Early Career Research Award

Discontinuous leading-strand synthesis: a stop–start story

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Abstract

Reconstitution experiments using replication proteins from a number of different model organisms have firmly established that, *in vitro*, DNA replication is semi-discontinuous: continuous on the leading strand and discontinuous on the lagging strand. The mechanism by which DNA is replicated *in vivo* is less clear. In fact, there have been many observations of discontinuous replication in the absence of exogenous DNA-damaging agents. It has also been proposed that replication is discontinuous on the leading strand at least in part because of DNA lesion bypass. Several recent studies have revealed mechanistic details of pathways where replication of the leading strand introduces discontinuities. These mechanisms and their potential contributions to observations of discontinuous replication *in vivo* will be discussed.

Introduction

In the 60 years since Watson and Crick published their model for the structure of the DNA double helix [1], a vast amount has been learnt about the elegant mechanisms by which DNA is duplicated. The general features of the replication process as we know it today are remarkably similar to those first proposed at the time the structure was published [2]. DNA synthesis is initiated once the two strands of the double helix have been unwound to expose the bases that are to be copied. The unwound single strands then serve as templates to generate two identical copies of the original DNA molecule in a semi-conservative replication reaction [3].

We now know that replication is carried out by large multisubunit molecular machines called replisomes (reviewed in [4]). These enzyme complexes contain all the activities required to catalyse semi-conservative DNA replication. The polymerization of new chains of DNA begins with the *de novo* synthesis of short RNA primers that are then extended by DNA polymerases with a strict 5′→3′ directionality. This unidirectional strand extension poses a major challenge to the replication machinery given the antiparallel arrangement of the two strands of the DNA double helix. A replisome can only synthesize one nascent strand continuously, termed the leading strand, as the DNA duplex is being unwound, given that the replication fork will be moving in the opposite direction to that of polymerization on the complementary strand, the lagging strand. To circumvent this issue, the lagging strand is synthesized in short DNA (Okazaki) fragments of 200–300 bp in *Saccharomyces cerevisiae* and 1000–2000 bp in *Escherichia coli* that are subsequently ligated to form a single continuous strand.

Experimental support for the semi-discontinuous replication model described above has been provided by numerous different *in vitro* replication systems [5–8]. The model is now well established as the mechanistic basis for replisome-catalysed DNA replication. Evidence for entirely semi-discontinuous replication *in vivo* is, however, somewhat less clear. In *E. coli*, there have been many reports of discontinuous replication operating on both the leading and lagging strands, both under normal growth conditions and following the exposure of cells to DNA-damaging UV radiation. Until relatively recently, it has been difficult to accommodate these observations within current models of replisome action. The findings that the *E. coli* replisome can reinitiate leading-strand synthesis downstream from either a stalled RNA polymerase [9], or leading-strand template lesion [10,11], now provide a potential framework by which some of the evidence for discontinuous replication may be

Key words: discontinuous replication, DNA damage, DNA replication, leading-strand priming, replisome.
Abbreviations: CPD, cyclobutane pyrimidine dimer; DSB, double-strand break; NER, nucleotide excision repair; Pol, polymerase; RNAP, RNA polymerase.
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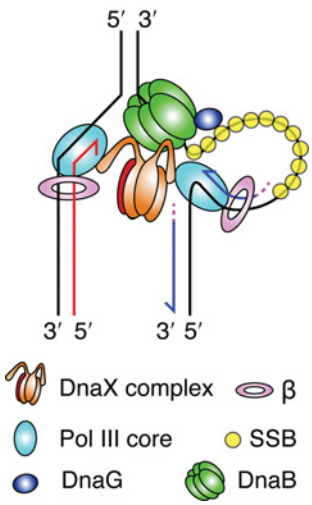
Figure 1 | Schematic representation of the *E. coli* replisome

The nascent leading strand is red and the lagging strand is blue. RNA primers on the lagging strand are represented as pink broken lines. For simplicity, the illustration shows a replisome with a DnaX complex containing two τ subunits and therefore two Pol III core complexes. Several studies indicate that replisomes can contain three copies of τ and Pol III core [17–19].



reconciled with our understanding of the mechanics of DNA replication. The present review evaluates the evidence for semi-discontinuous and discontinuous DNA replication in *E. coli*. The specific circumstances under which replication may become discontinuous are discussed, as are the putative replication mechanisms that could give rise to discontinuities in newly synthesized leading-strand DNA.

