the replisome could bypass lesions without stalling or decoupling was proposed by (Langston *et al.* 2014) In this pathway, a Pol ε encountering a lesion would stall (see figure 4a). A competing translesion synthesis (TLS) polymerase from solution then triggers dissociation of Pol ε from the DNA and PCNA to synthesize nucleotides across the lesion (see figure 4b,c). Meanwhile Pol ε is retained at the fork through its strong interaction with GINS, to assure outcompeting of the error-prone TLS polymerase downstream of the lesion (see figure 4d). A similar mechanism for lagging-strand lesions was proposed by Hedglin, Pandey, and Benkovic (2016). They propose stalling of pol δ at a lesion which eventually leads to dissociation. PCNA would stay bound to DNA and can recruit a TLS-polymerase to the DNA. A more comprehensive review on mechanisms of TLS polymerases at active replication forks was written by (Trakselis *et al.* ~~, Cranford, and Chu (~~2017)

Conclusion and Outlook

In this review we discussed the stochastic nature of DNA replication particularly in presence of lesions or roadblocks on DNA. Recent single-molecule studies have revealed the dynamic behavior of proteins involved in DNA replication. This has changed our view of the replisome from a neat and deterministic machine, to a more 'messy' and stochastic system of proteins (Åberg, Duderstadt, and van Oijen 2016; Geertsema and van Oijen 2013; Scherr, Safaric, and Duderstadt 2018; Xu and Dixon 2018). Proteins bind to the replisome through a plurality of weak interactions. This allows the replication system to adapt to its environment; proteins can be recycled or exchanged as necessary. It is likely that the multi-site exchange mechanism plays an important role during the bypass of replication blocks, as it assures that multiple molecular pathways are accessible to the replisome. Especially in the case of eukaryotic organisms many processes remain unclear and need further investigation. For example, we have not discussed the effect that various post-translational modifications could have in DNA repair and damage tolerance pathways. These modifications are likely to alter the kinetics of all the molecular interactions within the replisome. Further development of biochemical approaches and single-molecule imaging tools are needed to elucidate the rules by which proteins play their complex game of tag.

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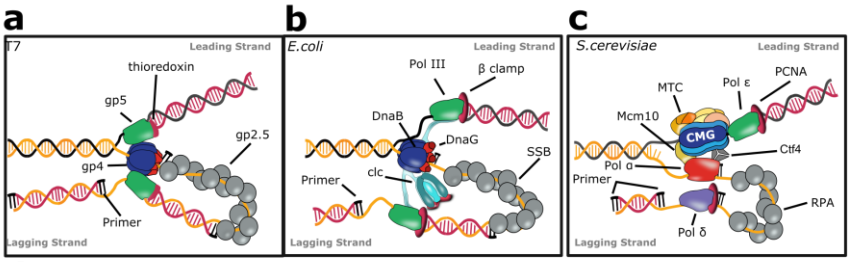
Figures



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Protein class	T7	E.coli	S. cerevisiae
Helicase	gp4	DnaB	CMG
Polymerase	gp5	αεθ	α, δ and ε
Primase	gp4	DnaG	α
Clamp / processivity factor	thioredoxin	β	PCNA
Clamp loader	-	$\tau_{(n)}\gamma_{(3-n)}\delta\delta'\chi\psi$	RFC
ssDNA binding protein	gp2.5	SSB	RPA
Accessory/unknown function	-	-	Ctf4, MTC, Mcm10

Fig. 1 Schematic representations of replisomes in viruses, bacteria and eukaryotes. (a) The T7 replisome. The helicase gp4 translocates in 5′ – 3′ direction on the lagging strand and synthesizes ribonucleotide primers. Two gp5 polymerases are bound to gp4 for Okazaki-fragment and continuous leading-strand synthesis, respectively. The gp2.5 protein covers exposed ssDNA regions. Figure adapted from Yao and O'Donnell (2010) (b) The *E. coli* replisome. The helicase DnaB translocates in 5′ – 3′ direction on the lagging strand. Associated with the helicase, three DnaG primase molecules synthesize ribonucleotide primers on the lagging strand. Two to three polymerases are tethered to the helicase via the clamp-loader complex (clc). The SSB protein covers exposed ssDNA regions. Figure adapted from Robinson and van Oijen.(2013) (c) The *S. cerevisiae* replisome. The CMG helicase translocates on the leading strand in 3′ – 5′ direction. Pol *α*, tethered to the helicase via Ctf4, synthesizes mixed RNA-DNA primers. The leading and lagging strand are synthesized by Pol ε and Pol δ respectively, both of which associate with the processivity factor PCNA, loaded by the RFC clamp loader (not shown). Additionally Mcm10 and the MTC complex interact with the helicase. RPA covers exposed ssDNA regions. Figure adapted from J. Sun *et al.* (2015). (d) Table of replisome components for the three organisms depicted in (a), (b) and (c). Adapated from Stratmann and van Oijen (2014).

