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# Choreography of bacteriophage T7 DNA replication

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The replication system of phage T7 provides a model for DNA replication. Biochemical, structural, and single-molecule analyses together provide insight into replisome mechanics. A complex of polymerase, a processivity factor, and helicase mediates leading strand synthesis. Establishment of the complex requires an interaction of the C-terminal tail of the helicase with the polymerase. During synthesis the complex is stabilized by other interactions to provide for a processivity of 5 kilobase (kb). The C-terminal tail also interacts with a distinct region of the polymerase to captures dissociating polymerase to increase the processivity to >17 kb. The lagging strand is synthesized discontinuously within a loop that forms and resolves during each cycle of Okazaki fragment synthesis. The synthesis of a primer as well as the termination of a fragment signal loop resolution.

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## Introduction

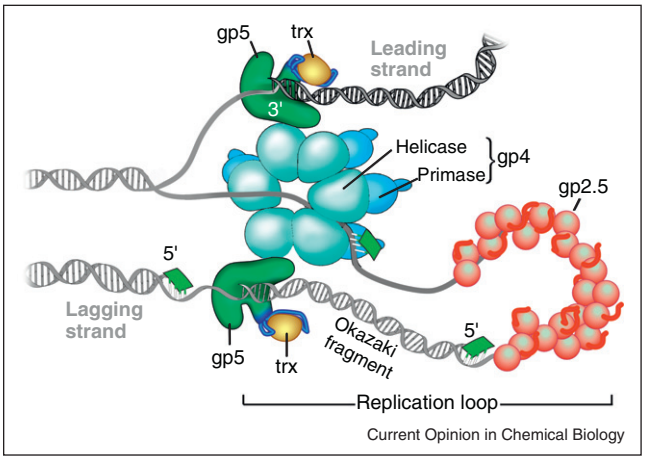
Bacteriophage T7 is a lytic phage that infects *Escherichia coli*. Its genome of 40 kilobase (kb) encodes approximately 50 proteins of which three, along with one host protein, can constitute a functioning replisome. The T7 replisome mediates coordinated DNA synthesis in a manner that mimics the fundamental steps found in higher organisms [1,2••]. The helicase domain of the hexameric gene 4 protein (gp4) unwinds dsDNA to provide a ssDNA template for gene 5 DNA polymerase (gp5) (Figure 1). The leading strand is synthesized continuously by gp5 in complex with its processivity factor *E. coli* thioredoxin (trx). The lagging strand is synthesized discontinuously to yield Okazaki fragments that are processed to form a continuous strand. The synthesis of each Okazaki fragment is initiated by the extension of tetraribonucleotides synthesized by the primase domain of gp4. The nascent Okazaki fragments are found within a replication loop that undergoes multiple rounds of formation and resolution during each cycle of synthesis of an Okazaki fragment. The gene 2.5 ssDNA-binding protein (gp2.5) is essential for protection of ssDNA produced during replication and for coordination of the synthesis of both strands [3]. The T7 replisome is a dynamic entity wherein each step is coordinated and proteins continuously enter and exit.

Other DNA replication systems are generally more complicated than the T7 system. Helicase and primase are found in separate proteins although they must physically interact to properly function. Additional proteins in these systems include processivity clamps, loading proteins for the helicase and processivity factor, and proofreading exonucleases. How does the T7 replisome function with only four proteins whereas others require far more? It is clear that the four proteins of the T7 replisome have usurped many of these functions. The limited number of proteins has made possible: (i) reconstitution of a replisome [2••], (ii) determination of the crystal structures of the proteins [4–6], and (iii) visualization of active replisomes by single-molecule techniques [7•,8•]. A more detailed review of T7 DNA replication is available [2••].

## Processivity of DNA polymerase

The binding of trx to a flexible loop (thioredoxin binding domain, TBD) located at the tip of the thumb of gp5 increases the rate of dNTP incorporation by 20–80-fold to yield a processivity of approximately 800 nucleotides (nt) per binding event [9–12]. Unlike ring-shaped processivity factors that encircle DNA, binding of trx to the TBD in gp5 reorganizes this flexible region to better grasp the DNA. Comparative analysis of gp5 and gp5/trx by small angular X-ray scattering and exonuclease footprinting show that the binding of trx increases protein surface interactions with the duplex portion of the primer-template [13•]. The extended surface renders gp5/trx more resistant to salt and facilitates sliding of the DNA in the binding cleft. These adjustments induced by trx binding most probably create a closed conformation of the binding crevice rather than the open complex shown in the crystallographic structure [4]. Single-molecule analyses reveal the enhancement of gp5 sliding on DNA by trx [14]. While gp5 alone frequently dissociates from dsDNA and rebinds, the binding of trx to gp5 suppresses such microscopic hopping so that gp5/trx can slide on the DNA in search for a 3′-terminus. The processivity of 800 nt resulting from the binding of trx does not explain the high processivity of >17 kb observed during movement of the