Replisome architecture and mechanism

It is necessary to first describe the architecture (Figure 1) and basic functioning of the *E. coli* replisome before discussing how the machinery responds to the obstacles that are encountered during chromosome duplication. Replication of the 4.6 Mb circular chromosome is initiated at a single origin of replication (*oriC*). The key step in replisome assembly is loading of the hexameric DnaB helicase around the lagging-strand template, on which it translocates with 5′→3′ directionality [12]. Loading is accomplished by the sequence-specific initiator protein DnaA. DnaA binding to specific sites in *oriC* leads to transient melting of the duplex to expose the ssDNA required to load DnaB from a complex with DnaC (reviewed in [13]). Once loaded, DnaB serves as the platform on to which the remaining replisome components assemble. A single DnaX complex, consisting of seven subunits ($\tau_2\gamma$ or τ_3, φ, ψ, δ and δ') binds to both DnaB and the replicative polymerase, Pol III core, via domains located in the τ subunit [14–16]. These interactions are critical for cementing the replisome together and ensuring that leading- and lagging-strand synthesis are coupled to DnaB-directed template unwinding. DnaX complexes can contain either two or three τ subunits, resulting in replisomes that contain either two or three copies of Pol III core at the replication fork [4,17–19]. The primase DnaG is responsible for repeatedly synthesizing short 10–12-nt RNA primers on the lagging-strand template that are then extended by Pol III core. The protein acts distributively [6] and is targeted to the replication fork via an interaction with DnaB [20]. In addition to its function at the heart of the replisome, the DnaX complex is responsible for assembling β clamps around newly primed DNA to enable processive primer extension by Pol III core.

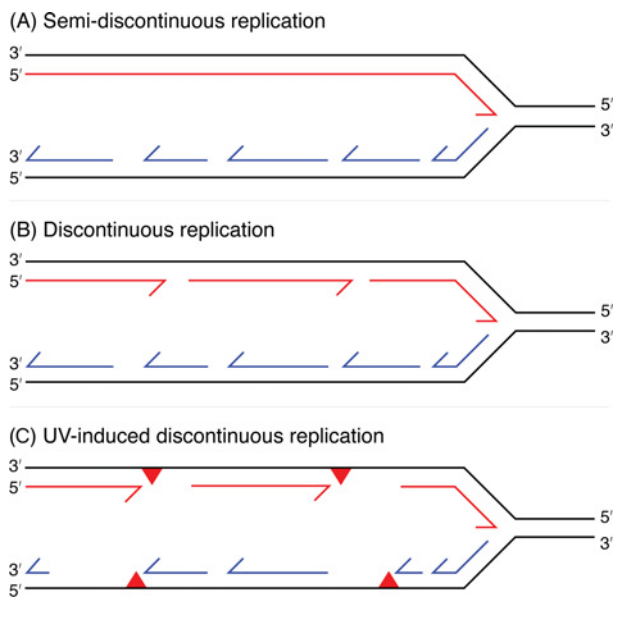
In vitro replication systems using either *oriC*-dependent replication initiation on covalently closed plasmid DNA molecules [8], or rolling-circle replication [6,21] revealed that the replisome catalyses rapid (~700 bp·s^{-1}) and highly processive semi-discontinuous replication (Figures 1 and 2A). In the rolling-circle system, leading strands in excess of 100 kb were synthesized [21], highlighting the continuous nature of leading-strand synthesis under these conditions. In contrast, lagging-strand synthesis generated much shorter products of approximately 1–2 kb. Furthermore, the size distribution of these products was influenced by the primase concentration [8], which is consistent with a discontinuous synthesis reaction that requires multiple rounds of primase-dependent initiation.

Evidence of both semi-discontinuous and discontinuous replication *in vivo*

Whereas the results of *in vitro* reconstitution experiments have shown that the *E. coli* replisome catalyses semi-discontinuous DNA replication on templates free from DNA lesions or protein obstacles, there have been numerous reports of discontinuous DNA replication *in vivo*, even in the absence of exogenous DNA-damaging agents [22–26] (Figure 2). Indeed, the pioneering work of the Okazakis and their colleagues found that almost all the nascent DNA synthesized in a variety of different *E. coli* strains consisted of short DNA fragments [27]. These experiments were conducted by pulse-labelling cells with [^{3}H]thymidine for various periods of time, gently isolating the chromosomal DNA so as to minimize strand breakages, denaturation of the DNA duplex and fractionation by alkaline sucrose gradient to determine the size of the newly synthesized ^{3}H-labelled DNA. The length of the pulse significantly influenced product size distribution. Short pulses of a few seconds resulted in fragments corresponding to 1–2 kb, whereas larger products began to appear with pulses over 10 s. These observations led to a model with discontinuous replication operating on both template strands.

Further support was lent to the discontinuous replication model by experiments conducted in both *E. coli* cells and T4 phage-infected *E. coli* cells, both of which had been disabled with respect to processing of discontinuously synthesized DNA fragments [27–29]. In both instances, all of the pulse-labelled nascent DNA fragments were small and persisted for longer than in wild-type cells, demonstrating that these small fragments were precursors for the longer

Figure 2 | Semi-discontinuous and discontinuous DNA replication
(**A**) Semi-discontinuous replication. The leading strand is synthesized continuously and the lagging strand discontinuously as Okazaki fragments. (**B**) Discontinuous replication where both nascent strands are synthesized in short fragments that will subsequently be ligated. (**C**) Several studies have indicated that replication may become discontinuous following UV irradiation. Red triangles represent polymerization-blocking lesions. Nascent strands are coloured as in Figure 1.



