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Response of the bacteriophage T4 replisome to non-coding lesions and regression of a stalled replication fork

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Abstract

DNA is constantly damaged by endogenous and exogenous agents. The resulting DNA lesions have the potential to halt the progression of the replisome, possibly leading to replication fork collapse. Here, we examine the effect of a non-coding DNA lesion in either the leading or lagging strand template on the bacteriophage T4 replisome. A damaged base in the lagging strand template does not affect the progression of the replication fork. Instead, the stalled lagging strand polymerase recycles from the lesion and initiates synthesis of the new Okazaki fragment upstream from the damaged base. In contrast, when the replisome encounters a blocking lesion in the leading strand template, the replication fork only travels approximately 1 kb beyond the point of the DNA lesion before complete replication fork collapse. The primosome and lagging strand polymerase remain active during this period and an Okazaki fragment is synthesized beyond the point of the leading strand lesion. There is no evidence for a new priming event on the leading strand template. Instead, the DNA structure that is produced by the stalled replication fork is a substrate for the DNA repair helicase, UvsW. UvsW catalyzes the regression of a stalled replication fork into a “chicken foot” structure that has been postulated to be an intermediate in an error-free lesion bypass pathway.

Keywords

T4 DNA replication; lesion bypass; fork regression; UvsW

Introduction

The bacteriophage T4 replisome has proven to be an excellent model system for the study of replisome-dependent coupled DNA replication¹. Despite the variation in number and nature of individual proteins between replication systems, the components that constitute an operative replication fork are faithfully represented in other more complex replication systems². Therefore, what has been learned from the T4 replisome can be extended to the replisomes of other organisms, including higher eukaryotes³.

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The T4 replisome is made up of eight proteins, which are thought of as three sub-complexes. The leading and lagging strand holoenzymes make up two of the sub-complexes. The holoenzyme is composed of the polymerase (gp43) and the clamp protein (gp45)⁴. The trimeric clamp protein encircles the DNA duplex behind the polymerase to increase its processivity from just a few bases incorporated per single binding event to several thousand⁵. The holoenzyme can be assembled through multiple routes via the action of the clamp loader protein, which uses the energy of ATP hydrolysis to chaperone the assembly process⁶⁻⁸. The third sub-complex is known as the primosome, which moves along the lagging strand DNA template in the 5' to 3' direction⁹. The primosome is composed of a hexameric helicase (gp41) and the oligomeric primase (gp61). The helicase is loaded onto the replication fork by the action of the hexameric helicase loader protein (gp59)^{10,11}. The helicase loader also acts to coordinate the assembly of the primosome with the initiation of leading strand synthesis by directly interacting with and inhibiting the activity of the leading strand polymerase¹²⁻¹⁴. Once the helicase is loaded, the gp59-polymerase interaction is broken and gp59 is displaced from front of the replication fork prior to the initiation of DNA synthesis¹⁵. The fate of gp59 has been somewhat controversial with several lines of data indicate that gp59 remains part of the replication fork, interacting with gp32 (single-stranded DNA binding protein) coated ssDNA on the lagging strand template^{16,17}. However, the importance of this interaction is not well understood, as severe dilution of gp59 following replisome assembly has little effect on the rates of leading and lagging strand synthesis or the overall size distribution of Okazaki fragments¹⁸.

The T4 replisome appears to be the most dynamic of the characterized replisomes with the helicase being the only protein that does not exchange with solution during coupled leading and lagging strand synthesis. The clamp, clamp loader, and single-stranded binding proteins enter and leave the replisome during each cycle of Okazaki fragment synthesis^{19,20}. The primase displays moderate processivity that is at least partially controlled by the efficiency of primer handoff²¹. The leading and lagging strand polymerases are 'dynamically processive' in that they participate in an active exchange mechanism with polymerases from solution¹⁸. This active exchange appears to be mediated through an interaction between the C-terminus of the polymerase and the clamp protein. These dynamic processes are very likely related to the mechanism of Okazaki fragment synthesis, which is initiated every 2–10 seconds²².

