The application of a minicircle substrate in the study of the coordinated T4 DNA replication

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A reconstituted in vitro bacteriophage T4 DNA replication system was studied on a synthetic 70-mer minicircle substrate. This substrate was designed so that dGMP and dCMP were exclusively incorporated into the leading and the lagging strand, respectively. This design allows the simultaneous and independent measurement of the leading and lagging strand synthesis. In this paper, we report our results on the characterization of the 70-mer minicircle substrate. We show here that the minicircle substrate supports coordinated leading and lagging strand synthesis under the experimental conditions employed. The rate of the leading strand fork movement was at an average of ~150 nucleotides/s. This rate decreased to less than 30 nucleotides/s when the helicase was omitted from the reaction. These results suggest that both the heloenzyme and the primosome can be simultaneously assembled onto the minicircle substrate. The lagging strand synthesized on this substrate is of an average of 1.5 kb, and the length of the Okazaki fragments increased with decreasing [rNTPs]. The proper response of the Okazaki fragment size toward the change of the priming signal further indicates a functional replisome assembled on the minicircle template. The effects of various protein components on the leading and lagging strand synthesis were also studied. The collective results indicate that coordinated strand synthesis only takes place within certain protein concentration ranges. The optimal protein levels of the proteins that constitute the T4 replisome generally bracket the concentrations of the same proteins in vivo. Omission of the primase has little effect on the rate of dNMP incorporation or the rate of the fork movement on the leading strand within the first 30 s of the reaction. This inhibition only becomes significant at later times of the reaction and may be associated with the accumulation of single-stranded DNA leading to the collapse of active replisomes.

The bacteriophage T4 DNA replication system has served as a successful working model for eukaryotic systems. A total of eight T4 proteins are required to reconstitute an in vitro replication fork that carries out efficient and coordinated leading and lagging strand synthesis (1–4). These eight proteins form the T4 DNA replisome that can be subdivided into one primosome complex and two heloenzyme complexes based on their different functionalities. These subassemblies are believed to be structurally and functionally integrated through protein-protein interactions within the replisome.

Three protein components are involved in the assembly of the heloenzyme complex: gp43 (the polymerase), gp45 (the clamp protein), and gp44/62 (the clamp loader protein complex). The gp43 polymerase catalyzes the addition of a nucleotide monophosphate onto the 3’ end of the nascent DNA chain and also contains an editing 3’–5’ exonuclease activity (5). In the presence of the clamp protein, gp43 switches from a distributive to a processive enzyme (6–8). gp45 is a trimeric protein that forms a sliding clamp circumscribing the primer/template junction and increases the binding between gp43 and the DNA template (7). gp45 itself is loaded onto DNA by gp44/62, a molecular motor protein that utilizes energy from ATP hydrolysis for clamp loading (9–11). The detailed kinetics of the multistep clamp loading process have been carefully studied by a series of pre-steady-state kinetics and stop-flow fluorescence resonance energy transfer experiments (12–14).

The primosome complex constitutes another important component of the T4 replisome consisting of the helicase (gp41), the primase (gp61), and the helicase accessory protein (gp59). gp41 helicase forms a ring-shaped hexameric structure in the presence of ATP/GTP (15). This gp41 hexamer is assembled on the lagging strand and unwinds the double-strand DNA in front of the moving fork in the 5’ to 3’ direction (16–18). In the presence of gp32, gp59 facilitates the loading of the helicase through its interactions with both proteins (19–22). gp61 is required to synthesize pentaribonucleotide primers for Okazaki fragment synthesis on the lagging strand. The recognition sequences for the T4 primase are 5’-GTT and 5’-GCT (23), with the former being the recognition site in vivo (24, 25). The primase activity is modulated by the presence of other proteins. For example, gp61 activity is greatly stimulated by the gp41 helicase (24) and is further enhanced by the presence of both gp59 and gp32 (26). Recently, strong evidence for the formation of a hexameric ring structure of gp61 within the primosome has been provided through both kinetic and biophysical studies (26).

The single-stranded DNA-binding protein, gp32, is an important component of the T4 replisome. It exhibits strong cooperative single-stranded DNA binding ability and is presumably functioning in stabilizing the loop structure formed during replication (27). gp32 also interacts with a number of other T4 replication proteins in the replisome including gp59 and gp61.
minicircle replication (33). Studies of the asymmetric and dimeric holoenzyme complexes (34) have been used to assess the feasibility of the minicircle substrate as a fork template. In this paper, we examine the recruitment or recycling of various protein components at the replication fork by using both the minicircle and the T7RFII M13 template.