DNA molecules observed during extended pulses. However, ssDNA discontinuities, which would give rise to fragmented nascent DNA, can also be generated as intermediates of DNA repair processes such as NER (nucleotide excision repair), BER (base excision repair) and MMR (mismatch repair). Nicks generated during these repair processes would have the potential to preclude the detection of larger DNA fragments synthesized by continuous replication of a nascent DNA strand. Several studies attempted to address this issue by selectively inactivating individual DNA repair pathways, but no significant differences in the size distribution of nascent DNA fragments were detected between wild-type and mutant cells [23,25,30,31].

In vivo evidence for semi-discontinuous replication is also widely available. Iyer and Lark [32] found that, in addition to the small (Okazaki) DNA fragments that were routinely observed, more than 50% of pulse-labelled DNA could be isolated as high-molecular-mass DNA. Their data also suggested that this population resulted from elongation of pre-existing material in the 5′→3′ direction, consistent with continuous leading-strand synthesis. A later study [33] found a significant proportion of [^{3}H]thymidine incorporated into long DNA molecules during a pulse in wild-type *E. coli*. When an *E. coli* strain that was unable to process and ligate discontinuously synthesized DNA fragments was investigated during the same study, the large nascent DNA was no longer observed and, in addition to small DNA fragments, an intermediate species became apparent. The authors proposed that replication was semi-discontinuous in wild-type cells, but discontinuous in the mutant strain, with one strand being synthesized in larger fragments than the other [33]. Small- and intermediate-sized fragments were also detected in *in vitro* replication assays using *E. coli* whole-cell extracts that were DNA-ligase-deficient [34]. The authors were able to show that the intermediate-sized fragments were complementary to the small fragments, consistent with an asymmetry of nascent-strand lengths at the replication fork [35]. Intriguingly, similar assays using an extract carrying a temperature-sensitive DnaG allele revealed that only the population of small DNA fragments was affected by a loss of primase activity [36]. This result is consistent with a model of semi-discontinuous replication where only one strand (the lagging strand) requires repeated priming for nascent DNA synthesis.

The response of the *E. coli* replisome to UV-induced DNA damage

The *in vivo* data pertaining to the replication mechanism of *E. coli* remain somewhat conflicting, and opinion is also divided regarding how the replication machinery responds to DNA lesions. The consensus view is that lagging-strand lesions are efficiently bypassed by reinitiating Okazaki-fragment synthesis downstream from a lesion (for details of the reaction, see [37–39]). The major difference between models relates to whether leading-strand template lesions constitute an absolute block to replication fork progression, or whether replication can be reinitiated, or restarted, downstream from a lesion before its removal. Rupp and Howard-Flanders [40,41] investigated the rate of nucleotide incorporation, and the size distribution of nascent DNA synthesized, in NER-deficient *E. coli* cells following UV irradiation. Using pulse labelling, they found that DNA synthesis was inhibited by UV treatment, but, crucially, it did not cease completely in the following minutes [40]. This was even the case with UV doses sufficiently high to generate a density of CPDs (cyclobutane pyrimidine dimers) that would have resulted in the replisome encountering multiple lesions within the first 1 min after irradiation. To explain these data, the authors hypothesized that the replisome could bypass template lesions with a short delay occurring at each lesion (Figure 2C) [40]. The idea was supported further by alkaline sucrose gradient analysis of nascent DNA from UV-irradiated cells, which showed that it was considerably shorter than in unirradiated cells, the length decreasing as the dose increased [40]. The data suggested that discontinuities were present in the nascent strands synthesized on UV-damaged DNA templates and could be explained if replication was being reinitiated beyond lesions in both the leading- and lagging-strand templates. Evidence of single-stranded DNA gaps in the nascent products from UV-irradiated cells added further weight to this model [42,43] (Figure 2C).