The opposite polarities of duplex DNA requires the leading and lagging strand polymerase to move in opposing directions relative to their respective templates. However, because the lagging strand template loops back upon itself, the lagging strand polymerase remains coupled to the replication fork and travels in the same apparent direction as the leading strand polymerase^{16,17,23}. Since the lagging strand polymerase remains with the replication fork, it must repeatedly release the lagging strand template and recycle to a newly synthesized primer during each cycle of Okazaki fragment synthesis. There are two mechanisms by which the lagging strand polymerase can release from its position on the lagging strand template. The first is termed the 'collision model'. In the collision model the lagging strand polymerase releases from its DNA template after completely copying the available template and colliding with the 5' end of the previously synthesized Okazaki fragment²⁴. The second mechanism is termed the 'signaling model'^{25,26}. In the signaling model, the polymerase does not need to reach the end of the available ssDNA template. If the synthesis of the RNA primer and the subsequent loading of the clamp onto that primer preceeds the collision of the polymerase with the previous Okazaki fragment, the polymerase will release its template and leave a ssDNA gap between the point of release and the previous Okazaki fragment²⁷. It is assumed that the gap will be filled in by a polymerase recruited from solution, possibly during the normal Okazaki fragment maturation process (i.e., following RNA primer removal by RNaseH1).

In vivo, the replisome is constantly faced with potential replisome stalling agents such as DNA lesions or other DNA-bound proteins 28. DNA lesions can be generated through intrinsic mutagens such as reactive oxygen species or extrinsic mutagens such as UV light, IR, and a variety of mutagenic chemicals. These lesions or blocks have the potential to disrupt the forward progress of the replication fork. Stalled replication forks have the demonstrated potential to cause illegitimate recombination and cellular dysfunction. When the *E. coli* replisome encounters a blocking lesion in the leading strand template, the polymerases uncouple and the lagging strand polymerase remains active long enough to synthesize an Okazaki fragment past the blocking lesion^{29–31}. On the other hand, a lagging strand blocking lesion does not impede the forward progress of the *E. coli* replisome due to the recycling of the stalled lagging strand polymerase to a newly synthesized primer^{30–32}. In this report we test the response of the bacteriophage T4 replisome to non-coding lesions locating in either the leading or lagging strand template. A lesion in the leading strand template stalls the leading strand polymerase but the helicase continues to unwind duplex DNA and the primase synthesizes at least one RNA primer beyond the point of the DNA lesion. The uncoupled replisome is destabilized compared to the coupled replisome with a disassembly rate of greater than 2 min^{-1} . The DNA structure produced when the T4 replisome encounters a leading strand lesion is a substrate for the DNA repair helicase, UvsW. UvsW catalyzes the ATP-dependent regression of a well-defined DNA fork that mimics that produced by the replisome. The regression product, often termed a chicken foot, is likely a necessary intermediate in the bypass and/or repair of the leading strand lesion. In contrast to a leading strand lesion, a lesion in the lagging strand template does not impede the forward progress of the replisome. The stalled lagging strand polymerase simply recycles to a new RNA primer, likely via the signaling mechanism, to reinitiate Okazaki fragment synthesis upstream from the lesion.

Results

Replisome-mediated DNA synthesis with a lesion located in the leading strand template

To observe the response of the T4 replisome to a lesion located in the leading strand template, large DNA substrates must be used. This is because the assembly of the primosome is slow and the leading strand holoenzyme must travel an average of 0.5–1 kb before the lagging strand initiates synthesis of the first Okazaki fragment 33. For this reason, we have employed a plasmid-based substrate containing a site-specific lesion located approximately 1.3 kb away from the initiation site of DNA synthesis.

Reactions were performed using a pGEM plasmid containing no lesion, an abasic lesion (abasic_{lead}-pGEM), and a sp9 lesion (sp9_{lead}-pGEM) (Fig. 1). The abasic site was chosen because it is one of the most commonly occurring DNA lesions, whereas the sp9 lesion was chosen because it was our expectation that the nine atom spacer would completely block translesion synthesis, which would simplify product analysis. In both reactions using abasic_{lead}-pGEM or sp9_{lead}-pGEM a prominent band is observed at the expected size corresponding to the position of the introduced lesion (4.9 kb; Fig. 2). In the case of the abasic lesion, a second stall site, approximately 3.6 kb away from the first stall site is also observed. To test if the second minor stall site was due to bypass of the first stall site via translesion synthesis, we repeated the reactions using a 10-fold higher concentration of dNTPs and performed a longer time-course. If bypass of the primary stall site is due to translesion synthesis, then we would expect that translesion synthesis would become more efficient at higher concentration of dNTPs. As shown in Fig. 3, using the abasic_{lead}-pGEM substrate there are many more bypass products in the reactions performed with 500 μM dNTPs than with 50 μM , indicating that indeed the greater than 5 kb fragments are produced as a result of translesion synthesis and not by any other mechanism, consistent with previous observations³⁴. There are at least five discrete stall sites observed, representing at least four

cycles of stalling, translesion synthesis, and stalling again, leading to a product of 19.3 kb long (close to the resolution limit of the gel). Importantly, the sp9 lesion completely stalls the leading strand polymerase with no translesion synthesis observed after 12 minutes at either concentration of dNTPs.