**Materials and Methods**

**T7 System**

- **DNA synthesis** requires the coordinated action of all the functional units within the replisome. It was first suggested by Alberts and co-workers (31) that the two T4 holoenzyme complexes must be coupled during replication in order to explain how the tethered holoenzyme complexes carry out the synthesis of anti-parallel DNA double strands. Their trombone model was later adopted in the *Escherichia coli* (32) and T7 replication system (33). Studies of the *E. coli* system have provided the most convincing evidence for the presence of asymmetric and dimeric holoenzyme complexes (34–36). A τ subunit has been shown to interact with both polymerases within the *E. coli* replisome (37). Although a direct physical link between the two polymerases has not been identified in the T7 system, dilution experiments did suggest that the lagging strand polymerase was highly processive and was recycled during repetitive cycles of the Okazaki fragment synthesis (38). Recent studies (39, 40) likewise support a dimeric polymerase model that the small size of the substrate may impose steric constraints on the loading of all replisome components. In particular, the simultaneous loading of both the holoenzyme and the primosome may be hindered on a 70-mer DNA substrate. If this be the case, the coordination between leading and lagging strand synthesis would be disrupted. In this paper, we report coordinated synthesis and responded to the variation of individual replisome protein concentrations. The protein concentrations that support coordinated synthesis agree well with their in vivo concentrations. Furthermore, we studied the effect of blocking lagging strand synthesis on the leading strand. Preventing primer synthesis by omitting gp61 did not affect the rate of the leading strand synthesis at early reaction times, but the effect became significant at later reaction times. A possible role of the inter-action between the accumulated ssDNA and T4 proteins such as gp32 is also discussed. In the accompanying paper (57), we examined the recruitment or recycling of various protein components at the replication fork by using both the minicircle and the T7RFII M13 template.

The T4 DNA replication system has been studied on a tagged replicative form II (TRFII) DNA template constructed on M13 ssDNA. Only recently was a minicircle substrate utilized for more quantitative analyses (39, 43). Compared with the TRFII M13 template, the minicircle system offers a number of advantages. With the minicircle substrate, the manipulation of the DNA sequence becomes possible. The design of the 70-mer minicircle substrate in this study enables the strand-specific incorporation of dGMP and dCMP into the leading and lagging strands, respectively. Two priming sites, 40 nucleotides apart on the lagging strand, are also present in this substrate. Another advantage of using a small circular substrate is that the smaller size of the minicircle provides a higher fork number/nucleotides ratio. As a result, a high concentration of the replication forks can be achieved in the reaction mixture. This allows the replication reactions to be carried out with the fork concentrations that are near or surpass the protein concentrations.

There are concerns, however, about using such a substrate. One of the potential problems with the 70-mer minicircle is that the small size of the substrate may impose steric constraints on the loading of all replisome components. In particular, the simultaneous loading of both the holoenzyme and the primosome may be hindered on a 70-mer DNA substrate (44). Should this be the case, the coordination between leading and lagging strand synthesis would be disrupted. In this paper, we assess the feasibility of the minicircle substrate as a fork template for the study of the coordinated T4 DNA replication.

We have monitored the replication fork movement under various experimental conditions. The rate of fork movement varied from the highest measured rate of 250 nt/s to an average rate around 150 nt/s. We have developed a dual-label method that enables us to quantify simultaneously the amount of leading and lagging strand synthesis in the same reaction mixture. This greatly reduces the experimental error associated with the study of coordinated synthesis in which leading and lagging strand synthesis is measured in separate reactions. Our results support the assembly of anti-parallel and functional holoenzymes on the 70-mer substrate that established coordinated strand synthesis and responded to the variation of individual replisome protein concentrations. The protein concentrations that support coordinated synthesis agree well with their in vivo concentrations. Furthermore, we studied the effect of blocking lagging strand synthesis on the leading strand. Preventing primer synthesis by omitting gp61 did not affect the rate of the leading strand synthesis at early reaction times, but the effect became significant at later reaction times. A possible role of the interaction between the accumulated ssDNA and T4 proteins such as gp32 is also discussed. In the accompanying paper (57), we examined the recruitment or recycling of various protein components at the replication fork by using both the minicircle and the T7RFII M13 template.

The minicircle was then annealed with a partially complementary 109-mer strand (5'AGA PGA TGA TAG GAA AAA TAG TGG GTA TGT GGA GGT TAT GGT GGA 3') which provides the template strand for lagging strand synthesis (Fig. 1).