That the replication machinery is able to reinitiate synthesis downstream of damage in both template strands has remained

controversial. This was in part due to a lack of mechanistic evidence for leading-strand template priming occurring outside the origin of replication. Furthermore, numerous studies have concluded that replication forks are arrested by the presence of DNA lesions, and that extensive replication fork processing and remodelling are required for DNA synthesis to resume. Typically, following UV irradiation of *E. coli* cells both proficient or deficient in NER, replication rates, as measured by labelled nucleotide incorporation, can be reduced by over 90 %. Replication rates then recover over a period of time that is comparable with the time taken to remove the CPDs from the chromosome [44,45]. These data have been used to support both models for replisome collisions with DNA lesions. On the one hand, as replication is not completely abrogated immediately following UV treatment, it has been argued that the replisome can replicate beyond these lesions with a delay occurring at each lesion [41]. The counter-arguments have focused on the lengthy recovery phase that occurs before normal replication rates are resumed. Numerous recombination and DNA repair proteins are required for this recovery [44,46,47]. To explain the inhibition of replication, and subsequent dependencies on these proteins for normal replication rates to be resumed, models have been developed whereby replication forks are arrested at the site of damage, then stabilized and remodelled to enable replication to proceed once the DNA lesions have been removed from the template (for comprehensive reviews of these pathways, see [46,48,49]).

Rudolph et al. [45] revisited the question of what happens to active replication forks following UV irradiation of *E. coli* cells. They showed that a large proportion of the observed nucleotide incorporation after irradiation was due to new rounds of replication initiation at *oriC.* Their data also suggested that UV-induced DnaA-independent establishment of new replication forks makes a contribution to the replication that was detected after UV irradiation. In the light of these findings, the authors used BrdU (bromodeoxyuridine) labelling and chromosome mapping to monitor the movement of pre-existing replication forks, rather than relying solely on global nucleotide incorporation. These experiments showed that movement of all pre-existing replication forks was significantly inhibited by a UV dose estimated to generate one CPD per 21 kbp (5 J/m^2) and demonstrated that replication forks are unable to advance significant distances following moderate UV exposure. Consistent with this finding, DnaC temperature-sensitive mutants that are unable to load DnaB, either at *oriC* or on to replication fork structures (see below for details), showed hardly any nucleotide incorporation following UV exposure, thus indicating that the bulk of observed nucleotide incorporation is the result of replisomes that have been reassembled, rather than existing forks [45]. Yet, despite this study providing the best evidence to date that replication forks make very little progress following UV irradiation, it does not rule out the possibility that a replisome is able to bypass DNA lesions before eventually coming to a halt. This is because the experiments were conducted by first irradiating cells and then adding labelled nucleotides. The authors acknowledge that this delay could have prevented detection of "residual synthesis associated with fork progression to the first blocking lesion" [45]. This is likely to be the case. For example, a replication fork would be expected to encounter a leading-strand CPD within 10–20 s of a UV dose of 12 J/m^2 (assuming that one CPD is generated every 9 kbp and the replisome moves at 1 kbp·s^{-1}) [45]. If, as Rupp and Howard-Flanders [40] first proposed, the replisome was able to skip over leading-strand lesions, it is certainly possible that several CPDs could have been bypassed without detection in the window between UV exposure and the initial uptake and incorporation of labelled nucleotides.

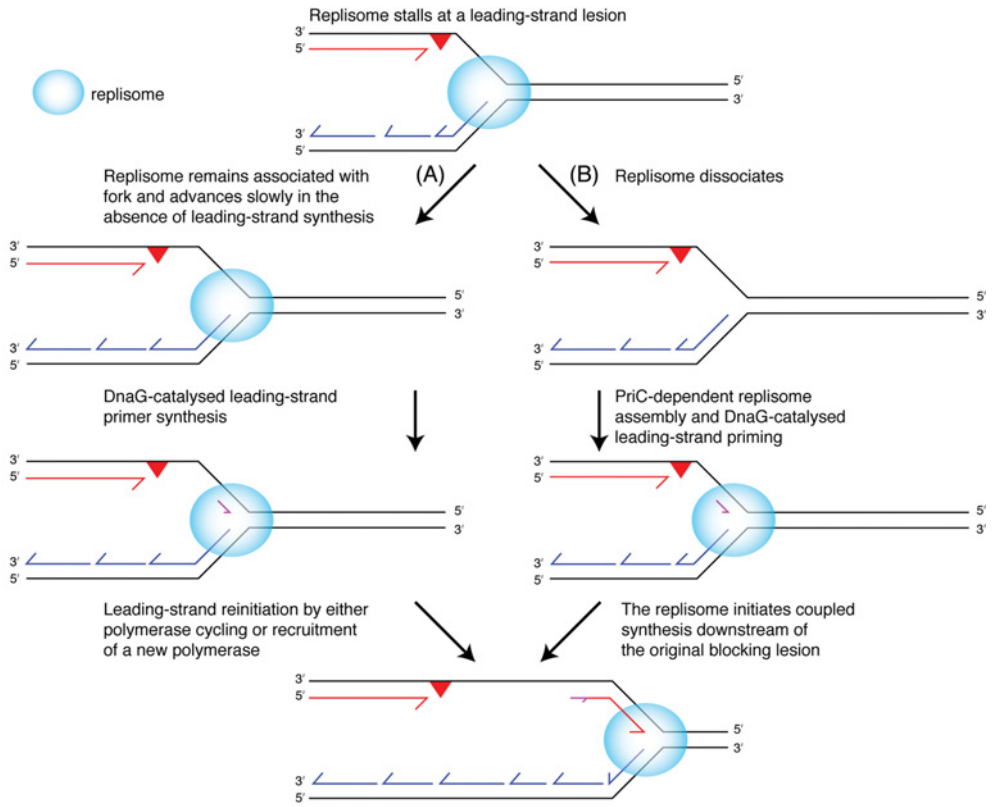
What actually arrests replication forks following UV exposure of *E. coli* cells?