It is clear in both reactions containing an abasic or sp9 lesion that lagging strand fragments are produced (running as a smear of less than 3.6 kb). Control reactions indicate that rNTPs and/or primase omission results in the disappearance of these products (data not shown). This indicates that the primosome has fully assembled and the lagging strand polymerase has started to produce Okazaki fragments prior to the encounter of the lesion by the leading strand polymerase. Quantitation of the leading and lagging strand products in Fig. 2 reveals that with both the abasic and sp9 lesion, there are more lagging strand products than leading strand (Fig. 4B). In standard reactions performed with non-lesion substrates, the leading and lagging strand polymerases are coupled and the amounts of leading and lagging strand are nearly equivalent (Fig. 4A). This differential increase in lagging strand products over leading strand indicates that the lagging strand DNA synthesis has gone beyond the point of the DNA lesion (i.e., the leading and lagging strand polymerases have become *uncoupled*). Since the positions of the replication initiation site and the leading strand sp9 lesion are known, we can determine the average number of nucleotides that have been incorporated into the leading and lagging strands. The number of bases incorporated into leading strand is the number of bases between the initiation site and the sp9 lesion (i.e., 1277 bases). Since the leading strand makes up 35.25% of the total replication products the total number of bases replicated is 3622 bases ($1277/0.3525$). The lagging strand total is then 2345 bases, 1068 bases of which come after the point of the DNA lesion (2345 minus 1277), which is approximately the length of a single Okazaki fragment. The above calculation assumes that all replication forks contain both leading and lagging strand holoenzymes, which appears to be valid based on the equivalent amounts of leading and lagging strand products when a lesion-free substrate it used (Fig 4A).

To confirm replisomal uncoupling, we probed the structure of the DNA products produced during a replication reaction using the sp9_{lead}-pGEM substrate. We utilized two restriction sites located on either side of the introduced DNA lesion (SapI and MfeI). Since both strands are labeled by the incorporation of dGTP [α -³²P], we can visualize the digestion products of the newly synthesized leading and lagging strands (Fig. 5A). The synthesis of an Okazaki fragment beyond the point of the leading strand lesion will produce a unique band of 1.74 kb in length when the MfeI restriction enzyme is used, whereas if lagging strand synthesis is strongly coupled with the leading strand synthesis then only a fragment greater than 3.6 kb will be observed. As shown in Fig. 5B, a band of approximately ~1.74 kb in length is readily apparent in the MfeI digested products, unequivocally demonstrating that an Okazaki fragment has been synthesized beyond the position of the leading strand sp9 lesion.

To determine the stability of the replisome stalled at a DNA lesion, we took advantage of the ability of the T4 polymerase to perform translesion synthesis across an abasic lesion. We performed a reaction where the replisome was allowed to encounter the abasic lesion under normal conditions and then performed a rapid 10-fold dilution into buffer containing all components except for the DNA helicase. Control reactions indicate that reducing the concentration of helicase to < 100 nM eliminates the formation of new replication forks (data not shown). The stability of the replisome can be measured by the appearance of translesion synthesis products after dilution. As seen in Fig. 6, dilution of the helicase virtually eliminates the appearance of translesion synthesis products, indicating that the stalled replisome is not stable enough to resume DNA synthesis following translesion synthesis by the polymerase without the need for reassembly. From this data we can

estimate that the half-life for replisome stability is less than 30 seconds and that for each translesion synthesis event the replisome must reassemble before replicating around the circular plasmid to encounter the lesion again.

Replisome-mediated DNA synthesis with lesion located in the lagging strand template

We next investigated the effect of an abasic or sp9 lesions in the lagging strand template. As shown in Fig. 7, neither the abasic or sp9 lesion causes a significant amount of stalling in the leading or lagging strand polymerases. This is consistent with a previous study that placed an abasic site near the end of the template for the first Okazaki fragment³⁴. The majority of the leading strand products are longer than the resolution of the gel and the amount of lagging strand products synthesized are comparable (i.e., leading and lagging strand synthesis remains coupled). However, a minor band corresponding to the expected length of a stalled leading strand product is present. This could be due to a small amount of linear, unligated pGEM substrate, which would give a product of similar length (since the site of the lesion and the end of the substrate would be located in the same place). However, on the basis of ethidium bromide stained agarose gels, there is no evidence that any linear plasmid exists. Another possibility is that a minor population of replisomes is highly coupled so that stalling of the lagging strand causes stalling of the leading strand as well. We ruled this out by carrying out the reaction in the absence of primase so that no lagging strand synthesis occurs. Even under this condition, the leading strand stall site is apparent (data not shown). Our favored explanation for the appearance of a stalled leading strand product is that the helicase, which travels on the lagging strand template, is not 100% efficient in translocating over the abasic and sp9 lesions and a small percent stalls or dissociates from the DNA substrate. This scenario is similar to that observed with the NPH-II helicase, which tracks along the DNA backbone, and has difficulty traversing abasic lesions³⁵.

