Two Distinct Triggers for Cycling of the Lagging Strand Polymerase at the Replication Fork*

Xiaojun Li and Kenneth J. Marians
From the Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

There are two modes of DNA synthesis at a replication fork. The leading strand is synthesized in a continuous fashion in lengths that in *Escherichia coli* can be in excess of 2 megabases. On the other hand, the lagging strand is synthesized in relatively short stretches of 2 kilobases. Nevertheless, identical assemblies of the DNA polymerase III core tethered to the β sliding clamp account for both modes of DNA synthesis. Yet the same lagging strand polymerase accounts for the synthesis of all Okazaki fragments at a replication fork, cycling repeatedly every 1 or 2 s from the 3'-end of the just-completed fragment to the 3'-end of the new primer. Several models have been invoked to account for the rapid cycling of a polymerase complex that can remain bound to the template for upward of 40 min. By using isolated replication protein-DNA template complexes, we have tested these models and show here that cycling of the lagging strand polymerase can be triggered by either the action of primase binding to the replication and synthesizing a primer or by collision of the lagging strand polymerase with the 5'-end of the previous Okazaki fragment.

The replisome of *Escherichia coli* is an extraordinary protein machine capable of coordinately synthesizing the nascent leading and lagging strands of DNA at a rate of nearly 1000 nucleotides/s while making only one misincorporation mistake for every 10^9 nucleotides polymerized (1). Efficiency of such a high degree bespeaks an ordered and cooperative process whereby individual catalytic activities operate synchronously, informed by the actions of one another. In support of this, several protein-protein interactions have been found to be crucial to replisome function by contributing both paths of information transfer and gains in enzymatic efficiency.

The τ subunit of the DNA polymerase III holoenzyme (Pol III HE) forms the central core of the replisome. Interactions between τ and the α subunit of the polymerase core hold the leading and lagging strand polymerases together (2). This ensures, as proposed originally by Alberts and colleagues (3), that when the lagging strand polymerase dissociates from a completed Okazaki fragment, it remains at the replication fork where it can be rapidly targeted to the next primer. An interaction between τ and DnaB, the replication fork DNA helicase (4), allows rapid replication fork movement (5) and, by preventing cycling of β from the core-β complex (6), defines which of the two polymerase assemblies becomes the leading strand polymerase (6, 7). The β clamp-loading γ-complex is anchored to the replication fork because τ and γ form a heterotetramer (8, 9). Interactions involving other fork components are important as well.

Primase is attracted to the replisome via an interaction with DnaB (10). The affinity of this interaction has been shown to govern Okazaki fragment size (11). An interaction between primase and the Pol III HE limits the size of primers synthesized at the fork to 12 nucleotides (12). Recent findings show that an interaction between primase, χ, a subunit of the clamp-loading γ-complex (13), and the single-stranded DNA-binding protein (SSB) may be important for the handoff of the primer from primase to the polymerase (14). This interaction could possibly account for the previously observed primer size limitation.

Perhaps the major gain of efficiency attained by the replisome is the repeated cycling of the lagging strand polymerase. This is a remarkable achievement, because to do so, another major gain in efficiency, that generated by β clamping α to the DNA topologically to form a polymerase with a processivity that can be measured in megabases, must be subverted to accommodate the relatively short size of Okazaki fragments synthesized on the lagging strand. This mechanism itself must also be highly efficient because the entire process of binding to the 3'-end of a primer, synthesizing an Okazaki fragment, and cycling to the next primer occurs in about 2 s. Considerable investigation using replication systems reconstituted with purified proteins has been devoted to attempting to define the lagging strand polymerase cycling mechanism.

Currently there are two models describing this process. It has been demonstrated that in both the cases of the *E. coli* (14) and bacteriophage T4 DNA polymerase holoenzymes (16, 17), the sliding clamp will not dissociate from the polymerase subunit when the polymerase is stalled because of the absence of substrate but does dissociate immediately when the polymerase encounters the 5'-end of a DNA strand annealed to the template, a situation identical to what happens when the lagging strand polymerase completes synthesis of the current Okazaki fragment and encounters the 5'-end of the penultimate fragment. These authors (14, 16, 17) suggested that it is this encounter that triggers cycling of the lagging strand polymerase. Alberts et al. (18) have argued as well that dissociation of the lagging strand polymerase would also be the signal for primase to initiate synthesis of the next primer.

We have shown that primase acts distributively at the replication fork (19), with a new primase molecule synthesizing each primer, and that it is the period of this cycle, set by the affinity of the primase-DnaB interaction (11), that governs the size of Okazaki fragments. Because essentially all variations in Okazaki fragment size could be understood as either a change in the frequency of primer synthesis or the efficiency with

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† The abbreviations used are: Pol III HE, the DNA polymerase III holoenzyme; SSB, the single-stranded DNA-binding protein; TFIIH, the DNA polymerase II holoenzyme; α, α subunit of the clamp; β, β subunit of the clamp; γ, γ subunit of the clamp.
which the primers were used to initiate lagging strand synthesis (20), we proposed that it was primase action that triggered cycling of the lagging strand polymerase (21).