The standard replication conditions and the filter binding assay— Replication reactions were carried out in a complex buffer containing 25 mM Tris-acetate (pH 7.5), 125 mM KOAc, and 10 mM Mg(OAc)2. The standard replication conditions used in all minicircle reactions consisted of 100 nM minicircle substrate, 240 nM each of gp43, gp45 (as trimer), and gp44/62, 600 nM each of monomer gp41, gp61, and gp59, 4.5 μM of gp32, 100 μM each of dCTP, dGTP, and dTTP, 2 mM ATP, 100 μM each of dATP, dGTP, and dTTP, [α-32P]dGTP (6.9 Ci/mmol), and [α-32P]dCTP (3000 Ci/ns), in a typical reaction volume of 100 μl. Unless otherwise noted, the DNA polymerase holoenzymes (gp43, gp45, and gp44/62) were first preincubated at 37 °C with the minicircle DNA template in the presence of 2 mM ATP for 30 s followed by the addition of the primosome (gp41, gp61, and gp59), and gp32 along with dNTPs, rNTPs, and 2 mM ATP. 10–20 μl aliquots were removed at various time points and quenched with an equal volume of 0.5 M EDTA. The quenched reaction aliquots were then spotted onto DE81 filter paper. All filter papers were allowed to air-dry and then washed in 30 ml ammonium formate buffer (pH 8.0) until no radioactivity was detected in the wash. The filter papers were washed twice with 95% ethanol and allowed to air-dry in a hood. The dried filter papers were placed in LSC vials with 5 ml of Ecoscint LSC mixture in each vial and counted with a Beckman LS6800 liquid scintillation counter.

The Minicircle Substrate—A linear 70-mer oligonucleotide (5'-CAC CAT AAT CCC TAC CCT CCG CAA TAT TCA CCA TCA ACC CTG CAC TTC TCA CTC CAC CCT CGC CCC CCA TAT TCA CGT TAT GGT GTA TA-3') was intramolecularly ligated under dilute conditions using a bridging 20-mer oligonucleotide (5'-GGT TAT GGT GGA GTG GTA TA-3') in a manner described previously (39, 43). The minicircle was then annealed with a partially complementary 109-mer strand (5'AGA PGA TGA TAG GAA AAA TAG TGG GTA TGT GGA GGT TAT GGT GGA 3') which provides the template strand for lagging strand synthesis (Fig. 1).

In *E. coli* DNA synthesis, the primosome (gp41, gp61, and gp59) and gp32 along with dNTPs, rNTPs, and 2 mM ATP. 10–20 μl aliquots were removed at various time points and quenched with an equal volume of 0.5 M EDTA. The quenched reaction aliquots were then spotted onto DE81 filter paper. All filter papers were allowed to air-dry and then washed in 30 ml ammonium formate buffer (pH 8.0) until no radioactivity was detected in the wash. The filter papers were washed twice with 95% ethanol and allowed to air-dry in a hood. The dried filter papers were placed in LSC vials with 5 ml of Ecoscint LSC mixture in each vial and counted with a Beckman LS6800 liquid scintillation counter (channel settings are based on an arbitrary scale of 0–1000). Channel 1 is set for 0–400 and channel 2 S. Alley, unpublished protocol.
2 for 400–1000). The specific activities of both tritium and $^{32}$P were calculated by direct counting of a known volume of the reaction mixture in LSC mixture. Control experiments showed that there was no quenching effect of $^{32}$P on the filter paper. However, there was a 70% quenching of tritium by the filter paper. This effect was corrected in the calculation of the tritium-specific activity. The spill-over of $^{32}$P radioactivity into channel 1 was determined to be 2.2% of the total $^{32}$P radioactivity. The amount of dNMP incorporation (cpm) in the leading and the lagging strand was calculated according to Equations 1 and 2.

$$^{32}P = 100 \times B/97.8 \quad \text{(Eq. 1)}$$

$$^{3}H = A - 2.2 \times B/97.8 \quad \text{(Eq. 2)}$$

where $A$ and $B$ are the counts in channel 1 and 2, respectively.

The Rate of the Replication Fork Movement—Unless otherwise specified, replication reactions were carried out under the standard conditions with either $[\alpha-^{32}\text{P}]$dGTP (3000 Ci/mmol) or $[\alpha-^{32}\text{P}]$dCTP (3000 Ci/mmol). Aliquots of the reaction mixture were sampled at various time intervals and quenched with an equal volume of 0.5 M EDTA, pH 8.0. The DNA products were analyzed either through 0.8% alkaline-agarose gel electrophoresis (30 mM NaOH and 5 mM EDTA) or through denaturing 8% PAGE. At the end of the separation, the alkaline gels were neutralized with 1 liter of TBE buffer, dried onto Whatman DE81 filter paper at room temperature for 12 h, and then dried under vacuum at 55 °C for 1 h. Autoradiography was obtained using a Molecular Dynamics Storm 860 PhosphorImager system (Amersham Biosciences). The length of the DNA was determined using Quantity One Quantitation Software (Bio-Rad) and comparing it to $^{32}$P-labeled DNA markers.

$[rNTPs]$ Dilution Experiment—Standard DNA replication reactions were carried out at various $rNTP$ concentrations (200, 50, and 12.5 $\mu$M). $[\alpha-^{32}\text{P}]$dCTP (3000 Ci/mmol) was included in the reactions for the detection of the lagging strand synthesis, and was added 1 min after the initiation of the reaction. The reactions were allowed to proceed for another 3 min before being quenched in equal volume of 0.5 M EDTA, pH 8.0. Reaction products were analyzed by electrophoresis on a 0.8% alkaline-agarose gel, and the sizes of the Okazaki fragments were analyzed as described above.