The coupling of the leading and lagging strand synthesis indicates that the lagging strand DNA synthesis is able to continue past the DNA lesion. This could be accomplished by the recruitment of a new lagging strand polymerase while the old one remains stalled at the lagging strand lesion or by recycling of the stalled polymerase to a newly synthesized RNA primer. To test between these two possibilities, we performed another dilution/trapping experiment where free polymerase in solution is brought to extremely low levels (Fig. 8). A short 14/19 primer/template that terminates with a 3' ddCTP is used to trap any remaining polymerase that is free in solution after the dilution (the trap is in 100-fold excess over diluted polymerase). Control experiments indicate that addition of the trap prior to addition of the polymerase results in no measurable DNA replication, confirming the effectiveness of the trap (data not shown). If the lagging strand polymerase is being recruited from solution, then the dilution/trapping scheme should eliminate lagging strand synthesis. On the other hand, if the lagging strand polymerase is being recycled from the lesion, then an Okazaki fragment cannot be synthesized. As shown in Fig. 8, dilution/trapping of solution polymerase has no effect on lagging strand DNA synthesis, demonstrating that the stalled lagging strand polymerase is indeed recycled from the lesion to a newly synthesized RNA primer.

Regression of a model stalled replication fork by UvsW helicase

The DNA fork structure that results from the synthesis of an Okazaki fragment beyond the point of the DNA lesion has been suggested to be a substrate for fork regression and subsequent error-free lesion bypass³⁶. Since the T4 replisome undergoes uncoupling to produce this structure (see Fig. 5A) we tested the ability of a T4 DNA repair helicase, UvsW, to catalyze fork regression *in vitro* using a well-defined forked DNA resembling that produced by the T4 replisome (Fig. 9A). Fork regression has been previously shown to be a UvsW-dependent process both *in vivo* and *in vitro* using an *in vivo* produced DNA structure termed

the 'origin fork'³⁷. The origin fork differs slightly from the DNA structure produced when the T4 replisome encounters a leading strand lesion in that the nascent lagging strand does not extend past the end of the nascent leading strand. Consistent with the results of Long and Kreuzer, UvsW catalyzes the regression of an uncoupled DNA fork in an ATP-dependent manner³⁷; Fig. 9B). We also found that addition of the T4 ssDNA binding protein (gp32) increases the fork regression activity of UvsW by approximately 2-fold (Fig. 9C). This result is surprising since gp32 has been shown to inhibit the annealing of complementary ssDNA strands³⁸ and the ssDNA binding rate of gp32 to ssDNA is extremely rapid³⁹ (1×10^{10} to $1 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1}$). The enhancement of fork regression activity by gp32 suggests that the two arms of the nascent leading and lagging strands are being unwound and annealed in a coupled manner (i.e, gp32 is being excluded from binding to the ssDNA intermediate). In this scenario, the function of gp32 is to increase the affinity of UvsW for the DNA substrate, perhaps while bound to the ssDNA in front of the leading strand lesion.

Discussion

DNA replisomes of all organisms constantly encounter DNA lesions, which may lead to stalling and replication fork collapse²⁸. Here we have shown that the T4 replisome reacts differently to lesions located in the leading and lagging strand templates. A leading strand lesion stalls the leading strand polymerase and causes replication fork collapse (Fig. 10A), whereas a lagging strand lesion poses no threat to the forward movement of the replisome and the stalled lagging strand polymerase rapidly recycles to a newly synthesized RNA primer to initiate another round of Okazaki fragment synthesis (Fig 10B). The stalling of the leading strand polymerase does not immediately inhibit the primosome since the helicase continues to unwind dsDNA, the primase synthesizes an RNA primer, and the lagging strand polymerase produces at least one Okazaki fragment beyond the point of the leading strand lesion before replication fork collapses. The DNA structure that results from this temporary uncoupling is a substrate for the DNA repair helicase UvsW, which catalyzes the ATP-dependent regression into a "chicken foot" intermediate.

