Molecular BioSystems

This article was published as part of the

Bruce Alberts Special Issue

Dedicated to Professor Bruce Alberts in commemoration of his 70th birthday and in recognition of his important contributions to science and education

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Repetitive lagging strand DNA synthesis by the bacteriophage T4 replisome†

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DOI: 10.1039/b812163j

Our studies on the T4 replisome build on the seminal work from the Alberts laboratory. They discovered essentially all the proteins that constitute the T4 replisome, isolated them, and measured their enzymatic activities. Ultimately, in brilliant experiments they reconstituted *in vitro* a functioning replisome and in the absence of structural information created a mosaic as to how such a machine might be assembled. Their consideration of the problem of continuous leading strand synthesis opposing discontinuous lagging strand synthesis led to their imaginative proposal of the trombone model, an illustration that graces all textbooks of biochemistry. Our subsequent work deepens their findings through experiments that focus on defining the kinetics, structural elements, and protein–protein contacts essential for replisome assembly and function. In this highlight we address when Okazaki primer synthesis is initiated and how the primer is captured by a recycling lagging strand polymerase—problems that the Alberts laboratory likewise found mysterious and significant for all replisomes.

Introduction

Bacteriophage T4 DNA replication is carried out by a dynamic multiprotein complex referred to as the replisome (Fig. 1). Eight proteins, which correspond to seven different activities, have been identified that together are able to reconstitute *in vitro* leading and lagging strand DNA synthesis.[1] Two holoenzyme complexes, each composed of the polymerase (gp43) and the clamp (gp45), are responsible for copying the leading and lagging strand templates.[2] The trimeric clamp protein stabilizes the polymerase on DNA during replication and is loaded by the clamp loader complex (gp44/62) in an ATP-dependent fashion.[3,4] The primosome is a subassembly of the replisome and is composed of a hexameric helicase (gp41) that unwinds dsDNA by translocating along the lagging stand template in the 5′ to 3′ direction[5] and an oligomeric primase (gp61) that synthesizes pentaribonucleotide primers at 5′-GTT and 5′-GCT sequences to initiate repetitive Okazaki fragment synthesis.[6,7] A helicase accessory protein (gp59) is required for the efficient loading of the helicase and may remain at the replication fork after the initiation of replication.[8–10] The functional importance of gp59 during active DNA replication is unclear. The single-stranded DNA binding protein (gp32) coats the ssDNA produced by the helicase[11] and is involved in coupling leading and lagging strand synthesis.[12,13]

The processivity of the lagging strand holoenzyme was established in landmark experiments carried out in the Alberts laboratory.[14] Alberts and coworkers

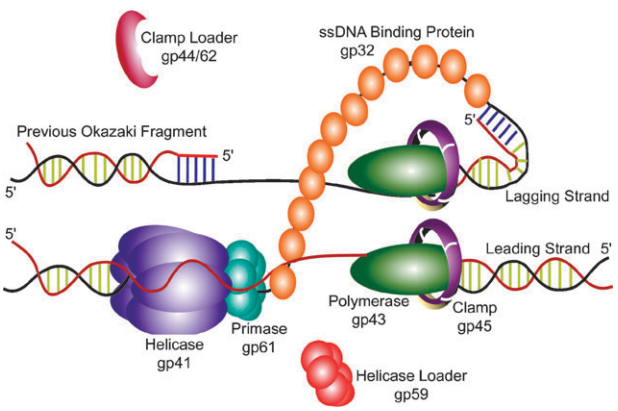

Fig. 1 The current model of the architecture of the bacteriophage T4 DNA replication complex is remarkably similar to the trombone model first proposed by the Alberts laboratory. The T4 replication complex is composed of eight proteins that interact to synthesize DNA. A helicase (gp41) and primase (gp61) form stacked rings that encircle the lagging DNA strand. This primosome complex is assembled with the aid of a helicase loader (gp59). The helicase unwinds duplex DNA ahead of the polymerase, while the primase synthesizes pentaribonucleotide primers for use by the lagging strand polymerase (gp43). Single strand regions of DNA created from helicase activity are bound by a single-stranded DNA-binding protein (gp32). The polymerases (one on the leading strand and one on the lagging strand) are responsible for nucleotide incorporation in the growing DNA strand, and interact with a number of proteins. A trimeric clamp (gp45), which is loaded by the clamp loader complex (gp44/62), binds to the polymerase and increases its processivity. The leading and lagging strand polymerases form a dimer.