The most straightforward interpretation of UV-dependent replication arrest in *E. coli* is that it is the UV-induced lesions themselves, such as CPDs, that are the replication-arresting obstacles. This assumption has formed the foundation of many models describing the fate of a replisome following UV exposure. The interpretation may, however, be an oversimplification of the situation. Transcription and DNA replication are not temporally separated in *E. coli* cells and therefore replication forks frequently encounter transcription complexes on the DNA template [50–52]. What's more, DNA regions coding for proteins and stable RNAs account for over 88 % of the genome [53], meaning that active transcription occurs across the vast majority of the chromosome. Estimates for the number of actively transcribing RNAPs (RNA polymerases) in a cell range from 400 to 9500, depending on the growth rate [54,55]. A significant proportion of UV-induced lesions are therefore likely to be encountered by a transcribing RNAP. As RNAP is stalled by DNA lesions in the template strand, forming complexes that are highly stable, it seems reasonable to consider the possibility that, following exposure of cells to UV radiation, replisomes might run into these complexes more frequently than they do naked lesions therefore complicating the interpretation that DNA lesions themselves form an absolute block to replication. There are several lines of experimental evidence that support this notion.

McGlynn and Lloyd [47] identified a strong correlation between levels of the stringent response signal molecule (p)ppGpp, which modulates the stability of RNAP, and the ability of Δ*ruvAC* cells to survive UV irradiation. These cells lack a functional RuvABC Holliday junction resolvase complex and are thus sensitive to UV light. Increased levels of (p)ppGpp dramatically enhanced survival of the Δ*ruvAC* strain, with decreased levels having the opposite effect, implying a link between RNAP stability and UV-irradiation-tolerance. This link was strengthened by the discovery of a group of suppressor mutations in RNAP that are known to destabilize transcription complexes [47,56]. The basis of this suppression was proposed to relate to the differential manner in which DNA damage is overcome depending on whether the replisome collides with a naked lesion or a stalled RNAP. That such a difference is observed is entirely consistent

Figure 3 | Lesion skipping and direct replication restart

Schematic diagram of how the *E. coli* replisome can reinitiate synthesis downstream of leading-strand lesions by damage skipping (**A**) [10,58], and direct replication restart (**B**) [57]. Reinitiating synthesis by either pathway generates a ssDNA gap in the newly replicated leading strand that must be repaired post-replicatively. Nascent strands are coloured as in Figure 1 and red triangles represent polymerization-blocking lesions.



with replication forks colliding more frequently with stalled RNAP following UV irradiation and lends support to the idea that replisome collisions with stalled transcription complexes account for at least some of the inhibition of replication seen following UV irradiation.

Replication mechanisms that give rise to leading-strand discontinuities

The *in vivo* evidence for discontinuous DNA replication in *E. coli*, in both the presence and the absence of exogenous DNA-damaging agents, clearly remains somewhat conflicting. However, the discoveries within the last 10 years of several novel replication mechanisms that generate leading-strand discontinuities following replisome collisions with either stalled RNAP or DNA damage has helped to rekindle the idea that discontinuous replication can indeed occur *in vivo*. Although such pathways are likely to occur more frequently when cells are exposed to exogenous mutagens such as UV, they would also be expected to function under normal growth conditions, where DNA lesions and stalled RNAP are still thought to be encountered by the replisome. The next section of the present review describes these mechanisms in detail and discusses the contexts within which they may be operative and the contributions that they may make to observations of discontinuous DNA replication.

Leading-strand lesion skipping

For many years, evidence for primer synthesis on the leading-strand template away from replication origins was not available. Models of DNA replication described a single priming event on the leading-strand at the origin of replication. Work from the Marians laboratory has now established that the *E. coli* primase, DnaG, can catalyse primer synthesis on the leading-strand template in a variety of different contexts (Figure 3), and that leading-strand priming can enable the replisome to effectively skip over leading-strand template lesions [10,57]. *E. coli* possesses multiple pathways to reassemble replication forks independently of the DnaA- and *oriC*-dependent pathway [11], which are discussed in detail in the next section. Using one such pathway, the PriC pathway, it was shown that the *E. coli* replisome could initiate coupled leading- and lagging-strand synthesis at a model replication fork structure lacking a nascent leading strand [57]. This observation could only be

accounted for if the leading strand was being primed *de novo* during the reaction. Leading-strand synthesis was found to be entirely dependent on DnaG and also required a functional interaction between DnaG and DnaB. This suggested that the primase was being targeted to the leading-strand template in a similar manner to which it is directed to the lagging-strand template during each cycle of Okazaki-fragment synthesis.