It is well-known that the leading strand polymerase and the replicative helicase are functionally coupled so that the presence of a fully active polymerase increases the rate of the helicase by up to 10-fold^{40,41}. The mere physical presence of the polymerase at the replication fork is not enough for this rate enhancement, the polymerase must incorporate dNTPs behind the moving helicase⁴⁰. Using a minicircle substrate, reducing the rate of the leading strand polymerase (by a reduction in the concentration of dGTP) reduces the rate of the helicase by a similar degree, reaching a value of ~40 bp/sec at the lowest dNTP concentration tested. The unwinding rate of the helicase alone has been determined to be 30 bp/sec⁴², suggesting that the unwinding rate of the helicase with a stalled polymerase behind it is between 30 and 40 bp/sec. The data presented here indicates that at least one Okazaki fragment is produced beyond the point of the DNA lesion, demonstrating that the primosome is able to function at a much less than optimal rate (30–40 bp/sec). However, the replisome as a whole is much more unstable when the leading strand polymerase is stalled at a lesion, indicating that the primosome cannot continue indefinitely without an actively replicating leading strand polymerase. We cannot unequivocally conclude if a particular component or the entire replisome disassembles after reaching a leading strand lesion but Alberts and colleagues has shown that the holoenzyme in isolation is extremely stable when stalled due to nucleotide omission⁴³. Additionally, single-molecule assays indicate that the T4 helicase alone has an average processivity ranging between 100 and 800 bp⁴², similar to the number of bases incorporated into the lagging strand beyond the leading strand lesion determined here. These two observations lead us to conclude that it is very likely that the helicase is the first component of the stalled/uncoupled replisome to disassemble. Moreover,

it is probable that it is this instability that limits continued lagging strand synthesis to only a single Okazaki fragment beyond the DNA lesion.

In contrast to the T4 replisome studied here, the *E. coli* replisome has been shown to highly stable when stalled at a leading strand lesion²⁹. A possible reason for this difference may lie in the differing ways that *E. coli* and T4 bypass DNA lesions. *E. coli* uses a specialized translesion polymerase to bypass non-coding lesions such as abasic sites⁴⁴. In recent years, a tool-belt model has emerged where the replicative and translesion polymerases simultaneously bind to the sliding clamp during normal DNA replication^{45,46}. When a lesion is encountered, the translesion polymerase switches places with the replicative polymerase and incorporates a very limited number of nucleotides at the site of the DNA lesion. After translesion synthesis, the high-fidelity replicative polymerase trades places with the translesion polymerase and presumably, due to the high stability of the replisome, coupled leading and lagging strand DNA synthesis resumes. Bacteriophage T4 codes for a single DNA polymerase, gp43, and no evidence exists for host cell translesion polymerases participating in T4 phage genomic replication⁴⁷. As a consequence of this, T4 phage is likely to be completely dependent on recombination-based pathways for the bypass and restart of replisome-dependent DNA synthesis (e.g. UvsW-catalyzed DNA fork regression). Considering this, it would be advantageous to have relatively rapid replisome disassembly when the leading strand polymerase stalls at a DNA lesion.

The non-coding sp9 lesion used here results in absolutely no leading strand DNA synthesis beyond the lesion. This result is in contrast to what would be expected if, like the *E. coli* replisome, the T4 replisome can restart DNA synthesis on the leading strand through primosome-mediated priming^{48,49}. It is unclear why the T4 replisome does not have this ability; however, it may be related to the very different situations that accompany the replication of *E. coli* and T4 genomic DNA. *E. coli* contains a single circular genome that must be completely copied prior to cell division. For this reason, it is likely that the *E. coli* replisome has been optimized for speed of completion. The ability to restart replication on the leading strand through DnaB-DnaG mediated priming allows leading strand ssDNA gaps that are left behind to be repaired in parallel. On the other hand, there are many copies of the T4 genome present in the host cell at all but the earliest stages of phage infection. A delay in replicating a single genomic copy should not pose as great of a constraint on the success of phage genomic replication when multiple copies are present.

In contrast to a lesion in the leading strand template, a lagging strand template lesion has no apparent affect on the leading strand polymerase or on continued DNA synthesis by the lagging strand polymerase. The data presented here indicates that the lagging strand polymerase simply recycles from the lagging strand lesion to a newly synthesized RNA primer to initiate another round of Okazaki fragment synthesis. While the mechanism for recycling the stalled lagging polymerase from the lesion is not completely certain, it very likely uses an identical mechanism to what is referred to as the 'signaling mechanism' (Fig. 10B). In T4 phage, the signaling mechanism relies on the loading of the clamp protein onto the newly synthesized RNA primer to signal the release of the lagging strand polymerase and its recycling to the clamp-loaded primer. We propose that one of the features of the signaling mechanism is to enable the lagging strand polymerase, which normally would have a high degree of stability at a non-coding lesion, to release from the site of the DNA lesion and continue to travel with the replication fork as normal.