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† This article is part of a *Molecular BioSystems* special issue dedicated to Professor Bruce Alberts on the occasion of his 70th birthday and in recognition of his important contributions to science and education.

demonstrated that Okazaki fragment size is not affected by the extreme dilution of polymerase, indicating that a new lagging strand polymerase is not being recruited from solution during each round of Okazaki fragment synthesis. These experiments led to the proposal of the trombone model as a physical mechanism to rationalize how the polymerase might remain with the replisome during the act of recycling. It is the discontinuous nature of lagging strand synthesis combined with the high processivity of the holoenzyme that requires the release of the lagging strand polymerase from a completed Okazaki fragment to be a regulated process.[14,15] Several mechanisms have been proposed to function as the trigger for the release and recycling of the lagging strand polymerase. Among them, two mechanisms have gained the most experimental support. In the first (the collision model), which has long been associated with the trombone model, the collision of the lagging strand polymerase into the 5′-end of the previous Okazaki fragment causes the release of the polymerase and acts as the trigger for RNA primer synthesis and polymerase recycling.[16] As a consequence, primer utilization (*i.e.*, primers used *versus* primers synthesized) should be highly efficient and the length of the Okazaki fragment is established by the travel of the leading strand holoenzyme.[14] Evidence in support of the polymerase release step comes from studies showing that the dissociation rate of the holoenzyme is greatly increased when it encounters a hairpin structure[17] or an annealed DNA or RNA.[18] In the second model (the signaling model), the lagging strand polymerase releases from the DNA as the result of one or more distinct events related to repetitive lagging strand DNA synthesis. These events could be the association of the primase with the replisome, the RNA primer synthesis, or the loading of the clamp onto the newly synthesized primer. The collision of the replicating lagging strand polymerase with the 5′-end of the previous Okazaki fragment is not required in this model, thus ssDNA gaps between Okazaki fragments are possible. In the *Escherichia coli* replication system it appears that both mechanisms are operable. Release of the lagging strand polymerase upon collision with the previous Okazaki fragment is controlled by the τ subunit of the γ complex, which increases the dissociation rate of the polymerase by over 300-fold.[19] Alternatively, it has been demonstrated that in the *E. coli* replication system, the association of the primase with the primosome can trigger the recycling of the lagging strand polymerase.[20–22]

Recycling of the lagging strand polymerase

We used several experimental approaches to investigate whether the length of Okazaki fragments could be manipulated in agreement with a signaling model.[23] These included (1) altering the rate of lagging, but not leading strand synthesis, intentionally uncoupling the two processes, (2) creating a dsDNA substrate with a single primase recognition site in order to measure accurately the response of Okazaki fragment size to variations in primase substrate (rNTP) concentration, and (3) changing the levels of clamp and clamp loader proteins to assess the effect on RNA primer utilization in Okazaki fragment synthesis.

We found first, the size of Okazaki fragments was reduced when the rate of the lagging strand polymerase was specifically lowered while maintaining a constant fork rate, an observation expected for a signaling model, but not the collision model. Second, the presence of ssDNA gaps was confirmed by our ability to extend Okazaki fragments that were synthesized by the slower moving lagging strand polymerase. These ssDNA gaps are not compatible with the collision model. Third, replication forks can transition from synthesizing long Okazaki fragments to shorter ones on a substrate containing only a single priming site, a result consistent with the signaling rather than the collision model, which does not predict fragment shortening. And fourth, dilution of clamp or clamp loader results in the formation of longer Okazaki fragments.[24] On the other hand, at higher clamp/clamp loader concentrations, RNA primers are utilized more efficiently for Okazaki fragment synthesis concomitant with shorter Okazaki fragments, indicating that the lagging strand polymerase releases more frequently. Together these two results suggest that the loading of the clamp on the RNA primer triggers the release of the lagging strand polymerase. The collective results indicate that collision with the end of the previous Okazaki fragment is not necessary for the release of the lagging strand polymerase. Rather, the loading of the clamp onto the newly synthesized RNA primer may serve as the signal for the release and recycling of the lagging strand polymerase. This signaling model requires that the length of Okazaki fragments results from kinetic control that is dictated by factors such as the rate of primase association with the replisome (in instances where it has dissociated), the priming rate of primase, the rate of clamp loading onto the RNA primer, and the rate of release of the lagging strand polymerase. As a consequence of signaling by clamp loading, ssDNA gaps will form between Okazaki fragments when the amount of lagging strand template produced during the previous round of lagging strand synthesis exceeds the ability of the lagging strand polymerase to replicate it before being signaled to recycle.