In this report, using isolated replisome complexes, we developed conditions to test each of these models. To test primase-directed cycling, conditions were developed where there was no Okazaki fragment for the lagging strand polymerase to run into downstream of the point of synthesis of the first primer at the replication fork, although there was available template. Under these conditions, the lagging strand polymerase started cycling immediately when primase was added, thus indicating that either the binding of primase to the replisome or the synthesis of a primer was a trigger for lagging strand polymerase cycling. This observation also explains how cycling of the lagging strand polymerase is initiated directly after replication fork assembly. To test the collision-directed cycling model, we developed conditions where, in the absence of primase, the only available primers for lagging strand synthesis were synthetic oligonucleotides annealed to the lagging strand template. In this situation, the lagging strand polymerase was shown to be able to find these primers and use them to initiate Okazaki fragment synthesis, thus validating collision of the polymerase with a 5′-end as a trigger for cycling of the lagging strand polymerase as well.

MATERIALS AND METHODS

DNA Replication Proteins and Assays—9X-type primosomal proteins were purified as described (22). Pol III HE subassemblies and subunits (the kind gift of Charles McHenry, University of Colorado) were as indicated (2). SSB was purified according to Minden and Marians (23).

Tailed form II DNA template was prepared according to Mok and Marians (24). Standard rolling circle reaction mixtures (12 μl) containing 50 mM Hepes-KOH (pH 7.9), 12.5 mM MgOAc, 10 mM dithiothreitol, 5 μM ATP, 200 μM KCl, 0.1 mg/ml bovine serum albumin, 1.1 μM SSB, 0.42 nM TFII DNA, 3.2 nM DnaB, 56 nM DnaC, 240 nM DnaG, 28 nM DnaT, 2.5 nM PriA, 2.5 nM PriB, 2.5 nM PriC, and 28 nM Pol III HE were incubated at 30 °C for 2 min. NTPs were added to final concentrations of 1 mM ATP, 200 μM GTP, 200 μM CTP, and 200 μM UTP, and dNTPs to 40 μM, and the reaction was then incubated for 2 min at 30 °C (stage 1). [α-32P]dATP (2000–4000 cpm/pmol) was added to the reaction mixture, and the incubation was continued at 30 °C for an additional 10 min (stage 2). Reactions were terminated by the addition of EDTA to 50 mM, and analysis was by alkaline-agarose gel electrophoresis as described (19). To form and isolate replisome complexes, reaction mixtures (120 μl) containing 50 mM Hepes-KOH (pH 7.9), 10 mM MgOAc, 10 mM dithiothreitol, 5 μM ATP, 80 mM KCl, 100 μg/ml bovine serum albumin (BSA), 550 nM TFI, 550 nM SSB, 7 nM DnaB, 56 nM DnaC, 240 nM DnaG, 38 nM DnaT, 2.5 nM PriA, 2.5 nM PriB, 50 nM PriC, and 28 nM Pol III HE were incubated at 30 °C for 2 min. EDTA was added to a final concentration of 20 mM, and the reaction was then incubated for 2 min at 30 °C (stage 1). [α-32P]dATP (2000–4000 cpm/pmol) was added to the reaction mixture, and the incubation was continued at 30 °C for an additional 10 min (stage 2). Reactions were terminated by the addition of EDTA to 50 mM, and analysis was by alkaline-agarose gel electrophoresis as described (19). To form and isolate replisome complexes, reaction mixtures (120 μl) containing 50 mM Hepes-KOH (pH 7.9), 10 mM MgOAc, 10 mM dithiothreitol, 5 μM ATP, 80 mM KCl, 100 μg/ml bovine serum albumin (BSA), 550 nM TFI, 550 nM SSB, 7 nM DnaB, 56 nM DnaC, 240 nM DnaG, 38 nM DnaT, 2.5 nM PriA, 2.5 nM PriB, 50 nM PriC, and 28 nM Pol III HE were incubated at 30 °C for 2 min. EDTA was added to a final concentration of 20 mM, and the reaction was then incubated for 2 min at 30 °C (stage 1). [α-32P]dATP (2000–4000 cpm/pmol) was added to the reaction mixture, and the incubation was continued at 30 °C for an additional 10 min (stage 2). Reactions were terminated by the addition of EDTA to 50 mM, and analysis was by alkaline-agarose gel electrophoresis as described (19).