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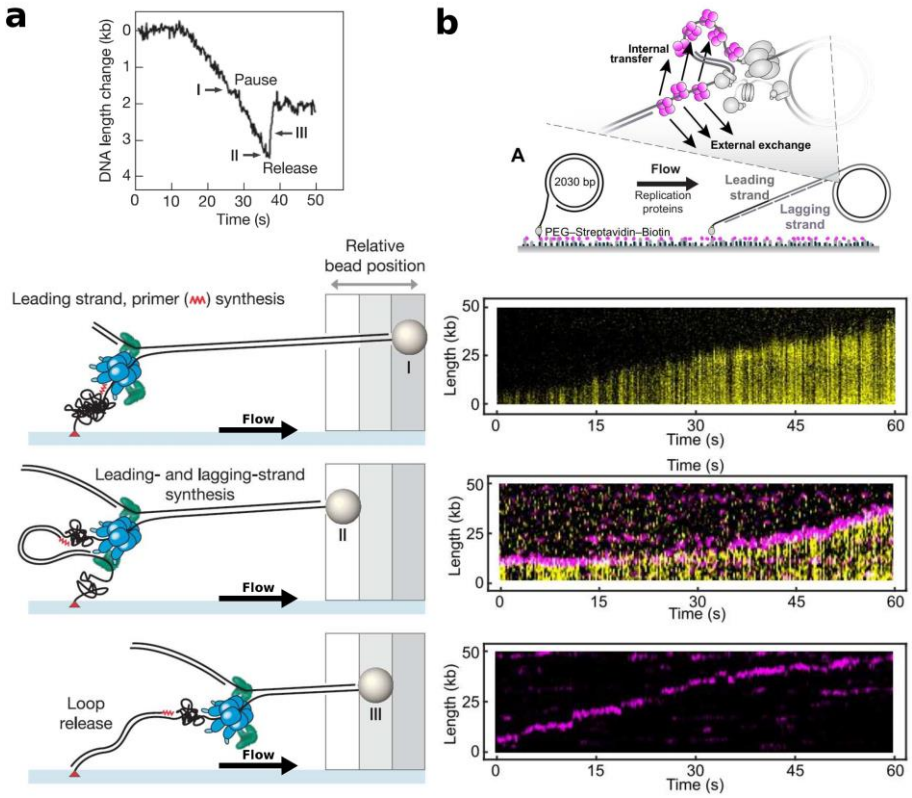

Fig 2 Single-molecule DNA replication experiments. (a) Tethered-bead assay. A bead attached to template DNA is hydrodynamically stretched and observed using bright-field microscopy. Loop formation causes shortening of the DNA and movement of the bead against the direction of flow. Pausing and abrupt lengthening of the DNA can be attributed to priming and loop release. Adapted from Lee *et al*. (2006) (b) The rolling-circle assay. A circular DNA template with a single-stranded overhang is attached to the surface of a microfluidic flow cell. Hydrodynamic elongation of created replication products allows visualization of fluorescently labelled SSBs (magenta) and fluorescent DNA stain (yellow). Adapted from Spenkelink *et al*. (2019).