Electron microscopy (EM) experiments carried out in the Griffith laboratory have elegantly described the DNA structures generated during coupled leading and lagging strand synthesis by the T4 replisome.[10,11,25] Importantly, these studies have confirmed the existence of a lagging strand loop, validating the original trombone model devised by Alberts. While the majority of DNA molecules produced in replication reactions contained no ssDNA gaps between Okazaki fragments, 17% contained one ssDNA gap between consecutive Okazaki fragments and 9% contained two or more.[11] The conclusion drawn from these data was that in 26% of all replisomes, two or more Okazaki fragments are being extended at the same time. In support of this, experiments using biopointers to determine the protein composition at the replication fork found that a significant fraction of replicating molecules contained two polymerases simultaneously extending two Okazaki fragments on the lagging strand template.[10] Presumably, one of these polymerases must be recruited from solution. While these EM data are consistent with the signaling model as described above, it

is also possible that the recruited polymerase is being used to initiate a new Okazaki fragment.

While our data clearly indicate a signaling mechanism, they do not preclude the collision model. In fact, if the polymerase reaches the end of the template before the signal is sent, the polymerase will likely release *via* collision and the usual nick between Okazaki fragments will result. Based on our computer simulations of lagging strand synthesis using a simple stochastic model incorporating the rate of primase association, primer synthesis and clamp loading onto the newly synthesized primer, ssDNA gaps occur ~45% of the time, whereas in the remaining 55% there is no separation between Okazaki fragments. Thus, polymerase release due to collision is a necessary part of the signaling model. However, even though the polymerase may release its template upon collision with the 5′-end of the previous Okazaki fragment, we do not observe any abortive or truncated Okazaki fragments when the concentrations of clamp and clamp loader are severely reduced.[26] This result indicates that even if the polymerase releases without a signal from a loaded clamp, the polymerase will not initiate DNA synthesis. A likely hypothesis to explain this observation is that the polymerase cannot gain access to the RNA primer due to shielding by the primase.

The signaling mechanism has several advantages over the collision model. First, the signaling model allows the initiation frequency of lagging strand synthesis (Okazaki fragment length) to respond to changes in cellular concentrations of replication proteins (*e.g.*, clamp, primase) and rNTPs. Second, because the signaling model requires an indirect primer handoff with the clamp protein loading onto the RNA primer before polymerase recycling, a clamp-loaded primer is usually available for a released lagging strand polymerase. This reduces the time needed for holoenzyme assembly and prevents the formation of abortive Okazaki fragments that could form when clamp loading is delayed. Third, the T4 primase synthesizes primers with a relatively slow rate of 1 primer per second.[27] In the signaling model, the priming step takes place in parallel with active lagging strand DNA synthesis, which decreases the time between the completion of one Okazaki fragment and the start of the next. Finally, the signaling model may provide a mechanism for bypassing sites of DNA damage located on the lagging strand template.[28,29] If the collision model were the only mechanism for the recycling of the polymerase, then a lesion in the lagging strand template would likely cause the collapse of the replication fork because the polymerase would not reach the 5′-end of the previous Okazaki fragment and would not recycle. On the other hand, the signaling model allows the lagging strand polymerase to recycle from the site of the DNA lesion and begin a new Okazaki fragment in normal fashion. This would leave a ssDNA gap between the DNA lesion and the previous Okazaki fragment that would, in the case of T4 phage, be repaired through homologous recombination.[30]

Primer handoff

As a central player of the replisome, ssDNA binding protein (gp32) is likely to be involved in the initiation of lagging strand synthesis. Gp32 is made up of three domains:[31] the N-terminal domain (domain B for "basic") is involved in cooperative ssDNA binding,[32,33] the core domain is responsible for the recognition and binding of ssDNA,[34] and the C-terminal domain (domain A for "acidic") interacts with other T4 proteins.[35]

We recently examined the relationship between the ssDNA binding protein, primase, clamp, and clamp loader during the initiation of Okazaki fragment synthesis by removing the protein interaction domain of gp32 (hereafter referred to as gp32-A) and observing the effect on primer synthesis, primer utilization, and primase processivity.[36] An inactive primase trap protein was used to determine the dependency of the rate of primase dissociation on the concentration of clamp and clamp loader proteins as well as the presence of intact gp32.