Southern Blotting—Gels were soaked in 1.5 liters of 0.25 M KCl for 30 min at room temperature, rinsed with H2O, and then soaked in 0.4 M NaOH for 10 min at room temperature. DNA was bound to an Amersham Pharmacia Biotech N+ membrane by upward capillary transfer for 3 h at room temperature. The membrane was washed twice with 100 ml of 2× SSC and then incubated in 10 ml of prehybridization solution (5× SSC, 50 μg/ml Denhardt’s solution, 0.5% SDS, 100 μg/ml calf thymus DNA) for 2 h at 45 °C. 32P probes were then added, and the incubation was continued for an additional 12 h. The membrane was then washed twice in 800 ml of 0.1× SSC, 0.1% SDS and autoradiographed. The probe to detect leading strand DNA was 5′-ATGAGGATCCAT-TCGTTTGTGAATATCAAG-3′, and the probe to detect lagging strand DNA was 5′-CTTGATATCCACAAAAGGATGGATCCT-3′. These represent sequences about the BamHI site in f1R229-A/33 DNA (24).

RESULTS

A Primase-Directed Trigger for Cycling of the Lagging Strand Polymerase—To assess whether primase could direct cycling of the lagging strand polymerase at the E. coli replication fork, we asked whether the lagging strand polymerase could cycle off the 3′-end of the nascent DNA if there was no 5′-end available from the previous Okazaki fragment for it to collide with. If such a collision served as the only trigger for cycling, the answer should be no. On the other hand, if cycling could be triggered by some other event, such as the association of primase with the replisome, then lagging strand polymerase cycling should be observed.

The test that we developed is described in Fig. 1. The lagging strand polymerase has to cycle from the 3′-end of the just-completed Okazaki fragment to the new primer for synthesis of the next Okazaki fragment (Fig. 1A). Under most experimental conditions, there will always be another Okazaki fragment downstream from the active lagging strand polymerase. Thus, it becomes difficult to distinguish one trigger event from another. However, we established conditions where the lagging strand polymerase bound to the first primer synthesized at the replication fork had only template available downstream from it; no previous Okazaki fragments existed. When primase synthesized the second primer, the lagging strand polymerase was therefore presented with a choice (Fig. 1B): either cycle to the new primer, thereby producing small Okazaki fragments of normal size or continue synthesis of the first Okazaki fragment until it ran off the end of the template, thereby producing an

![Image](322x470 to 540x729)
Okazaki fragment that was unusually long. Systems to assess the action of replication forks in vitro have been developed for the replication machinery from bacteriophages T4 (18) and T7 (25), as well as that from E. coli (24). Specialized TFII DNAs are utilized as templates that support rolling circle DNA synthesis in the presence of all the replication fork components (Fig. 2). These replication systems accurately mimic their counterparts in vivo. Moreover, the products of rolling circle DNA replication are cleanly resolved by denaturing alkaline-agarose gel electrophoresis into a large leading strand population that barely enters the gel and a population of rolling circle DNA replication are cleanly resolved by denaturing alkaline-agarose gel electrophoresis as described under “Experimental Procedures.” B, replisome complexes were isolated as described under “Experimental Procedures.” Reaction mixtures (336 μl) containing isolated replisome complexes (280 μl) were incubated in either the presence or absence of primase at 30 °C. Aliquots (48 μl) were withdrawn at the indicated times, and the DNA products were analyzed by alkaline-agarose gel electrophoresis as described under “Experimental Procedures.”}

Fig. 2. Isolated replisome complexes generate normal replication products. The diagram at the top of the figure illustrates the rolling circle DNA replication reaction. The black box on the tail of the TFII represents the primosome assembly site. The stippled regions of the template strands are regions of homology between the primer and the viral DNA that is used to make the TFII. (\(+\)) and (\(-\)) refer to the viral strand sense of the DNA template. A, standard rolling circle replication reactions were incubated in either the presence or the absence of primase at 30 °C. Aliquots (2 μl) were withdrawn at the indicated times, and the DNA products were analyzed by alkaline-agarose gel electrophoresis as described under “Experimental Procedures.” B, replisome complexes were isolated as described under “Experimental Procedures.” Reaction mixtures (336 μl) containing isolated replisome complexes (280 μl) were incubated in either the presence or absence of primase at 30 °C. Aliquots (48 μl) were withdrawn at the indicated times, and the DNA products were analyzed by alkaline-agarose gel electrophoresis as described under “Experimental Procedures.”

Incubation of the Pol III HE, SSB, the preprimosomal proteins (PriA, PriB, PriC, DnaT, DnaB, and DnaC), and the TFII DNA template in the presence of 5 μM ATP results in the formation of a stable replisome complex on the DNA that can be isolated by chromatography through a Biogel A50M column equilibrated with the same reaction buffer (2). Assays designed to detect both free Pol III HE and DnaB (at ∼1 MDa and 312 kDa, respectively, the largest proteins in the reaction mixture) have demonstrated that the protein-DNA complex isolated in the void volume is completely separated from free DNA replication proteins (2). This complex has been shown to be capable of supporting coupled leading and lagging strand synthesis that is resistant to 100-fold dilution. Thus, it contains a dimeric DNA Pol III HE and at least the DnaB protein. (The presence of the other preprimosomal proteins has not been assessed.)