It has now been shown that leading-strand priming can also occur following a collision between the replisome and a leading-strand lesion [10]. The replisome was seen to transiently stall immediately after encountering a site-specific leading-strand CPD before leading-strand synthesis was resumed downstream in a DnaG-dependent manner. These experiments showed that, *in vitro,* a single leading-strand lesion does not form an absolute block to replication fork progression, and that the replisome can essentially skip over the damage and continue synthesis downstream (Figure 3A). Very recent data have shown that uncoupled replication, where template unwinding and lagging-strand synthesis occur in the absence of leading-strand synthesis, continues downstream of the damage at a reduced rate compared with normal fork movement [58]. This uncoupled synthesis exposes the region of ssDNA on the leading-strand template that is needed for primer synthesis. Primer synthesis and subsequent β-clamp assembly are then required before leading-strand synthesis can be reinitiated. The kinetics with which these reactions proceed appear to play a key role in determining how far the replisome advances before coupled synthesis is resumed and therefore they influence the size of the ssDNA gap left in the leading-strand daughter. Under less favourable conditions, the replisome was seen to frequently reach the end of a linear DNA template, up to 6 kbp downstream of the damage, without reinitiating leading-strand synthesis. Elevating the concentration of DnaG and including a functional, but non-replisome-associated, clamp loader increased the proportion of replisomes that reinitiated leading-strand synthesis before the template was completely unwound [58].

The efficiency with which the replisome skips over DNA lesions *in vivo* has not been determined. Data showing that replication fork progress is severely inhibited by UV irradiation would seem to suggest that it must be an inefficient process at best. However, as discussed above, these experiments cannot distinguish between replication forks that have arrested because they collided with a naked DNA lesion, or forks that collided with nucleoprotein complexes, such as DNA repair intermediates, or RNAPs stalled at lesions. Therefore the current data cannot exclude the possibility that both leading and lagging strand lesions are 'skipped' over *in vivo*. If the replisome is indeed capable of skipping over leading-strand damage by repriming, the leading strand will effectively be synthesized discontinuously. Such a mechanism could help to explain the longstanding observations that: (i) replication does not appear to halt completely following UV irradiation of *E. coli* [40], and (ii) that all nascent DNA synthesized after irradiation is short and contains single-stranded gaps [42]. Lesion skipping should also be functional in the absence of exogenous damaging agents, helping to ensure that replisomes are not significantly delayed when endogenously generated DNA lesions are encountered, which will be of particular importance during periods of rapid bacterial growth.

Origin-independent replisome assembly and leading-strand repriming

In the absence of leading-strand reinitiation downstream of a block, DNA unwinding and lagging-strand synthesis (uncoupled replication) can continue for considerable distances, forming a long stretch of ssDNA on the leading-strand template. How far the replisome can travel in this situation is still not fully understood. *In vitro* studies showed that uncoupled replication can proceed for many kilobases [10,37,58]. Data from an *in vivo* study suggested that uncoupled products in excess of 1 kb could be formed [59]. These experiments may actually underestimate the potential extent of uncoupled replication due to the limited length of the plasmid used in the analysis. What happens to the replisome if it fails to reinitiate synthesis on the leading strand is not known, but it has often been assumed that it will disassemble and/or dissociate, leaving the characteristic stalled replication fork structure illustrated in Figure 3, which contains an extensive region of ssDNA on the leading-strand template downstream from the blocking lesion.

Replication forks containing ssDNA gaps on the leading-strand template are the preferred substrate for PriC-catalysed replisome assembly [60] (Figure 3B). PriC directs the assembly of DnaB on to the lagging-strand template in an origin-independent assembly reaction. Approximately 20 bases of ssDNA are required for this reaction. If insufficient ssDNA is present at the fork junction, the 3′→5′ helicases Rep or PriA can unwind the 5′ end of the last Okazaki fragment to permit PriC replisome assembly [61]. Once the replisome is assembled, replication is restarted directly via leading-strand priming [57]. Direct replication restart enables coupled synthesis to resume downstream of a blockage without the prior removal of the original fork stalling lesion. The leading strand is effectively synthesized discontinuously leaving a gap behind the restarted fork that is likely to be similar to those generated following lesion skipping. Direct replication restart is another mechanism that could give rise to discontinuities in leading-strand DNA and facilitate continued replication fork progression following UV irradiation.

Does leading-strand priming occur following replisome assembly at recombination intermediates?