**Figure 1**




Model of the T7 replisome. The hexameric helicase domain of gp4, encircling the lagging strand, unwinds the duplex DNA to provide two single-stranded DNA templates. Gp5/trx bound to the helicase catalyzes the polymerization of nucleotides on the leading strand. The ssDNA extruded by the translocating helicase forms a replication loop in which the primase domain of gp4 catalyzes the synthesis of a tetraribonucleotide (green) to serve as a primer for the lagging strand gp5/trx that is also bound to the helicase. Gp2.5 coats the exposed ssDNA.

replisome. As discussed below this enhanced processivity derives from two modes of gp5/trx-helicase interaction.

## DNA polymerase–helicase interactions for leading strand synthesis

T7 DNA polymerase interacts with DNA helicase, an essential interaction to coordinate nucleotide polymerization with unwinding of the DNA. T7 gp4 is present as a mixture of hexamers and heptamers in the absence of DNA but assembles onto ssDNA as a hexamer, raising the possibility that loading onto DNA involves the loss of a subunit [15,16]. In other replication systems a helicase loading protein is required for the assembly of the helicase. Once loaded onto ssDNA the helicase translocates 5′ to 3′ using the energy of hydrolysis of dTTP. Upon encountering duplex DNA its continued movement unwinds the duplex. Structural determinants in the nucleotide and the nucleotide binding pocket of gp4 responsible for the dTTP requirement have been identified [17,18].

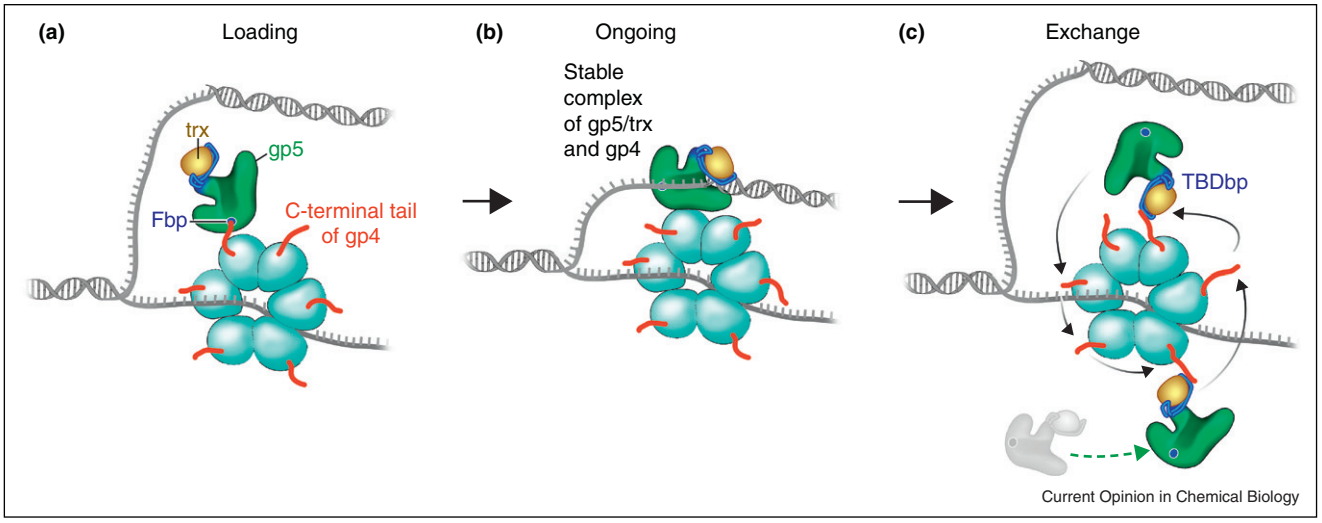
Gp4 has an acidic C-terminal tail that has multiple interactions with gp5/trx. One of these interactions is essential for the initiation of leading strand synthesis together with gp5/trx. A solvent exposed patch consisting of four basic amino acids is located on the surface of gp5 facing the duplex region of the DNA at a replication fork [4]. This basic patch is adjacent to the template strand as it exits the active site of gp5. Neutralization of the basic charges eliminates the ability of the polymerase to mediate strand-displacement synthesis with gp4 [19]. Binding studies show that altered polymerase is defective in binding to gp4 and that the acidic C-terminal tail of gp4 is involved in this interaction. However, the altered polymerase is able to replace wild-type gp5 once DNA synthesis is underway for strand-displacement synthesis. The ability of DNA polymerase in solution to exchange with the replicating DNA polymerase is medicated by interaction between the C-terminal tail of gp4 and the TBD as discussed below. Since the basic patch in the TBD is retained in the altered polymerase, it can bind to the C-terminal tail of gp4 and then exchange with the replicating DNA polymerase. The results suggest that the interaction between the C-terminal tail of the helicase and the basic patch of DNA polymerase is crucial for loading of gp5/trx onto the replication fork to initiate strand-displacement synthesis (Figure 2a).