To be able to perform the experiment described in Fig. 1, these isolated replisome complexes should synthesize only leading strand DNA if supplied with dNTPs and a high concentration of ATP and both leading and lagging strand DNA if primase and the other three NTPs are also provided. We therefore tested the activity of isolated replisome complexes in comparison with standard replication reactions (Fig. 2). In each case, the reaction was followed kinetically for the first 2 min of incubation in both the presence and absence of primase. The results with isolated replisomes (Fig. 2B) were comparable with those observed with standard reactions (Fig. 2A). The replication forks formed in either case moved at roughly the same rates in a manner that was not influenced by the presence of primase. In addition, only leading strand synthesis was observed in the absence of primase (Fig. 2, A and B, lanes 1–4), whereas both leading and lagging strand synthesis was observed in the presence of primase (Fig. 2, A and B, lanes 5–8). These data confirmed that the isolated replisomes behaved in the expected manner. This is consistent with our previously published observations (2).

To develop the conditions required for the experiment described in Fig. 1, we took advantage of the facts that during rolling circle DNA synthesis, the nascent leading strand is the lagging strand template, and, as shown in Fig. 2, that the
presence of primase is not required to assemble replisomes on the template that are capable of coupled leading and lagging strand synthesis (2, 19). Thus, isolated replisome complexes were recovered by gel filtration as described above, followed by a 3-min incubation in the presence of deoxynucleotides, β, and SSB. Because the latter two proteins act distributively at the replication fork, they are not recovered in the fractions containing the isolated replisome and thus must be restored to subsequent replication reactions.

Because these replication forks synthesize DNA at about 700 nucleotides/s (24), this initial 3-min incubation generates a long lagging strand template in excess of 100 kb in length. The addition of primase and the synthesis of the first primer on the lagging strand template now presents the lagging strand polymerase with the cycling dilemma described in Fig. 1. If collision with the previous Okazaki fragment was required to effect polymerase cycling, we would expect to observe the synthesis of an exceedingly long Okazaki fragment before any typically sized Okazaki fragments of 2 kb in length were evident. On the other hand, if cycling of the lagging strand polymerase could be triggered by the binding of primase to the replisome, we would expect to observe only the synthesis of Okazaki fragments 2 kb in length.

The results of such an experiment are shown in Fig. 3. Rolling circle replication reactions using isolated replisomes were initiated in the absence of both primase and (α-32P)dATP and incubated for 3 min. The reaction mixture was then divided into two, primase was added to one half, labeled was added to both halves, and aliquots of the reaction mixtures were withdrawn during the subsequent 2 min of incubation. Only two distinct types of DNA species were observed (Fig. 3A): large leading strand DNA that migrated to the exclusion limit of the gel and a population of typically sized Okazaki fragments centered about 1.8 kb in length. (Note that because the radioactivity present in the Okazaki fragment population is distributed over a large region of the gel, the lagging strand products can be difficult to visualize early in the reaction, even though the leading strand DNA, which is concentrated in a tight band, can be visualized easily.) There was no indication of any additional DNA population representing a nascent lagging strand increasing synchronously in length to large size as the incubation progressed.

To prove that the 3′-OH end of the first Okazaki fragment was, in fact, free and available for extension by the HE, we repeated the experiment shown in Fig. 3A but this time added back additional HE with the primase. If, in these experiments, the association of primase with the fork directed the lagging strand polymerase in the replisome to dissociate from the 3′-OH end of the first Okazaki fragment and move to a new primer terminus, then the additional HE should be able to detect the free 3′-OH end and extend it the length of the available template. This proved to be the case (Fig. 3B).

In the presence of primase and additional holoenzyme, a new DNA species was now evident that increased in length as a function of time (Fig. 3B, lanes 7–10). The appearance of this DNA product was dependent on the presence of primase, indicating that primers had to be synthesized and arguing that it represented elongation from the 3′-OH end of the first Okazaki fragment.

An alternative explanation, that primase was binding to the SSB-coated single-stranded DNA tail and synthesizing primers that were elongated by the free HE added to the reaction, can be discounted. (i) The specific activity of primase-catalyzed primer synthesis on single-stranded DNA in the absence of DnaB is one three-hundredth of that in its presence (10), (ii) there is no free DnaB present in the reaction mixture to associate with the single-stranded tail, and (iii) primase-catalyzed priming on single-stranded DNA is completely inhibited by the presence of SSB, in both the presence and the absence of DnaB (26–28).

In addition, to ensure that we had not created conditions where unused TFII DNA templates were somehow supporting new initiation events, we used Southern blotting to determine the strand sense of the new population of DNA (Fig. 4). The probe for leading strand DNA detected the large DNA population that barely entered the gel but did not detect the population of DNA that increased in size (Fig. 4A). On the other hand, the new primase-dependent population of DNA that increased in size was detected by the probe for lagging strand DNA (Fig. 4B). The band observed at 6.5 kb in Fig. 4B represents (−) strand DNA of the TFII template.