Pre-steady-state DNA Replication Reactions—Pre-steady-state reactions were performed using a KinTek rapid chemical quench-flow instrument. All concentrations are after initiating the reaction. The reaction buffer was as described above. One sample syringe contained 100 nm minicircle template, 4.5 $\mu$M gp32, 1 mM ATP, 100 $\mu$M dNTPs, $[\alpha-^{32}\text{P}]$dGTP (3000 Ci/mmol), and 0.5 M gp59. The other sample syringe contained 240 $\mu$M gp43, 240 $\mu$M gp45 (as trimer), 240 $\mu$M gp44/62, 600 $\mu$M gp41 (as monomer), and 600 $\mu$M gp32. The reaction mixtures were pre-equilibrated at 37 °C. The reactions were initiated by rapid mixing of the contents in two syringes. The replication reaction was allowed to proceed for various time lengths, after which it was stopped by rapid addition of quench solution (pH 8.0, 500 mM EDTA). Reaction aliquots were spotted onto DE81 filter paper for determining the amount of incorporation of radioactivity or analyzed by electrophoresis for assessing the rate of the fork movement as described above.

RESULTS AND DISCUSSION

The Rate of the Fork Movement—One of the concerns with using a 70-mer minicircle (Fig. 1) substrate is whether it has sufficient space for the proper assembly of the replisome (44). The rate of the leading strand fork movement is a useful parameter for assessing the replisome assembly. Based on earlier results about the structure and stoichiometry of the T4 replisome, we set the following standard reaction condition to study replication on the 70-mer minicircle substrate: 100 nm minicircle substrate, 240 nM each of the holoenzyme components (gp43, gp45 trimer, and gp44/62), 600 nm each of the primosome components (gp41, gp61, and gp59, monomer concentration), and 4.5 $\mu$M gp32 with the stoichiometry of DNA: gp43:gp45:gp44/62:gp41:gp61:gp59:gp32 of 1:2.4:2.4:2.4:6:6:6:45. The rate of the leading strand fork movement was measured under these conditions. Fig. 2A shows a gel of the leading strand fork movement (lanes 1–3). The highest fork rate measured was around 250 nt/s with a KinTek rapid quench instrument (data not shown). The average rate by manual mixing was 150 nt/s (Fig. 2A). The higher rate probably reflects the capture of earlier events by the rapid quench method. The average rate is similar to the fork rate observed on a TRFII M13 template synthesized in our laboratory (57). Taken together, it seems that the fork rate on a minicircle substrate approaches that observed on a larger circular substrate. Other published reports describing T4 DNA replication on a 70-mer minicircle template showed that the fork extended only at a rate of 50 nt/s. Moreover, this rate was not affected by the presence or absence of the primosome, indicating that the loading of the primosome onto the 70-mer minicircle was hampered (44). We note that these reactions were carried out at lower concentrations of both the minicircle substrate and the T4 replication proteins. It therefore appears that for the 70-mer minicircle substrate, higher concentrations of both the minicircle and the replication proteins are needed to assemble the functional replisome.

The need for higher protein component concentrations for the minicircle substrate suggests decreased protein/protein and protein/DNA affinities within the assembled replisome. In particular, the assembly of the primosome and its contacts with the holoenzymes might be altered. We next determined the effect of the primosome components on the rate of the fork movement to test for the capability of the minicircle substrate to accommodate both the holoenzyme and the primosome complexes. The rate of the fork movement was measured for the omission of either gp59 alone or both gp41 and gp59. The order of addition of proteins features the assembly of the holoenzyme prior to that of the primosome. If the size of the minicircle substrate is not big enough for loading both the holoenzyme and the primosome, one would expect that the latter would not assemble. This was clearly not the case under our experimental conditions. Omission of either gp59 or both gp41/gp59 markedly decreased the rate of the fork movement, as compared with the reaction in which all eight proteins were present (Fig. 2, A and B).