The activities of both polymerase and helicase are reciprocally enhanced by their interactions. Unwinding of dsDNA by gp4 is 10-fold slower than translocation on ssDNA as measured by pre-steady state kinetics [20]. DNA synthesis catalyzed by gp5/trx at the replication fork increases the rate of unwinding to 114 base pairs per second, equivalent to that observed for translocation of gp4 on ssDNA [21]. Gp5/trx not only destabilizes the duplex to facilitate forward Brownian push of the helicase but it also prevents backward slipping of the helicase by synthesizing the complementary strand [21,22]. Gp4 variants defective in ssDNA binding as well as unwinding can mediate efficient strand-displacement synthesis in the presence of gp5/trx, thus confirming the supporting role of gp5/trx [23].

Interactions with gp4 enable gp5/trx to increase its processivity from incorporation of about 800 nt on ssDNA template per binding event to >17 kb during leading strand DNA synthesis on duplex DNA [7•]. A major source for the increased processivity is the interaction that couples helicase unwinding to the polymerization of nucleotides by gp5/trx (Figure 2b). This interaction occurs only when gp5/trx and gp4 are functioning together during leading strand DNA synthesis. This stable interaction can be observed by surface plasmon resonance when gp5/trx is bound to a primer-template in the presence of the dideoxynucleoside 5′-triphosphate corresponding to the next incoming dNTP [24]. The sites of this interaction have not been identified but it does not involve the acidic C-terminal tail of gp4. This stable interaction provides for a processivity of around 5 kb [25•].

In addition to the two modes of binding of gp5/trx and gp4 aforementioned – a mode for loading and the one for ongoing DNA synthesis – a third mode of interaction allows for exchange of DNA polymerases during DNA synthesis (Figure 2c). The C-terminal tail of gp4 interacts

**Figure 2**




Three modes of interactions between gp4 and gp5/trx during leading strand synthesis. **(a)** Gp5/trx is loaded onto the hexameric gp4 through an interaction of the acidic C-terminal tail of gp4 with a basic patch at the front side (Fbp) of gp5. **(b)** A high affinity complex between the two proteins coordinates their activities during DNA synthesis. **(c)** Gp5/trx temporarily dissociated from gp4 or present in solution is retained with gp4 through an interaction between two basic loops in the trx binding domain (TBDbp) of gp5 and the acidic C-terminal tail of gp4.

not only with the basic patch adjacent to the template, as in the loading mode, but also with two basic loops located within the TBD of gp5 [24]. These two solvent exposed loops are partially configured by the binding of trx to the TBD. In addition, one lysine in trx located in proximity to one of the two loops also contributes to the interaction [26].