The data presented in Figs. 3 and 4 argue that the association of primase with the replication fork can trigger cycling of the lagging strand polymerase. Under the conditions of the experiment presented in Fig. 3A, no long Okazaki fragment is observed, even though there is template available, with isolated replisomes where the only available DNA polymerase is that which is present at the replication fork. This indicates that the lagging strand polymerase cycles immediately to the 3′-end of the second primer synthesized by primase, probably as soon as it becomes available, and does not continue synthesis of the first Okazaki fragment to the end of the template. This is supported by the fact that we can, in fact, observe extension of the first Okazaki fragment beyond typical size if additional,
The experiment described in the legend to Fig. 3 was repeated except that [$\alpha^{-32}$P]dATP was not included in the reaction mixture. The gels were then processed for Southern blot analysis as described under "Experimental Procedures." A, Southern blot hybridized with a probe to detect leading strand DNA. The major species detected is leading strand DNA produced by rolling circle DNA replication. B, Southern blot hybridized with a probe to detect lagging strand DNA. Visible on the blot are (−) strand template DNA (in all lanes) and the novel DNA species, seen increasing in size as a function of time. Note that because the Okazaki fragments are distributed over a large region of the gel, they only appear faintly in this exposure.

free Pol III HE is added to the reaction mixture (Fig. 3B).

An alternative explanation of the data was considered. It was possible that the first primer synthesized when primase was added to the reaction might not be used by the lagging strand polymerase yet still remain bound to the template. Under these circumstances, if the lagging strand polymerase then bound to the second primer made, it might synthesize DNA until it encountered the first primer and then cycle as a result of a collision-directed trigger. To address this issue, we tested the following prediction. If cycling was being induced by a collision with the first, unused primer, then when free HE was added back to the reaction, the long lagging strand product observed should originate from the 3′-OH end of a primer and not the 3′-OH end of an Okazaki fragment.

We therefore compared the size of the long lagging strand product made under the normal reaction conditions to that made when the ratio of NTPs to dNTPs in the reaction mixture was decreased. We have shown previously that this causes an increase in the size of Okazaki fragments made at the replication fork as a result of a decrease in the size of the primers synthesized from 10–12 to 8–9 nucleotides. This decrease in primer size leads to destabilization of the primers, thereby generating longer distances on the lagging strand template between successful initiations; hence, larger Okazaki fragments are formed (19, 20). Thus, if free HE was elongating from the 3′-OH of a primer, there should not be any difference in size of the long lagging strand product when the NTP/dNTP ratio was decreased, whereas if the free HE was elongating from the 3′-OH end of an Okazaki fragment, the size of the long lagging strand DNA should be larger at all time points examined when the NTP/dNTP ratio was changed, reflecting the larger size of the first Okazaki fragment.

The experiment described in Fig. 3 was therefore repeated. Under standard conditions, Okazaki fragments made by isolated replisome complexes were about 2.5 kb in length (Fig. 5A, lane 8). The addition of free HE to the reaction resulted in the appearance of the long lagging strand DNA, which was about 10.5 kb in length at the earliest time point (Fig. 5A, lane 8). In contrast, when the dNTP concentration was increased 4-fold (we have demonstrated previously that this has no effect on the rate of replication fork progression (19)), Okazaki fragment size increased to about 6 kb (Fig. 5B, lane 8), and at the earliest time point after addition of the free HE, the long lagging strand DNA was about 16.5 kb in length (Fig. 5B, lane 5). This size increase compared with that observed under standard reaction conditions was preserved at all times sampled (Fig. 5).

We conclude that it is the 3′-OH end of the first Okazaki fragment and not that of an unused primer that is bound by the free polymerase, and thus the lagging strand polymerase had to have dissociated from the first Okazaki fragment in response to either the binding of primase to the replication fork or the synthesis of the primer for the second Okazaki fragment.

A Collision-directed Trigger for Cycling of the Lagging Strand Polymerase—To assess whether the collision of the lagging strand polymerase with the 5′-end of the previous Okazaki fragment could cause the polymerase to cycle to the primer for the next Okazaki fragment, a situation had to be developed where the action of the lagging strand polymerase could be observed in the absence of primase. We reasoned that we could create such a situation by first generating a long lagging strand template using isolated replisome complexes as we did in the experiments above that addressed primase-directed cycling and then annealing a short oligonucleotide composed of a sequence that was present only once per genome copy to act as primers for Okazaki fragment synthesis. This procedure should lead to the scenario shown in Fig. 6.