As shown in Fig. 2A (lanes 4–9), when gp59 was lowered to 10 nm or was omitted from the reaction, two populations of forks were observed that moved at different rates. The major population moves slowly at a rate of less than 30 nt/s. The minor population moves at a much faster rate of ~150 nt/s. In
order to test whether or not the slower moving fork was due to the absence of the helicase in the replisome, both gp59 and gp41 were omitted from the reaction mixture, and the leading strand fork movement was measured under this condition. The faster moving fork fraction was now completely abolished, and all the forks moved at a rate of less than 30 nt/s (Fig. 2B). This result indicated that the faster moving minor population had gp41 in its replisome spontaneously loaded in the absence or at low concentrations of gp59. Correspondingly, the slower moving forks were defective in gp41 loading and performed DNA synthesis at a much slower rate. This result not only reinforces the role of gp59 in gp41 loading but also demonstrates the assembly of an intact replisome containing both the holoenzyme and the primosome complexes on the 70-mer minicircle DNA in the presence of all eight T4 proteins. Therefore, the strains mentioned above, if present, are not severe enough to affect the proper assembly of the replisome on the minicircle. Note also that the fast moving forks assembled in the absence of gp59 still travel at the same rate as the completely assembled forks, suggesting that gp59 does not affect the fork movement rate on the leading strand.

**Effect of rNTPs Concentrations on the Size of the Okazaki Fragments**—It has been shown in the E. coli replication system that the size of the Okazaki fragments can be altered under conditions that modify the priming on the lagging strand (49). For example, in both the T4 and the E. coli systems, an increase of the size of the Okazaki fragments was observed as the rNTPs concentration decreased (49, 50). A rationale for this response is a slowing in the priming frequency at different rNTPs concentrations (51). Because Okazaki fragment synthesis can only initiate when there is an RNA primer available, a decrease in the primer synthesis rate leads to an increase in the size of the Okazaki fragment.

We studied the size variation of the Okazaki fragments upon dilution of rNTP concentration to test whether or not such a response existed with the minicircle substrate. As shown in Fig. 3, decreasing the rNTPs concentration from 200 to 12.5 μM caused a shift of the average Okazaki fragment size from 1.3 to 2.5 kb. Furthermore, Okazaki fragments of 5–6 kb long were observed at 12.5 μM rNTPs. Such long Okazaki fragments were absent at 200 μM rNTPs.

As presented in the accompanying paper (57), varying gp45, gp44/62, or gp61 concentrations also has a marked effect on the length of the Okazaki fragments in the minicircle system. Priming events had been proposed to govern the repetitive cycle of the Okazaki fragment synthesis through temporally and spatially controlled protein-protein interactions within the replisome (49). The correctly assembled replisome therefore should have the ability to respond to different conditions that modulate the priming events. These results provide additional

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**Fig. 2. The leading strand fork rate.**

A, the leading strand fork rate on the minicircle template. Reactions were carried out under the standard conditions indicated under “Materials and Methods” with [α-32P]dGTP to monitor the leading strand synthesis. Three gp59 concentrations were used in these reactions. Lanes 1–3, 600 nM gp59; lanes 4–6, 10 nM gp59; and lanes 7–9, 0 nM gp59. For each reaction, 3 aliquots were taken at 20, 40, and 60 s. The samples were treated and analyzed on an alkaline-agarose gel as described under “Materials and Methods.”

B, the leading strand fork rate on the minicircle template in the absence of gp41 and gp59. The reaction was carried out under the standard conditions indicated under “Materials and Methods” with [α-32P]dGTP to monitor the leading strand synthesis. Both gp41 and gp59 were omitted from the reaction. Five aliquots were withdrawn from the reaction at 20, 40, 60, 90, and 120 s (lanes 1–5). The samples were treated and analyzed on an alkaline-agarose gel as described under “Materials and Methods.”

**Fig. 3. Effect of rNTPs concentrations on the size of the Okazaki fragments.** Reactions were carried out as described under “Materials and Methods.” [α-32P]dCTP was added into the reaction mixture at 60 s for the measurement of the lagging strand synthesis. Three rNTPs concentrations, 200, 50, and 12.5 μM (lanes 1–3), were used in these reactions. Aliquots were analyzed on an alkaline-agarose gel.
The minicircle system would support coordinated leading and lagging strand syntheses and turned to further optimize the concentrations of the component proteins.

Effects of Varying the Holoenzyme Components—When the holoenzyme components were varied individually, they revealed marked effects on the amount of DNA synthesis as well as on the coordination between leading and lagging strand synthesis (Table I). In these experiments, five gp43 concentrations (24, 56, 120, 240, and 480 nM) and three gp45 (as trimer) and gp44/62 concentrations (28, 84, and 252 nM) were studied. When the concentration of either gp43, gp45, or gp44/62 was decreased, the amount of synthesis of both strands decreased correspondingly (Fig. 6).

For leading strand synthesis, a 9-fold dilution of [gp45] from 252 to 28 nM decreased the rate proportionally 10-fold from 1.9 to 0.20 pmol/s (Fig. 6A). For the clamp loader protein, however, the rate of leading strand synthesis decreased by only 2-fold (from 2.13 to 1.23 pmol/s) after a 9-fold dilution from 252 to 28 nM.
Reactions were carried out in the presence of [3H]dGTP and 480 nM (value of 0.08 by fluorescence resonance energy transfer (52) providing a 240, and 480 nM gp43, respectively; and 0.0036, 0.078, 0.75, 1.66, and 2.23 pmol/s for 30, 60, 120, 240, and 480 nM holoenzyme, respectively.