The presence of an Okazaki fragment beyond the point of the leading strand lesion leaves open the possibility of an intramolecular "template switch" mechanism for lesion bypass^{36,38}. It was for this reason we tested the ability of UvsW to catalyze fork regression on a large substrate designed to mimic the uncoupled DNA fork produced by the T4

replisome. It was recently shown by Long and Kreuzer that UvsW promotes fork regression using a coupled replication fork where the nascent leading strand was extended past the position of the nascent lagging strand 37. Here, we demonstrate that the substrate specificity of UvsW is flexible enough to handle both types of stalled replication fork. However, based on recent results from the Kreuzer laboratory, while fork regression appears to be the dominant mechanism for the processing of a stalled replication fork, it is unlikely that the chicken foot structure is used to directly bypass the lesion. Instead, the chicken foot is a substrate for nucleolytic degradation by either the T4 Mre11/Rad50 homologs (gp46/47) or the Holliday junction endonuclease (gp49). In this scenario, the purpose of fork regression is to move the DNA lesion back into duplex DNA where it can be repaired through conventional pathways (e.g., base and nucleotide excision repair).

In general, the results reported here using the bacteriophage T4 system are in accordance with those obtained using the *E. coli* replisome 29·32·30·31. This similarity in mechanism highlights the conservation of fundamental properties between replisomes of all organisms and supports the notion that the bacteriophage T4 replisome is an excellent model for the investigation of DNA replication. Through this work and others, it has become clear that the T4 replisome is dynamic system with several proteins entering and leaving the complex during active DNA replication. It is likely that this flexibility has evolved, at least in part, to deal with DNA lesions and road blocks that would potentially interfere with the completion of DNA replication.

Materials and Methods

[α -³²P] dGTP, was purchased from PerkinElmer Life Sciences. Unlabeled ribonucleotides were purchased from Roche Applied Science. Bacteriophage T4 replication proteins gp41, gp61, gp43 exo⁻, gp44/62, gp45, gp32, and primase trap protein were purified as described previously 50·51. The sequence of the oligonucleotides used to make the lesion containing cassettes were: 5'-GGCCTAGCGATCAXTGAATCAG-3' (top strand, where X denotes either the abasic or sp9 lesion) and 5'-GCGCCTGATTCACTGATCGCTA-3' (bottom strand)

Construction of DNA lesion containing substrates

A large dsDNA substrate containing either an abasic or sp9 lesion was constructed via cassette ligation (Fig. 1). A total of 1 milligram of pGEM_nick plasmid 14 was digested with 200 units each of MluI and NotI enzymes in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, and 10 mM DTT in a volume of 1 ml for 15 hours at 37°C (a 10-fold over-digest). The digestion reaction was heat denatured at 65°C for 30 minutes followed by a 50-fold dilution into the ligation buffer containing a 10-fold molar excess of the 5' phosphorylated dsDNA cassette containing either abasic or sp9 lesions and T4 DNA ligase (100 Weiss units). The ligation reaction was allowed to proceed for 24 hours at room temperature. The ligation reaction was then concentrated in an YM-30 centricon and loaded onto a 1% TAE-agarose gel containing 10 mg/ml ethidium bromide (to promote supercoiling of the ligated plasmid). The supercoiled band was gel extracted using Qiagen gel extraction kit. Finally, commercially available nicking enzymes (New England Biolabs) were used to create initiation sites for DNA synthesis. The enzyme Nb.BbCvI introduces a nick such that the lesion is located in the leading strand template, whereas when Nt.BbCvI is used the lesion is located in the lagging strand template. The nicking site is approximately equidistant from the site of the DNA lesion in either direction. The overall yield for the lesion containing substrate synthesis was 2.1%.