As demonstrated in other systems (29), even though the lagging strand template will be coated with SSB, the oligonucleotide will anneal if added in considerable excess over the DNA. The issue therefore becomes whether the lagging strand polymerase in the replisome, which, up until the point of addition of the oligonucleotide would not be engaged in DNA synthesis (Fig. 6(ii)), could encounter the 3′-OH end of an oligonucleotide annealed to the nascent leading strand (Fig. 6(i)) and
This possibility seemed likely, although diffusion of both the polymerase and the primer template represented by the site of an oligonucleotide bound on the lagging strand template should be restricted compared with the situation where the polymerase was free and the primer was bound to a small circular phage DNA in solution, there should still be a significant probability that productive encounters would occur. Once bound to a primer on the lagging strand template, the lagging strand polymerase will certainly be capable of synthesizing DNA.

The test of collision as a trigger for cycling now presents itself. The first time the lagging strand polymerase binds an oligonucleotide and synthesizes nascent lagging strand DNA, it will eventually run into another oligonucleotide primer bound to the template (Fig. 6, (iii) and (iv)). If the annealing process has been 100% efficient, this encounter should occur once every 6.5 kb. If the collision with another primer on the template strands dissociation of the polymerase from the nascent DNA, it will be free to rebind to yet another primer, and so on (Fig. 6, (v)). Cycling of the polymerase would therefore be manifested as the appearance of a discrete 6.5-kb band on the denaturing gels. If the polymerase could not cycle at this point (Fig. 6, (vi)), such a band would not be observed because of the disproportionate distribution of radioactivity that would occur; essentially all of the radioactive label would be incorporated into the nascent leading strand.

Accordingly, replisome complexes were isolated and incubated to generate a long leading strand (which is the lagging strand template) in the absence of primase as in Fig. 3. [α-32P]dATP was then added along with a 4000-fold excess (over the TFII template) of a 30-nucleotide-long oligonucleotide that anneals to one position on the 6.5-kb viral template DNA, and the incubation was continued for an additional 10 min. The DNA products were analyzed as described under “Experimental Procedures.” B, PhosphorImager traces of the lanes of the gel shown in panel A. PSL, photostimulated luminescence.
length (Fig. 7, A, lane 3, and B). Thus, the lagging strand polymerase was clearly able to detect and bind the oligonucleotide primers on the lagging strand template, to synthesize nascent lagging strand DNA, and to respond to the collision with the 5'-end of a downstream oligonucleotide primer by disengaging from the 3'-OH end of the just-synthesized nascent DNA and cycling to the 3'-OH of another oligonucleotide primer bound to another region of the template. Whereas it is possible that the synthesis observed was a result of free polymerase being generated in the reaction because the replisome falls apart, this is very unlikely. For example, in the experiments shown in Figs. 3 and 5, no long lagging strand product is observed in the absence of added free polymerase even after 10 min of incubation (data not shown).

Remarkably, the efficiency of the oligonucleotide collision-directed cycling of the lagging strand polymerase was very high. PhosphorImager analysis indicated that the amount of radioactivity in all of the oligonucleotide-dependent DNA species amounted to 40% of that present in the leading strand DNA (Fig. 7B). The multigenome-length oligonucleotide-dependent DNA species clearly arose as a result of the lagging strand polymerase binding an oligonucleotide on a region of the template where the annealing reaction had not been 100% efficient. In addition, the weaker series of bands clearly represent annealing of the oligonucleotide to secondary sites on the template. The first DNA species in this series would be shorter than unit length because there would be a high probability that the polymerase would encounter another oligonucleotide bound at the primary site before synthesizing one genome length of lagging strand DNA.

Could this system be used to distinguish which cycling trigger held primacy at the replication fork? The addition of both the oligonucleotide and primase to the reaction resulted in a reduction of most of the multigenome-length DNA species. The full-length DNA species was still present, although it was also reduced in intensity compared with the reaction that contained oligonucleotide alone. In addition, a smear was now present starting at the position of the full-length DNA and continuing to a position equivalent to that of DNA about 0.5 kb in length (Fig. 7, A, lane 4, and B).

Unfortunately, these results cannot be used to determine which cycling trigger is primary. The same prediction, which is confirmed by the data, is made in either case. If primase-directed cycling held sway, then one would predict that the majority of Okazaki fragments would be shorter than unit length because, at the high concentration of primase used, a primase molecule would, in most instances, bind and trigger a cycling event while the lagging strand polymerase was synthesizing DNA from either another primase-synthesized primer or from an oligonucleotide that had been annealed to the template. Because the oligonucleotides are, for the most part, one genome length apart on the template, this would lead to shorter fragments overall. When thought about in terms of the collision model, ultimately, one predicts the same: that most of the Okazaki fragments would be shorter than unit length. In this view, the primer synthesized by primase is likely to be either upstream or downstream from an oligonucleotide annealed to the template. Thus, even if collision was the only trigger for cycling of the lagging strand polymerase, the bulk of the Okazaki fragments would be shorter than unit length.