...the rate of lagging strand synthesis decreased by... 

...decreased only slightly at 120 nM gp43... 1.71, and 2.13 pmol/s for 28, 84, and 252 nM gp45, respectively; 0.42, 0.92, 1.38, 1.45, and 1.56 pmol/s for 24, 56, 120, 240, and 480 nM gp43, respectively; and 0.16, 0.36, 0.89, 1.67, and 1.96 pmol/s for 30, 60, 120, 240, and 480 nM holoenzyme, respectively. The rates of the lagging strand synthesis are as follows: 0.005, 0.26, and 1.88 pmol/s for 28, 84, and 252 nM gp45, respectively; 0.53, 1.43, and 2.00 pmol/s for 28, 84, and 252 nM gp44/62, respectively; 0.21, 0.81, 1.23, 1.36, and 1.53 pmol/s for 24, 56, 120, 240, and 480 nM gp43, respectively; and 0.0036, 0.078, 0.75, 1.86, and 2.23 pmol/s for 30, 60, 120, 240, and 480 nM holoenzyme, respectively.

The effect of changing gp45 levels contrasted sharply with that of gp44/62 (Fig. 6D) and gp43 (Fig. 6F). A 9-fold dilution of...
the kinetics at 0 (Œ 49834 range of gp43 levels from 56 to 480 nM. Apparent respectivly; 0.0054, 0.81, 0.81, 0.79, and 0.68 pmol/s for 0, 114, 285, 570, and 1140 n M gp61, respectively; 0.06, 1.67, 1.86, 1.73, and 0.88 pmol/s

lagging strand synthesis, respectively. Aliquots were withdrawn and spotted onto DE81 filter paper. After washing and drying, the tritium and32P on the minicircle template were carried out under the standard conditions with 0 (concentrations for gp32 were all in "Materials and Methods." The concentrations for gp32 were all in µM. The rates of the leading strand synthesis are as follows: 0.26, 0.91, 1.21, and 1.65 pmol/s for 0, 144, 296, and 600 nM gp41, respectively; 0.64, 0.86, 1.01, 0.89, and 0.76 pmol/s for 0, 114, 285, 570, and 1140 nM gp61, respectively; 0.4, 1.84, 1.67, 1.64, and 0.99 pmol/s for 0, 150, 300, 600, and 1200 nM gp59, respectively; and 0.19, 0.45, 0.71, 1.1, and 1.23 pmol/s for 0, 1.2, 2.4, 4.8, and 7.2 µM gp32, respectively. The rates of the lagging strand synthesis are as follows: 0.0045, 1.04, 1.25, and 1.55 pmol/s for 0, 144, 296, and 600 nM gp41, respectively; 0.0054, 0.81, 0.81, 0.79, and 0.68 pmol/s for 0, 114, 285, 570, and 1140 nM gp61, respectively; 0.06, 1.67, 1.86, 1.73, and 0.88 pmol/s for 0, 150, 300, 600, and 1200 nM gp59, respectively; and 0.0089, 0.21, 0.53, 0.84, and 0.79 pmol/s for 0, 1.2, 2.4, 4.8, and 7.2 µM gp32, respectively.

When the holoenzyme concentration was varied as a whole, i.e. all components gp44/62, gp45, and gp43 were kept at a fixed ratio of 1:1:1 but their level was changed (30, 60, 120, 240, and 480 nM), the effect on the leading and lagging strand synthesis was largely determined by the effect of gp45 (Fig. 6, G and H) which parallels the behavior noted in Fig. 6, A and B. A decrease by a factor of 10 was observed in the rate of the leading strand synthesis as the holoenzyme concentration dropped from 240 to 30 nM. At 30 nM, the lagging strand synthesis was almost completely eliminated. These results further demonstrate that gp45 is the limiting factor in the assembly of the holoenzyme complex.

Effects of Varying the Primosome Components—Next, the primosome components were varied individually to study their effects on the leading and lagging strand synthesis. As expected, when the concentration of either of the primosome components (gp41, gp61, gp32, and gp59) decreased, the lagging strand synthesis also decreased. When they were omitted from the reaction mixture, lagging strand synthesis was largely or entirely eliminated (Table II). All the concentrations below represent the monomeric protein concentrations.