Replisome-mediated DNA synthesis

Replication reactions were carried out in a standard replication buffer [25 mM Tris acetate (pH 7.8), 125 mM KOAc, and 10 mM Mg(OAc)₂] at 37°C. The standard conditions used to assemble and initiate the replication reaction consisted of 2 nM lesion substrate, 200 nM each of gp43, gp45 (as trimer), and gp44/62, 600 nM each of monomer gp41, gp61, and gp59, 4 μM of gp32, 100 μM each of CTP, GTP, and UTP, 2 mM ATP, 50 μM each of dATP, dGTP, dCTP, and dTTP with 25 μCi of [α -³²P] dGTP, in a typical reaction volume of 45 μl. In the polymerase dilution/trapping experiment (Fig. 8), the reaction was diluted 10-fold into buffer containing all components at standard concentrations except for the helicase and polymerase with a 14/19 trap concentration of 2 μM. 10 μl aliquots were removed at the indicated time points and quenched with a half volume of 0.5 M EDTA. The quenched reactions were analyzed with alkaline agarose gel electrophoresis. The quenched reactions were run at 40 V for 24 hours in 30 mM NaOH and 1 mM EDTA buffer. The gel was dried and exposed overnight to a phosphorimager plate and analyzed using a Molecular Dynamics Storm 800 Phosphorimager system (Amersham Biosciences).

To determine the stability of the stalled replisome, a replication reaction using the pGEM_nick substrate with an abasic lesion in the leading strand template was initiated in with the standard conditions but at 20 seconds after the start of the reaction, the reaction was diluted 10-fold into buffer containing all components except gp41. Control reactions indicate that at 60 nM helicase no new replication form (data not shown). Reactions were processed and analyzed as above except that the volume loaded onto the alkaline agarose gel was doubled.

Restriction enzyme digest to determine degree of replisome uncoupling

DNA synthesis reactions under the standard conditions were carried out using the pGEM_nick plasmid containing an sp9 lesion in the leading strand template. After the reaction had been allowed to proceed for 12 minutes, it was quenched in Qiagen PB buffer and purified using a Qiaquick PCR purification column. The purified reaction was then treated with either SapI or MfeI for 60 minutes at 37°C. Reactions were quenched with EDTA and loaded onto a 1% TBE-agarose gel. The gel was run for 4 hours at 80V, followed by drying onto DEAE paper and exposing to the storage phosphor screen.

UvsW catalyzed regression of a model replication fork

The model uncoupled replication fork was constructed as described⁵². The pG68 and pG46 plasmids were generously provided by Dr. Leonard Wu (University of Oxford, UK). The basis of this assay is the movement of the EcoRI restriction sites from the plasmid arms into the regressed arm of the DNA fork, allowing regression to be monitored by the appearance of an 86 bp digestion product (see Fig. 9). The fork regression reactions were carried out at 37°C in the standard replication buffer containing 0.2 mM ATP, 2 nM DNA, and UvsW concentrations indicated in the figure legend. The reaction was initiated by the addition of UvsW and allowed to proceed for 15 minutes before quenching by the addition of 1 mM ATP γ S and 10 μM ssM13 DNA (bases). EcoRI restriction enzyme was then added to the quenched reaction and the digest was carried out for 1 hour at 37°C. The digestion products were analyzed using 10% polyacrylamide electrophoresis in 1X TBE (35 mA for 2 hours), exposed overnight to a phosphorimager plate, and analyzed using a Molecular Dynamics Storm 800 Phosphorimager system (Amersham Biosciences).

Abbreviations

kb   kilobasepairs

ssDNA	single-stranded DNA
dsDNA	double-stranded DNA
rNTP	ribonucleotide triphosphate
T4	bacteriophage T4

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Figure 1. Design of plasmid-based lesion containing leading and lagging strand template lesions
 (A) The pGEM-ori(Y) vector was mutated to add a BbvCI nicking site at position 1890, which serves as the initiation site for DNA replication. The plasmid is then digested with MluI and NotI restriction enzymes, followed by ligation of a dsDNA cassette containing either abasic (B) or sp9 (C) lesions. The ligated product is gel purified and treated with either Nb.BbvCI or Nt.BbvCI to place the lesion in the leading and lagging strand templates, respectively.

Figure 2. DNA replication products with lesions located in the leading strand template

The synthesis products are separated via alkaline agarose electrophoresis. Reactions performed with the nicked pGEM plasmid containing no lesion (pGEM), an abasic lesion (abasic_{lead}-pGEM) and an sp9 lesion (sp_{lead}-pGEM) are shown. The four lanes for each reaction are time points of 0.5, 1, 1.5, and 2 minutes. Due to the rolling circle nature of the non-lesion containing substrate, many more DNA synthesis products are produced, therefore the signal is much higher and the exposure has been decreased compared to the abasic and sp9 substrates.

Figure 3. DNA replication products with lesions located in the leading strand template at normal and high concentrations of dNTPs

The replication reactions with abasic_{lead}-pGEM and sp9_{lead}-pGEM DNA substrates were carried out using the dNTP concentrations indicated on the figure at time points of 3, 6, 9, and 12 minutes.