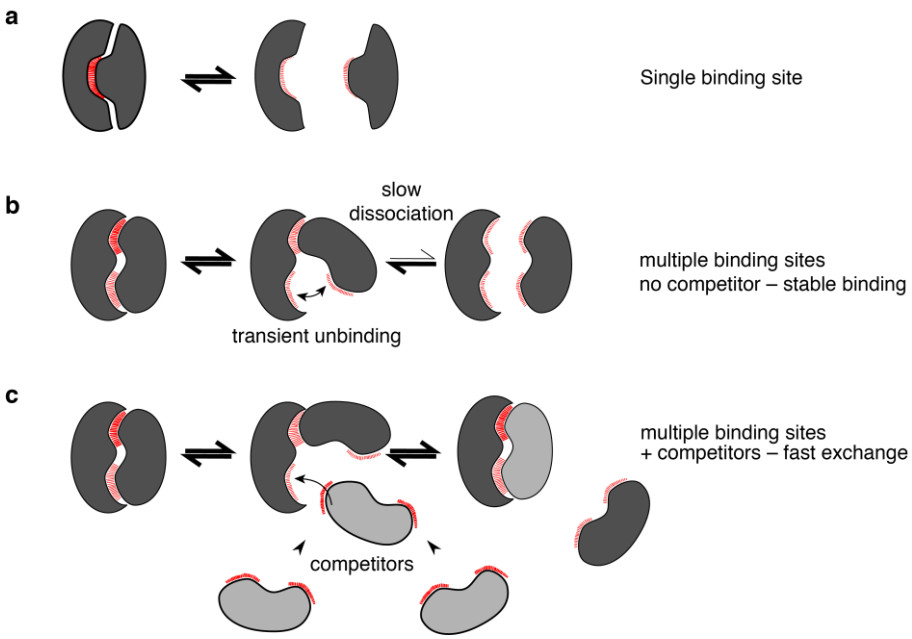

Fig 3 Concentration-dependent exchange. (a) Binding via one unique binding site. The kinetics are determined by association and dissociation rates alone. (b) The presence of multiple binding sites leads to a two-step dissociation process, determined by two separate rates. Complete dissociation becomes less likely compared to (a). (c) Competitor molecules in solution influence the overall dissociation rate. The competitor molecule binds at the transiently vacated binding site and then fully displaces the first molecule.

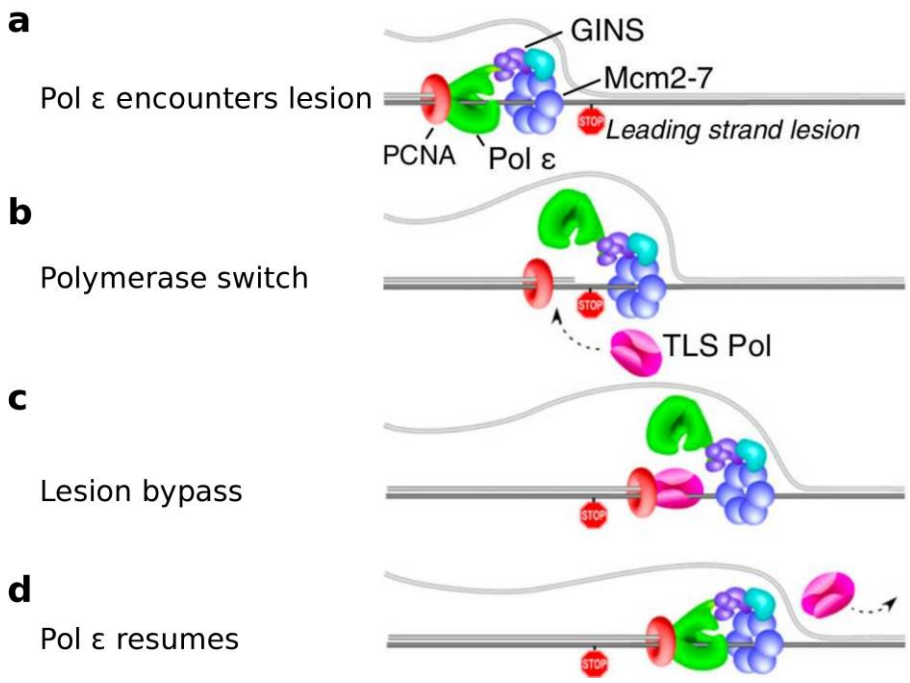

Fig. 4 Polymerase switching. Proposed model for dynamic bypass of lesions. (a) Pol ε encounters a lesion (depicted as stop sign) and cannot incorporate nucleotides opposite to the damaged template. (b) While Pol ε is retained at the elongating fork through its strong interaction with CMG a TLS polymerase binds to the PCNA left at the lesion and extends the aborted primer across the lesion. (c) After lesion bypass Pol ε rapidly replaces the TLS polymerase. (d) Normal replication resumes. Figure from Langston *et al.* (2014).