Between 100 and 600 nM, the effects of gp41, gp61, and gp59 were similar on the leading strand synthesis (Fig. 7, A, C, and E). For example, 150 nM of gp59 induced an optimal synthesis rate at the leading strand (1.8 pmol/s), and a further increase to 600 nM did not change the rate significantly. The same pattern was observed with gp61. For gp41, varying the level from 144 to 600 nM only induced less than a 2-fold increase in the leading strand rate. For all three proteins, coordinated synthesis was

Collectively, our results indicate that gp45, when compared with gp43 and gp44/62, has a much more significant effect on lagging strand synthesis. In the accompanying paper (57), we demonstrated that the clamp protein was recruited from solution during repetitive Okazaki fragment synthesis. Presuming that the clamp protein is somehow involved in RNA primer capture, a decrease in the gp45 level would severely affect the efficiency of primer utilization during the lagging strand synthesis. The difference in the stringency for the requirement of gp44/62, was only slightly disrupted at 84 nM gp44/62 when a 9-fold change in gp43 levels (480 to 56 n M). The lagging strand rate decreased by a factor of 10 was observed in the rate of the leading strand synthesis as the holoenzyme concentration dropped from 240 to 30 nM. A decrease by a factor of 10 was observed in the rate of the leading strand synthesis as the holoenzyme concentration dropped from 240 to 30 nM. At 30 nM, the lagging strand synthesis was almost completely eliminated. These results further demonstrate that gp45 is the limiting factor in the assembly of the holoenzyme complex.

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Effects of Varying the Primosome Components—Next, the primosome components were varied individually to study their effects on the leading and lagging strand synthesis. As expected, when the concentration of either of the primosome components (gp41, gp61, gp32, and gp59) decreased, the lagging strand synthesis also decreased. When they were omitted from the reaction mixture, lagging strand synthesis was largely or entirely eliminated (Table II). All the concentrations below represent the monomeric protein concentrations.

Between 100 and 600 nM, the effects of gp41, gp61, and gp59 were similar on the leading strand synthesis (Fig. 7, A, C, and E). For example, 150 nM of gp59 induced an optimal synthesis rate at the leading strand (1.8 pmol/s), and a further increase to 600 nM did not change the rate significantly. The same pattern was observed with gp61. For gp41, varying the level from 144 to 600 nM only induced less than a 2-fold increase in the leading strand rate. For all three proteins, coordinated synthesis was

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maintained between -100 and 600 nM. The only notable difference on leading strand synthesis was when any of these proteins was omitted from the reaction. Omission of gp41 from the reaction decreased the rate of the leading strand synthesis to 0.26 pmol/s and could be attributed to the slowing of the replication fork in the absence of gp41. Because gp59 functions primarily through facilitating gp41 loading, its absence also inhibits leading strand synthesis (0.40 pmol/s). Changing the levels of gp61, on the other hand, did not change significantly the rate of the leading strand synthesis over the entire concentration range studied. These results support the observation that among the primosome components, only the helicase, gp41, is needed to establish the rapid and processive replication by the holoenzyme (17).

The effects of gp41, gp61, and gp59 were also quite similar on the lagging strand synthesis (Fig. 7, B, D, and F). There was virtually no lagging strand synthesis when either gp41 or gp61 was omitted from the reaction. The residual rate observed was probably due to the misincorporation of [32P]dGTP into the lagging strand. Lagging strand synthesis was observed at a very low rate of 0.06 pmol/s in the absence of gp59, a synthesis possibly induced by spontaneous gp41 loading. The rate of lagging strand synthesis increased from a residual rate of 0.0045 pmol/s in the absence of gp41 to 1.04 pmol/s at 144 nM gp41. Further raising the gp41 concentration 4-fold to 600 nM increased the rate of the lagging strand synthesis by only...
The results suggest that, in the presence of the primosome, the function of the leading strand holoenzyme is not inhibited, nor is it stimulated, by the assembly of the third member of the replisome complex, the lagging strand holoenzyme.

The inhibition of leading strand synthesis in the absence of lagging strand after 1 min of reaction can be seen in the relationship of accumulated ssDNA in the reaction mixture which could interact with and sequester the T4 replication proteins (44). Because the fork rate remains constant under these conditions, the inhibition must be due to a decrease in the amount of active complexes in the reaction mixture. Because no significant amount of active complex formation after 1 min of the reaction was observed ($t_{1/2} = 24$ s, see accompanying paper (57)), we suggest that under our conditions it is primarily the collapse of the active forks that contributes to the inhibition of the leading strand synthesis. With the accumulation of ssDNA, gp32 is most likely to be the protein that is trapped. Our DNase I footprinting experiments show that among the four primosome proteins (gp41, gp61, gp32, and gp59), only gp32 forms a stable complex with ssDNA and protects it from nuclease digestion (data not shown). Although gp41, gp61, and gp59 are all known to bind ssDNA, they may have fast off rates so that no footprinting was observed.