Figure 4. Quantification of leading and lagging strand replication products produced in reactions with pGEM (A) or sp⁹lead-pGEM (B)

The reactions were carried out as described in “Materials and Methods”. The error bars represent the standard error for three individual time points. Below each chart is a cartoon depicting the type of fork structure that produce the observed levels of leading and lagging strand DNA synthesis. The black, blue, and red colored lines represent the template, nascent leading strand, and nascent lagging strand, respectively.

A

uncoupled

SapI = 0.97 kb + ~3.6 kb

MfeI = 1.74 kb + > 3.6 kb

coupled

SapI = 0.97 kb + ~3.6 kb

MfeI = >3.6 kb?

Figure 5. Restriction digest assay to determine uncoupling of replication fork

(A) A schematic showing the position of restriction sites in relation to the leading strand sp9 lesion. The black, blue, and red colored lines represent the template, nascent leading strand, and nascent lagging strand, respectively. If the replisome becomes uncoupled and the nascent lagging strand extends past the nascent leading strand, then treatment of the replicated product with the MfeI restriction enzyme should produce two fragments with apparent mobilities of 1.8 kb and greater than 3.6 kb. If the replisome is coupled and the

nascent lagging strand stops before or at the same point as the leading strand, then treatment of the replicated product with the MfeI restriction enzyme will only produce a single product with an apparent mobility greater than 3.6 kb. Treatment with SapI should produce 0.9 kb and 3.6 kb fragments for both coupled and uncoupled DNA replication products. (B) Native gel electrophoresis of MfeI and SapI restriction digestion products. The open circle, linear, 1.8 kb, and 0.9 kb fragments are circled.

Figure 7. DNA replication products with lesions located in the lagging strand template
 Reactions were carried out using standard conditions as described in “Materials and Methods”. Reactions performed with the abasic_{lag}-pGEM and sp9_{lag}-pGEM DNA substrates are shown (for the pGEM reaction see Fig. 2). The four lanes for each reaction are time points of 3, 6, 9, and 12 minutes.

Figure 8. Recycling of lagging strand polymerase

(A) A schematic illustrating the assembly and dilution scheme used to determine the recycling properties of the lagging strand polymerase. Q represents quench. Details can be found in the text. (B) Reactions were carried out as described in "Materials and Methods". The products of DNA replication using pGEM and sp9_{lag}-pGEM substrates analyzed using alkaline agarose electrophoresis. The four lanes for each reaction are time points of 3, 6, 9, and 12 minutes.

Figure 9. ATP-dependent UvsW-catalyzed DNA fork regression

(A) A Schematic of the DNA substrate used to monitor fork regression. The asterisk represents a 5' ^{32} P label. The black, blue, and red colored lines represent the template, nascent leading strand, and nascent lagging strand, respectively. (B) A native PAGE gel of the fork regression reactions. Reactions were carried out as described in "Materials and Methods". Lanes 1 through 4 contain ATP/EcoRI, UvsW/ATP/EcoRI, UvsW/EcoRI, and UvsW/ATP, respectively. (C) A plot of percent forks regressed versus concentration of UvsW without (black diamonds) and with the T4 SSB (gp32) (black squares).

Figure 10. Proposed models for the response of the T4 replisome to lesions in the leading and lagging strand templates

(A) An illustration depicting the action of the replisome upon encountering a leading strand lesion. The leading strand polymerase stalls at the lesion, the helicase travels past the lesion, the primase synthesizes an RNA primer, and the clamp protein is loaded onto that primer. The stalling of the leading strand polymerase, combined with the continued travel of the helicase causes the leading strand template to loop out, similar to what occurs on the lagging strand during normal Okazaki fragment synthesis. Presumably, the leading and lagging strand loops are rapidly coated with gp32 SSB, which prevents the annealing of the loops. Loading of the clamp onto the RNA primer triggers the lagging strand polymerase to recycle

and initiate the synthesis of a new Okazaki fragment beyond the point of the DNA lesion. Following Okazaki fragment synthesis the replication fork becomes unstable and the replication fork collapses. (B) An illustration depicting the action of the replisome upon encountering a lagging strand lesion. The most helicases travel past the lesion and the primase synthesizes a primer, followed by clamp loading. The lagging strand polymerase recycles and begins the synthesis of an Okazaki fragment. When the lagging strand polymerase stalls when it reaches the lesion but the helicase/primase and leading strand polymerase continue uninterrupted. When the primase synthesizes another RNA primer and the clamp is successfully loaded, the stalled lagging strand polymerase recycles away from the DNA lesion and initiates synthesis of a new Okazaki fragment. The replication fork continues and only a ssDNA gap is left behind.