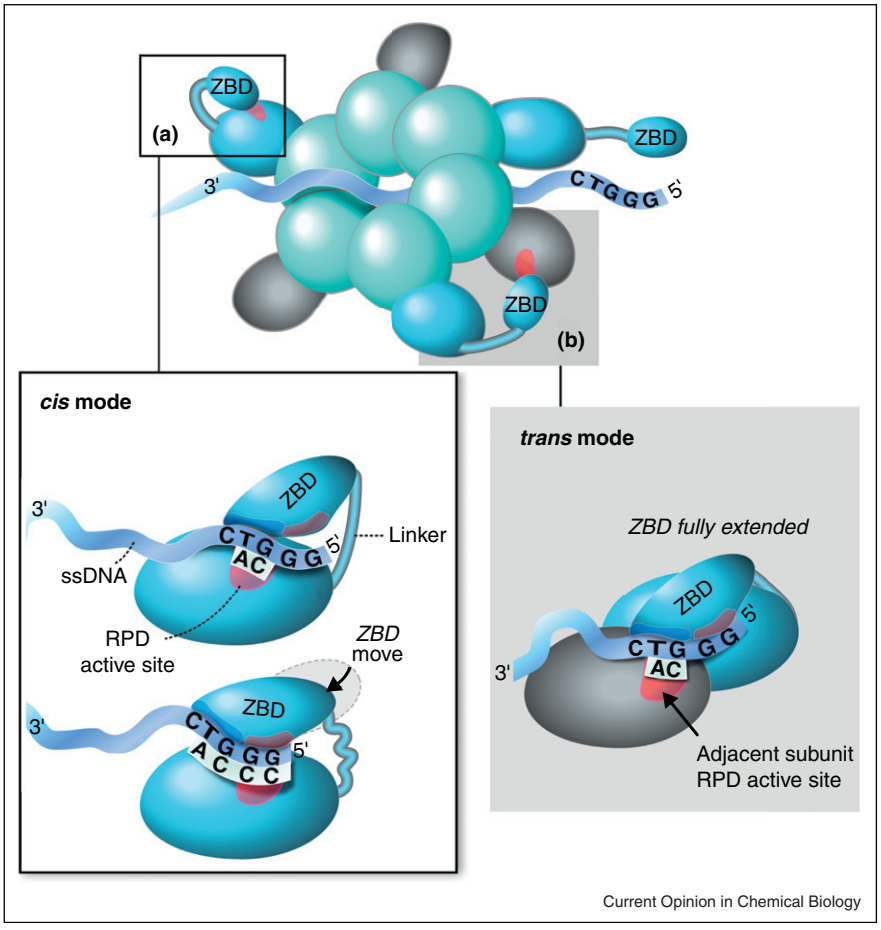
The C-terminal tail of gp4 binds to these two loops and provides for backup DNA polymerase in the event the replicating gp5/trx dissociates. Elimination of the TBD in gp5 decreases the binding affinity to gp4 by 90-fold [24]. This interaction also allows for the capture of the replicating gp5/trx upon dissociation and its return to the primer-template. The ability of the hexameric gp4 to retain the dissociating replicating polymerase increases the processivity from 5 kb to >17 kb [25•]. Abolishment of the charges in the two loops or removal of the C-terminal tail of gp4 reduces the normal high processivity to 5 kb as measured by single-molecule techniques [25•]. Gp4 allows for the exchange of gp5/trx in solution with the replicating gp5/trx at the replication fork without affecting processivity. In the cell there is most probably an abundance of DNA polymerase thus assuring that there is always a polymerase bound to the helicase ready for exchange in the event the replicating polymerase dissociates. Exchange of gp5/trx in the T7 replisome was initially demonstrated in ensemble experiments [27] and later confirmed quantitatively using single-molecule measurements [28].

## Synthesis and delivery of primers during lagging strand synthesis

Synthesis of the lagging strand requires the synthesis of oligoribonucleotides by the primase domain of gp4. These oligoribonucleotides are then used as primers to initiate the synthesis of Okazaki fragments. Like other prokaryotic primases, T7 primase catalyzes the synthesis of short oligoribonucleotides from specific sequences (primase recognition sites) on the template [29]. At the basic recognition sequence 5′-GTC-3′ T7 primase synthesizes the diribonucleotide 5′-pppAC-3′ that is then extended to the functional tetraribonucleotide 5′-pppACCA, pppACCC, or pppACAC-3′ provided the complementary sequence is present in the template [30] (Figure 3a). A stable complex between the primase and its recognition sequence is formed only when the ribonucleotides necessary for tetraribonucleotide formation are present [31•]. Consistent with previous kinetic data [32], the binding affinity is relatively weak (Kd ~20 μM). Although the primase binds to its recognition sequence in the presence of preformed tetraribonucleotides, its binding is less stringent than with ribonucleotides. The results suggest that conformational changes that occur during synthesis of functional primers enable the primase to form a specific complex with DNA.