**DISCUSSION**

Synthesis of the nascent leading and lagging strands at the replication fork is efficiently coordinated with replication fork progression. In studies with replication forks reconstituted with purified bacteriophage T4 proteins, Alberts and colleagues (3) noted that even when reaction conditions restricted the fraction of active templates severely, both the nascent leading and lagging strands were still made. They suggested that this implied the existence of a mechanism to ensure the rapid retargeting of the lagging strand polymerase to the replication fork after the synthesis of an Okazaki fragment was completed and the polymerase dissociated from the template.

In the original formulation of the trombone model of a replication fork (18), the solution to this problem was the proposal that the replication fork polymerase was dimeric, with two active catalytic centers, one synthesizing the leading strand and the other synthesizing the lagging strand. The lagging strand polymerase was proposed to be physically tethered to the leading strand polymerase, creating a loop in the lagging strand template. This resulted in the juxtaposition in space of the site of termination of synthesis of the current Okazaki fragment and synthesis of the primer for the next Okazaki fragment. Because the lagging strand polymerase did not leave the replication fork after it completed synthesis of an Okazaki fragment and dissociated from the lagging strand template, the time required to find the next primer to initiate Okazaki fragment synthesis decreased dramatically. The loop in the lagging strand template alternated between growth (during synthesis of the Okazaki fragment) and complete release (when the lagging strand polymerase moved to the new primer). The model derived its name from the trombone slide-like action of this loop.

One key aspect of this model has been proved correct for replication forks formed in vitro using either E. coli, bacteriophage T4, or bacteriophage T7 replication proteins. That is, that there are two polymerases at the replication fork and that the lagging strand polymerase is physically anchored to the fork. The mechanism of coupling, however, is only clear in the E. coli system where the polymerases are coupled via interactions between the τ and α subunits of the Pol III HE (2, 21). Coupling in the bacteriophage T7 (25, 30) and bacteriophage T4 (31) systems may be mediated via interactions between the polymerases and the helicases. On the other hand, the trigger that initiates cycling of the lagging strand polymerase has remained elusive. Alberts et al. (18) suggested originally that this would be the release of the lagging strand polymerase from the template when it collided with the 5'-end of the previous Okazaki fragment. This collision trigger, however, made it difficult to explain how, when the replication fork was first established, one could account for the synthesis of the first Okazaki fragment, because there would not be any other fragment downstream for the lagging strand polymerase to collide with.

Support for the collision trigger was provided when both the Alberts (16, 17) and O'Donnell (15) labs demonstrated that the sliding clamp dissociated from the polymerase with first order kinetics when the polymerase encountered secondary structure ahead of it, as it would when it encountered the previous Okazaki fragment, but dissociated very slowly when it was stalled by deoxynucleotide omission. Thus, a central tenet of the trombone model, that cycling of the lagging strand polymerase could be triggered by its encounter with the 5'-end of the previous Okazaki fragment, appeared to receive strong support.

As a result of our studies with the E. coli system, we proposed a different trigger for lagging strand polymerase cycling based on the cyclical association of primase with the replication fork (21). We showed that the leading and lagging strand polymerases were physically coupled at the replication fork (32), as proposed by the trombone model, and that this coupling was mediated by the τ subunit of the polymerase (2), as predicted by biochemical analyses of the Pol III HE in solution.
Okazaki fragment synthesis was quite high, each time a primer was synthesized (19). Nevertheless, the fraction of primers synthesized that were used for initiation of Okazaki fragment synthesis was quite high, indicating an efficient coupling of primer synthesis to initiation of nascent DNA synthesis (20). Okazaki fragment size was shown to vary inversely with the concentration of primase (19), suggesting that it was the association of primase with the fork, mediated by its interaction with DnaB (10), that was the controlling feature (21). In addition, all variations in Okazaki fragment size that resulted from alterations in reaction conditions could be shown to be a function of either the frequency of primer synthesis or the efficiency with which primers were used to initiate the synthesis of Okazaki fragments (20). Thus, we proposed that it was either the binding of primase or the synthesis of a new primer that acted as the trigger for cycling of the lagging strand polymerase (21).

In support of this, we were able to construct mutant primases that were altered in the affinity of their interaction with DnaB and that, as a consequence, at equivalent concentrations programmed the fork to synthesize Okazaki fragments of different size than the wild-type primase (11). These mutant primases did not have defects in either their ability to synthesize primers or to interact with the Pol III HE. Okazaki fragment size still varied inversely with the concentration of the mutant primase, and the alteration in Okazaki fragment size could not be attributed to changes in either the rate of generation of template (i.e., the rate of replication fork progression) or the efficiency with which primers were used to initiate the synthesis of the lagging strand products.