Replication forks that stall at DNA lesions can collapse to generate a one-ended DSB (double-strand break) [62,63]. Break processing in bacteria is catalysed by AddAB- or RecBCD-type helicase/nuclease complexes (reviewed

in [64,65]). The enzyme complexes convert the DSBs into 3′-terminated ssDNA extensions on to which RecA-nucleoprotein filaments are assembled. RecA catalyses a homology search in the intact chromosome to generate a D-loop recombination intermediate. D-loops are then targeted by PriA, which, together with PriB and DnaT, constitute a second origin-independent replisome assembly system in *E. coli* (reviewed in [66]). As with PriC-catalysed assembly, a DnaB hexamer is loaded around an exposed region of ssDNA on the lagging-strand template and a replisome is subsequently assembled. The 3′ end of the invading strand is extended by the leading-strand polymerase to enable the resumption of replication. This entire reaction has been reconstituted *in vitro* using purified proteins from *E. coli* and plasmid DNA replication templates [67]. Replication was found to be almost entirely dependent on the primase DnaG. At the time, this was a surprising finding given that DnaG was known not to be required for replisome assembly in other reconstituted systems and that the 3′ end of the invading RecA filament should have been able to prime leading-strand synthesis. The authors noted that DnaG could have been required to prime leading-strand synthesis, which now seems a likely explanation. Why leading-strand priming should be required at such D-loops is not known. Perhaps the presence of RecA inhibits the elongation of the 3′ end of the invading joint molecule leading to a requirement for leading-strand priming. Regardless of why primase was required for robust replication, the data suggest that leading-strand synthesis can be reinitiated by repriming at recombination intermediates, providing yet another potential mechanism by which the leading strand may be synthesized discontinuously.

Co-directional collisions between the replisome and RNAP may give rise to leading-strand discontinuities

Collisions between the replisome and transcribing or stalled RNAPs occur frequently during bacterial growth [51]. This is because replication and transcription are not temporally separated, the replisome moves considerably faster than RNAP, and RNAPs stall frequently during transcription elongation. The orientation of transcription relative to replication fork progression helps to limit the conflict between replication and transcription. In *E. coli*, all ribosomal RNA operons and many highly transcribed genes are co-oriented with the direction of replication. This reduces the number of head-on collisions between the replisome and RNAP, which tend to be highly deleterious as displacement of RNAP in this orientation is relatively inefficient [68]. Co-directional collisions occur frequently, especially so in highly transcribed genes, where arrays of RNAPs are traversing the genome. As such, the replisome employs multiple strategies to ensure its passage around the chromosome.

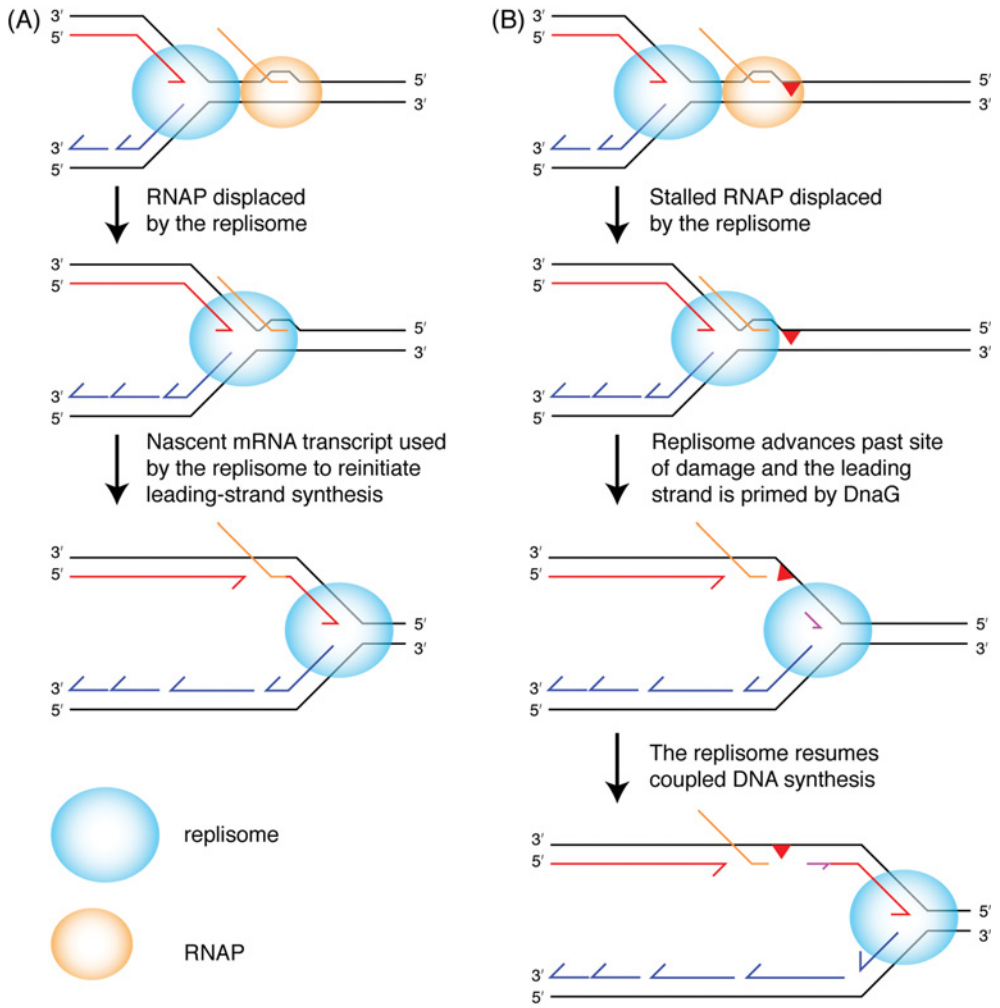
The O'Donnell laboratory demonstrated that a single RNAP, stalled co-directionally with replication fork movement, presents little obstacle to replisome progression [9] (Figure 4A). Leading-strand synthesis was arrested at the RNAP stall site, but the RNAP was swiftly displaced and the replisome then utilized the nascent mRNA transcript as a primer to reinitiate leading-strand synthesis. Consequently, the replication products contained a leading-strand discontinuity. The efficacy of this mechanism *in vivo* has not been determined, but if it does operate, it is easy to see how it could contribute to observations of discontinuous DNA replication. The experiments described above were conducted using a minimal replisome, which lacked DnaG and therefore only catalysed leading-strand synthesis. In the light of the evidence that leading-strand synthesis can be reinitiated downstream of a block by DnaG-dependent repriming [10,57], it would be interesting to discover whether such repriming could also occur following a co-directional collision with RNAP, for example if primer synthesis occurred downstream of the nascent mRNA transcript. This would be particularly useful if RNAP was stalled by DNA damage (Figure 4B) as, in this scenario, the replisome would be unable to extend the nascent mRNA due to the presence of the lesion.