The interaction between the primase and the hexameric helicase results in a structure in which the primase domain of one subunit interacts with that of an adjacent subunit (Figure 3b). This arrangement allows the zinc-binding motif of one subunit of gp4 to interact with the

**Figure 3**




Two distinct modes involved in primer synthesis by the primase domain of gp4. Synthesis of tetraribonucleotides at the primase recognition sequence requires interactions between the zinc-binding domain (ZBD) and RNA polymerase domain (RPD) of the primase domain of gp4. **(a)** Diribonucleotide pppAC is synthesized from the 5′-GTC-3′ basic recognition sequence through interactions between the ZBD and RPD in the same subunit (*cis* mode). Extension of the diribonucleotide to the functional tetraribonucleotide is facilitated by shortening the linker between the ZBD and RPD. **(b)** In the hexameric gp4 structure, the ZBD in one subunit interacts with the RPD in an adjacent subunit to catalyze the synthesis of a primer (*trans* mode).

RNA polymerase catalytic site on another subunit to catalyze primer synthesis in a '*trans* mode' [33]. Results obtained by modifying the length of the linker connecting the RNA polymerase domain and the zinc-binding domain suggest a model in which the primase catalyzes the synthesis of pppAC in a *cis* mode and the extension occurs in a *trans* mode [34]. The *trans* mode of primer synthesis could function as a brake to stop helicase activity and hence leading strand DNA synthesis [7•]. This pause would prevent leading strand synthesis from outpacing lagging strand synthesis.

Once primers are synthesized they are delivered to gp5/trx to initiate the synthesis of an Okazaki fragment. Tryptophan 69, located in the N-terminal region of the RNA polymerase domain of the primase plays a critical role in the delivery of primers to gp5/trx [35]. Substitution of lysine for Trp69 results in a primase that can synthesize but fails to stabilize the tetraribonucleotide.

Gp4 exists in two molecular weight forms *in vivo*, a 56-kDa and a 63-kDa form [36]. The 56-kDa form arises from an internal initiation codon and ribosome binding site located within the 63-kDa form [37]. The 56-kDa gp4 lacks the N-terminal 63 residues that comprise the zinc-binding domain and therefore cannot catalyze the *de novo* synthesis of tetraribonucleotides. However it can deliver a wide range of preformed oligonucleotides, including tRNA, to gp5/trx for use as primers [35]. Suppressor phages grown with the 56-kDa gp4 reveal alteration in gene 5.5 that encodes a protein that potentially regulates transcription of both host and phage [38], suggesting alterative mechanisms to acquire primers from a RNA transcript (B. Zhu *et al.*, unpublished).

## Regulation of lagging strand synthesis

Lagging strand synthesis is considerably more complex than leading strand synthesis yet both proceed at the same rate with a reconstituted T7 replisome [3]. The association of both the leading and lagging strand DNA polymerase with the gp4 must play a critical role in this coordination. For the parallel progression with the leading strand, the lagging strand polymerase forms a replication loop (Figure 1). The nascent Okazaki fragment is located within the loop and the eventual length of the Okazaki fragment is, on average, 0.8 kb [8•]. The mechanisms regulating loop size, the use of primase recognition sites, and the length of Okazaki fragments have been difficult to dissect. Visualization of the formation and release of the replication loop by single-molecule analysis has provided insight into this complex process [8•]. Analysis of the distributions of loop sizes and lag times between loops reveals that initiation of primer synthesis (signaling model) and the completion of an Okazaki fragment (collision model) each serve as a trigger for loop release. The presence of two triggers may represent a fail-safe mechanism ensuring the timely reset of the replisome after the synthesis of every Okazaki fragment.

The primase of gp4, like the helicase, is stimulated by interaction with gp5/trx. Primer synthesis during leading strand synthesis is approximately 10-fold greater relative to gp4 alone on ssDNA [39•]. Single-molecule experiments show that in the presence of ATP and CTP, precursors for primer synthesis, leading strand synthesis periodically halts for approximately 6 s [7•]. This cessation of DNA synthesis is the time, determined by kinetic studies, required to initiate and synthesize a primer by gp4 [32]. These results are compatible with a model in which the initiation of primer synthesis halts the movement of the helicase to stop leading strand synthesis during the lengthy period of primer synthesis. However, in ensemble experiments the rate of leading strand synthesis is not affected by the presence of a primase recognition site on the lagging strand [40]. Single-molecule FRET studies suggest that gp4 maintains contact with the primase recognition sequence and the primer, resulting in a priming loop [39•]. In these studies no pause was observed during primer synthesis with leading synthesis slowed by the rate of helicase movement. The basis for the difference in these studies is not known. It is possible that different phases in primer synthesis and loop formation are being observed with a priming loop preceding the larger replication loop.