We found first, that replisomal DNA synthesis in the presence of gp32-A results in a 2.5-fold increase in the average length of Okazaki fragments and a more broadly distributed range of lengths as compared to reactions performed with wt-gp32. Examination of the primers produced during coupled replisomal DNA synthesis indicates that the source of the Okazaki fragment lengthening is a combination of reduction in total priming and lower primer utilization. Second, the dissociation rate of primase is dramatically increased in the presence of gp32-A, indicating that an interaction between gp32 and a replisomal protein (presumably primase) is responsible for the moderate processivity of the primase. It is likely that the decrease in primase processivity is directly responsible for the reduced amount of total priming activity in reactions containing gp32-A. Third, we found that high levels of the polymerase accessory proteins, clamp and clamp loader, decreased the dissociation rate of the primase by two-fold. The most likely mechanism for a clamp/clamp loader-induced increase in primase processivity involves the handoff of the RNA pentamer from the primase to the polymerase. The primase protein must release the RNA primer so that the clamp loader can recognize the primer/template and chaperone the clamp protein into position on the RNA/DNA duplex. Once the primer is released, the primase could continue with the replisome or dissociate into solution.

Therefore, we propose a "timing" mechanism, where the primase is bound to the RNA primer within the replisome for a limited time (Fig. 2). The initiation of an Okazaki fragment cycle occurs when the primase encounters a priming site and synthesizes a RNA pentamer. Not every priming site is used as a template for primer synthesis and the mechanism behind the selection is unclear, but it is likely to be a stochastic process. The primase remains bound to both the helicase and the RNA primer while the helicase continues to unwind the duplex DNA. The production of ssDNA by the helicase produces a second loop between the helicase and primase. Presumably this loop becomes coated with gp32 in the same manner as the lagging strand loop. Alternatively, it is possible that the replication fork pauses during primer synthesis (as it does in the T7 system[37]), although we have no evidence for this. Next, the clamp and clamp loader enter the replisome from solution and displace the primase from the RNA primer, which causes the release of the priming loop. The primase remains bound to the