Conclusions and perspectives

The *E. coli* replisome catalyses rapid and processive semi-discontinuous replication on unmodified DNA templates. Yet, because of the wide array of obstacles that the replisome must negotiate *in vivo*, it appears that replication may become discontinuous on both the leading and lagging strands. For almost 40 years, observations of discontinuous replication both under normal laboratory conditions and following exposure of *E. coli* to UV could not be reconciled with the mechanics of replisome action. The discoveries that DnaG can prime the leading-strand template away from the origin of replication [57], that the replisome can skip over leading-strand lesions by repriming, and that the replisome can utilize mRNA as a primer following a co-directional RNAP collision, provide three potential mechanisms by which replication can become discontinuous in both strands, at least temporarily. Although all three pathways are likely to occur more frequently following the introduction of DNA damage, they would still be expected to function to some extent in the absence of exogenous agents, where DNA lesions are still present and RNAPs, both stalled and elongating, are still encountered by the replisome. It is, however, questionable that the replisome encounters, and discontinuously bypasses, leading-strand obstacles with the required frequency to explain some of the observations of discontinuous synthesis, especially those that found all of the nascent DNA synthesized in *E. coli* to be of Okazaki-fragment size. Perhaps there are additional pathways that enable the replisome to synthesize the leading-strand discontinuously, or alternatively, some of these observations could have arisen due to uncharacterized DNA repair pathways or aspects of sample processing that have yet to be identified. Regardless, further work is still required to determine whether the leading-stand

Figure 4 | Leading-strand reinitiation following a co-directional replisome–RNAP collision

(**A**) The replisome displaces the stalled RNAP and then uses the nascent mRNA as a primer to reinitiate replication [9]. (**B**) RNAP stalled at a lesion is displaced by the replisome. If the replisome remains associated with the fork, leading-strand priming downstream of the damage could enable coupled synthesis to resume before damage removal. mRNA is orange and nascent DNA strands are coloured as in Figure 1 and red triangles represent polymerization-blocking lesions.



is synthesized with discontinuities in the absence of an encounter between the replisome and a DNA lesion or a stalled RNAP.

Questions remain about the fate of the replisome once it has encountered a leading-strand lesion. Whereas there are now several mechanisms that describe lesion bypass by repriming, it is not known whether or how frequently repriming occurs *in vivo*, either in the presence or absence of exogenous DNA-damaging agents. It is quite clear that replication fork progression is severely inhibited following UV exposure, but despite a wealth of data, the possibility that the replisome is able to rapidly bypass several lesions before coming to a halt cannot be excluded. Recent advances in single-cell imaging and single-molecule techniques should help us to uncover more precise details about how the replisome responds to DNA damage in the leading-strand template.

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