## Role of gene 2.5 ssDNA-binding protein

T7 single-stranded DNA-binding protein (gp2.5) has multiple roles. Like gp4 it has an acidic C-terminal tail that is essential for T7 DNA replication [41]. In a reconstituted replication system it is essential for the coordination of leading and lagging strand synthesis [3]. The C-terminal tails of gp4 and gp2.5 are similar in length and composition and chimeric proteins in which the tails have been switched support the growth of T7 [42,43]. Like other ssDNA-binding proteins gp2.5 has an OB-fold consisting of anti-parallel β-sheets that form a barrel with a well-defined cleft [6]. Structural and mutagenesis data show that ssDNA binds within the cleft via stacking and electrostatic interactions [44•]. Cross-linking studies show that in the absence of ssDNA the C-terminal tail resides within the DNA binding cleft most likely to protect from random binding of charged molecules and to coordinate the DNA and protein interaction of gp2.5.

Not surprisingly, gp2.5 physically binds to gp5/trx through interactions of the acidic C-terminal tail with both the basic patches located in the TBD [24] and on the front of gp5 [19]. Despite the similarity of the C-terminal tails, sequential bindings of gp4 and gp2.5 to gp5/trx in a polymerizing mode suggest that they interact with gp5/trx in a distinct manner [45]. Gp2.5 enables gp5/trx to catalyze strand-displacement DNA synthesis at a nick in DNA, a process that involves the C-terminal tail [46]. It has been postulated that the binding of gp2.5 to the ssDNA extruded by the helicase results in condensation of the DNA with the increasing mass leading to resolution of the replication loop [3].

Interestingly, the C-terminal tails of both gp4 and gp2.5 bear a phenylalanine at the last position. A screening for suppressors of gp2.5 lacking the C-terminal phenylalanine identified residues that map in proximity to aromatic residues and to residues in contact with DNA in the crystal structure of gp5/trx bound to DNA [47]. Gp2.5 lacking the C-terminal phenylalanine has a lower affinity for gp5/trx relative to the wild-type gp2.5 and this affinity is partially restored by the suppressor mutations in gp5 [46].

## Additional factors contributing to T7 DNA replication

T7 phage derives most of the nucleotides needed for DNA replication from breakdown of host DNA to deoxynucleoside 5′-monophosphates. Host enzymes can convert these nucleoside monophosphates to the di and triphosphate nucleotides but may not be sufficient to meet the demand for the rapid DNA synthesis that occurs in phage-infected cells. A screening for host proteins essential for phage growth identified *E. coli* CMP kinase that also phosphorylates dCMP as an essential protein [48]. Gene 1.7 encodes a nucleotide kinase that phosphorylates dTMP and dGMP to dTDP and dGDP and subsequently, albeit at a slower rate, to the nucleoside triphosphate [49]. The ability of gp1.7 to phosphorylate dideoxythymidylate explains the sensitivity of *E. coli* harboring gene 1.7 to dideoxythymidine; *E. coli* thymidylate kinase cannot phosphorylate dideoxythymidylate [50]. Increased dTTP is beneficial for DNA synthesis mediated by gp5/trx and unwinding by gp4 helicase.

## Conclusions

Protein interactions occur within the T7 replisome in a coordinated manner. Binding of trx reconfigures the TBD in gp5 to enhance contact with DNA and with gp4 and gp2.5. Interactions of gp5/trx with the helicase domain of gp4 involve loading of the helicase, coordination of polymerization and unwinding, and exchange of gp5/trx during leading strand synthesis. Reciprocal stimulation of the polymerase and helicase results in highly processive synthesis. Synthesis of oligonucleotides by the primase domain of gp4 not only provides primers for the synthesis of Okazaki fragments but also signals the formation and resolution of replication loops on the lagging strand. The C-terminus of gp2.5 modulates ssDNA binding and its interaction with gp5/trx contributes to coordinated DNA synthesis. Besides these four key components, other proteins also contribute to the efficiency of replication. These proteins include those necessary for the processing of Okazaki fragments and for supplying nucleotides to the replisome. Structural information on the intact replisome as well as its subassemblies would improve greatly our understanding of a functional replisome.

## Acknowledgements

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