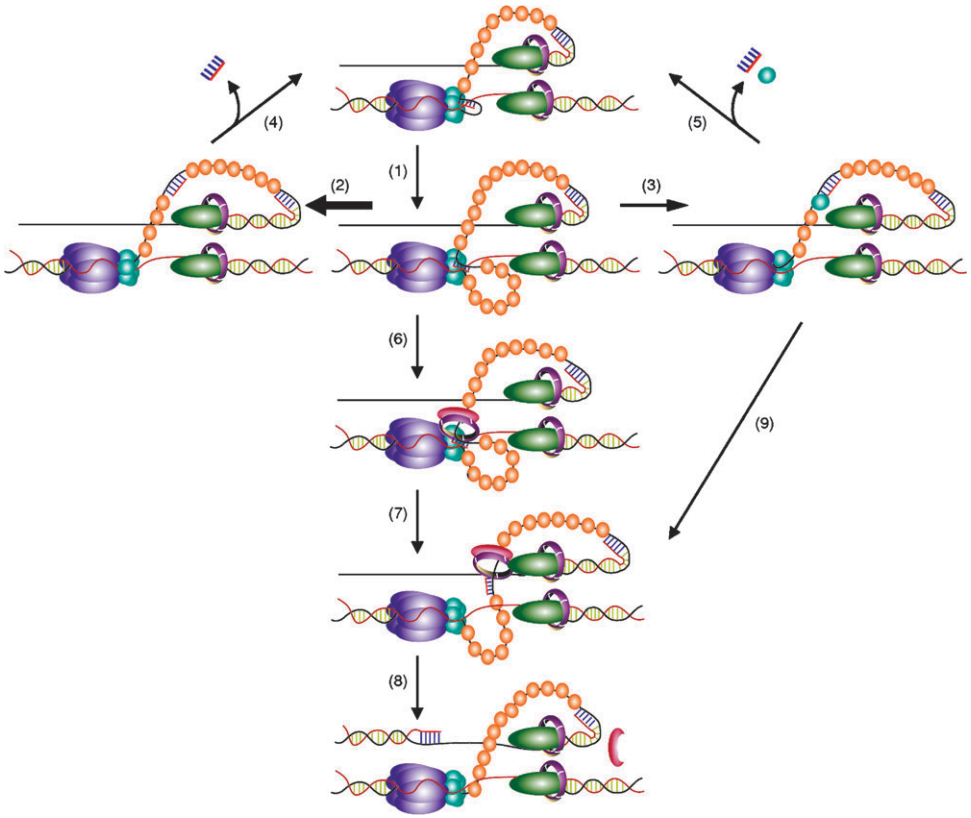

Fig. 2 Model for the initiation of lagging strand DNA synthesis in the T4 replisome. Initiation of lagging strand synthesis begins with the synthesis of a RNA pentamer by primase. During primer synthesis, the lagging strand polymerase is synthesizing an Okazaki fragment, causing an increase in size of the lagging strand loop. In addition, the helicase continues to unwind the DNA duplex, creating a second loop (1). If the clamp loader protein does not load the clamp in time, the primase either releases the primer and remains with the replisome (2) or releases from the helicase and dissociates from the replisome (3). The relative rates for steps 2 and 3 are indicated by the arrow size. The thermal stability of a naked pentamer primer generated by route (2) is very short and the primer will dissociate into solution (4). If the primase dissociates with the primer, they may both dissociate into solution and a new primase subunit must be recruited from solution to reset the replisome and allow for another round of RNA primer synthesis (5). If the clamp loader does load the clamp (6), the primase will release the RNA primer and remain bound to the helicase (7). The successful loading of the clamp onto the RNA primer causes the lagging strand polymerase to release the lagging strand template and recycle to the new clamp-loaded RNA primer (8). Although unlikely, our data can not rule out the possibility of the clamp loader complex loading a clamp onto a primer retained by monomeric primase thereby triggering recycling of the lagging strand polymerase (9). An identical color scheme for the proteins was employed as in Fig. 1.

helicase ready for the next round of primer synthesis. Once the clamp loader has guided the clamp onto the primer/template, the lagging strand polymerase releases the lagging strand template and recycles to the newly synthesized RNA primer. It is conceivable that the release of the priming loop is a physical signal for the recycling of the lagging strand polymerase. It is unclear at this time if proximity is sufficient to explain how the lagging strand polymerase efficiently locates and binds the primer/clamp to re-establish the replication loop or if specific protein–protein interactions aid in this process. If the clamp and clamp loader proteins are delayed in their entry into the replisome and fail to displace the primase from the RNA primer, then the primase will either release the primer and remain with the replisome or will dissociate from the replisome and remain with the primer. A functional interaction between gp32 and the primase strongly favors the pathway where the primase remains with the replisome.

Future directions

Despite our better understanding of repetitive lagging strand DNA synthesis, some outstanding questions remain regarding the process, which will require future investigations to answer. In the signaling mechanism, we believe that the signal for "premature release" of the lagging strand polymerase before completion of the Okazaki fragment synthesis is the loading of a clamp protein onto the next RNA primer. The physical mechanism by which the normally processive lagging strand polymerase releases the current Okazaki fragment and recycles to begin synthesizing the next Okazaki fragment is unknown. The means of transmitting this signal from the clamp/clamp loader/RNA primer to the lagging strand polymerase is also unknown. Presumably, the signal must be transmitted through protein–protein interactions within the replisome or through some constrained geometry of the DNA. Using a model substrate, we have observed a minor amount of intramolecular polymerase "hopping" from one clamp-loaded primer/template to

another (a reaction analogous to polymerase release and recycling).[38] However, the low efficiency of this process indicates that some feature(s) of the replisome or geometry of the DNA that is not present in our model substrate is required for efficient signaling. In general, the location of the DNA strands, how they wrap around and transverse individual proteins (with the exception of the polymerase) is unknown. Once the lagging strand polymerase receives the signal to recycle and releases the Okazaki fragment, it must locate the clamp/clamp loader/RNA primer to begin synthesis of another Okazaki fragment. The types of protein/DNA gymnastics required to accomplish efficient transfer of the primer to the polymerase are yet to be discovered. Finally, retention of the primase within the replisome is important for efficient repetitive lagging strand syn thesis. Helicase and primase form an important subassembly within the replisome; however, the coupling of primase and helicase activities is poorly understood.

The elucidation of the various processes underlying the recycling of the lagging strand polymerase, the primer handoff pathway from primase to polymerase, and the integration of the holoenzyme and primosome subassemblies to form an active replisome would provide substantial insights into how this marvelous machine functions. Due to the broad similarity between the replication system in T4 and those in *E. coli* and eukaryotes, both in terms of key protein units as well as their behavior, the T4 system serves as a paradigm for understanding DNA replication in general.

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