To determine the nature of the trigger for cycling of the lagging strand polymerase, in the current report we devised tests for each of the two models described above. We took advantage of the following: (i) that primase acts distributively at the replication fork (19), (ii) that its presence is not required for proper assembly of the leading and lagging strand polymerases with the replication fork helicase, DnaB, to form the central core of the replisome (2), and (iii) that during rolling circle DNA replication, the nascent leading strand is the lagging strand template. For these reasons, we could isolate a replisome complex capable, in the absence of primase, of leading strand synthesis that is coupled to the action of DnaB and of coupled leading and lagging strand synthesis in the presence of primase. Importantly, all free replication proteins could be removed from the replisome complex by gel filtration chromatography.

Thus, by initiating DNA synthesis with isolated replisomes in the absence of primase, a long leading strand was synthesized. This allowed us to test each of the models by creating situations where cycling could only be observed that was triggered by one or the other mechanism. To test primase-directed cycling, we focused on the first Okazaki fragment made after the long leading strand was established and primase was added to the reaction. At the time of synthesis of the first primer at the replication fork, the lagging strand polymerase, which was present at the replication fork because of its physical linkage to the leading strand polymerase but inactive, binds the 3'-end of the primer and starts to synthesize the first Okazaki fragment. Primase then dissociates from the fork, a new molecule associates, and the second primer is synthesized. The lagging strand polymerase could now either continue synthesis of the first Okazaki fragment or cycle to the second primer. It should be noted that because of the presence of SSB coating the long lagging strand template, primase can only synthesize primers at the replication fork via its interaction with DnaB.

If the only trigger for cycling was the encounter with the 5'-end of the downstream Okazaki fragment, then synthesis of the first Okazaki fragment should continue, producing a long, nascent lagging strand easily visible on the alkaline-agarose gels used to analyze the DNA products. On the other hand, if either primase binding or primer synthesis could trigger cycling, the lagging strand polymerase would cease synthesis of the first Okazaki fragment and move to the new primer, leading to the production of typically sized Okazaki fragments. This is precisely what we observed.

It was clear that the lagging strand polymerase had initiated synthesis of the first Okazaki fragment and cycled off it, because if free Pol III HE was added subsequent to the addition of primase, synthesis of a long Okazaki fragment could be observed, indicating that the 3'-OH end of the first Okazaki fragment was vacant. Thus, cycling of the lagging strand polymerase can be triggered by the action of primase. How this occurs is unknown, although an obvious pathway of signal transduction would be through DnaB to the polymerase via its interaction with the 7 subunit (5, 7).

To test the collision model for lagging strand polymerase cycling, we eliminated primase from the reaction altogether. Instead, after generation of the long lagging strand template a short oligonucleotide that anneals to the lagging strand template at one site per genome length was provided. Thus, similar to the test of primase-directed cycling, the once dormant lagging strand polymerase was now presented with the opportunity of binding to a 3'-OH end and synthesizing nascent lagging strand DNA. The only way in which this could occur repetitively was for the lagging strand polymerase to dissociate from the nascent DNA once it completed synthesis by colliding with the 5'-end of another, downstream oligonucleotide on the template.

Although we were initially uncertain as to whether the lagging strand polymerase could "see" primers on the lagging strand template that had not been synthesized by primase, this concern proved unfounded. Not only could the polymerase detect and bind the primers, but, given the efficiency of the reaction, it also clearly could cycle from 3'-end of nascent Okazaki fragment to the 3'-OH end of another oligonucleotide bound to the template. That a collision was occurring between the polymerase and the 5'-end of a downstream oligonucleotide was clear because the size of the oligonucleotide-directed Okazaki fragments was one genome length. In addition, these fragments could be joined together by DNA ligase, indicating that there was no gap present (data not shown).

The demonstration that two triggers for cycling of the lagging strand polymerase exist provide interesting insight to replication fork function. Primase-directed cycling reveals the mechanism required for synthesis of the first Okazaki fragment during the establishment of a replication fork. In our test of the collision-directed cycling model, we found that the lagging strand polymerase could efficiently find oligonucleotides annealed to the lagging strand template in the absence of primase, even if those primers were only 12 nucleotides long (data not shown). This rules out any concerted exchange of the primer terminus from primase to the lagging strand polymerase that is mediated by protein-protein interactions as being required for lagging strand synthesis, as has been suggested previously (14), although such an exchange is likely to result in an increase in the efficiency of lagging strand synthesis.

We were unable to develop evidence that could be used to argue for the primacy at the replication fork of one or the other trigger for cycling of the lagging strand polymerase. Certainly,
the initial trigger is the association of primase with the replication fork. Does the trigger mode now switch as soon as normal, i.e. with repeated cycling, Okazaki fragment synthesis commences? This remains to be determined. It could be that the presence of two triggers for lagging strand polymerase cycling is a safety measure that has been designed into the system to guarantee that all the DNA becomes replicated, ensuring, for example, that a lagging strand polymerase that becomes frozen at the nick between Okazaki fragments can still be induced to cycle by the binding of primase to the replication fork.

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