Several lines of evidence suggested that gp32 was involved in the organization of the replisome. Indeed, gp32 has interactions with a number of T4 replication proteins (22, 28–29) and has an active role in facilitating holoenzyme assembly and improving its stability (53, 54). An earlier study on gp32 demonstrated the importance of this protein on the replication of the minicircle (39). Data generated in this study show that lowering [gp32] decreased significantly the rate of not only the lagging strand synthesis (0.0089 pmol/s) and decreased the leading strand rate by 5-fold. The trapping of gp32 from the replisome could disrupt the protein-protein interactions within the replisome and lead to its collapse. Further studies will likely reveal the detailed functions of gp32 within the replisome.

Results from the above experiments indicate that the rates of the leading and lagging strand synthesis vary at different protein concentrations, and the coordinated synthesis can be supported only within certain protein concentration ranges. Specifically, about 200 nM each of the holoenzyme components (gp43, gp45, and gp44/62) and between 100 and 600 nM each of the primosome components gp41, gp61, and gp59 are needed for coordinated synthesis on 100 nM of minicircle template. Higher concentrations of primosome proteins generally inhibit replication, whereas lower concentrations of replisome proteins disrupt coordinated synthesis. Our results also suggest that the trapping of proteins such as gp32 by ssDNA could result in the collapse of the active replisome. DNA replication requires the coordinated actions of multiple macromolecular com-

TABLE III

| Cellular concentrations of T4 replication proteins |
|-----------------------------------------------|
|                                             |
| Molecules/cell$^b$ | Cellular concentrations$^a$ | Experimental concentrations$^c$ |
|---------------------|---------------------------|-------------------------------|
| Single-stranded binding protein (gp32)         | 10,000 | 8.5 μM | 4.5 μM |
| Polymerase (gp43) | 790   | 676 nm | 240 nm |
| Clamp protein (gp45) | 10,200 | 8.7 μM | 720 nm |
| Clamp loader protein (gp44) | 2,900 | 2.5 μM | 960 nm |
| Clamp loader protein (gp62) | 700   | 600 nm | 240 nm |
| Helicase (gp41) | 810   | 600 nm | 600 nm |
| Primase (gp61) | 170   | 146 nm | 100–600 nm |

$^a$ Copy numbers of cellular replication proteins are from Burke et al. (55).

$^b$ The cellular protein concentrations are calculated based on a cell volume of $1.94 \times 10^{-12}$ ml. The in vivo replication fork concentration is estimated to be 61 nm (55).

$^c$ Optimal protein concentrations determined in this work. For multimeric proteins, the concentrations listed represent the monomeric concentrations.

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3 F. T. Ishmael and M. A. Trakselis, unpublished results.
CONCLUSIONS

Reconstituted in vitro T4 bacteriophage DNA replication system with eight replication proteins on a 70-mer minicircle template is shown here to be active and capable of coordinated synthesis of both the leading and lagging strand. The coordination between leading and lagging strand synthesis was shown by the identical rate of the nucleotide incorporation into both strands, as well as by the presence of a distinct lag phase of lagging strand synthesis. Under optimal conditions, the rate of the replication fork extension averages at 150 nt/s among different measurements with the highest measured rate of 250 nt/s. The average fork rate is similar to that measured on a TRF II M13 replication fork in our laboratory. It is possible that the small size of the minicircle substrate may create strains within the replisome. However, these strains, if present, are not severe enough to cause the collapse of the replisome complex. Our results have shown that both the holoenzyme and the primosome can be loaded simultaneously on the 70-mer minicircle DNA. Furthermore, the replication fork responds to the change in rNTP concentrations by producing longer Okazaki fragments at lower [rNTPs]. This result suggests functional lagging strand synthesis executed by the replisome complex reconstituted on the minicircle substrate. As shown in the accompanying paper (57), the size of the Okazaki fragments also varies with varying concentrations of certain other replisome proteins. All of these results suggest that a 70-mer minicircle template is a useful substrate for the study of the coordinated DNA synthesis in the T4 system.

Both leading and lagging strand synthesis respond to the variation in concentration of individual replisome protein components. We have identified the concentration ranges of various replisome components within which the coordinated synthesis can be supported. The concentration effect on the strand synthesis was most significant for gp45 among the three holoenzyme components, suggesting that gp45 is the limiting factor in holoenzyme assembly. This result is also in agreement with the distributive nature of gp45 during the repetitive lagging strand synthesis. The effects of gp41, gp61, and gp59 were very similar on both strand synthesis as well as on the coupling between leading and lagging strand, providing kinetic evidence for a 1:1 stoichiometry of these three proteins during primosome assembly. Our determined protein concentration ranges generally agree with those found in vivo, suggesting that optimal system equilibria are achieved during coordinated replication.

Blocking lagging strand synthesis by omitting gp61 or rNTPs from the reaction mixture did not inhibit the leading strand synthesis during early stage of the reaction (0–30 s). Neither the rate of dNMP incorporation nor the rate of leading strand fork movement was affected. The inhibition became significant after 1 min when the reaction proceeded into the steady-state phase. We suggest that the accumulation of ssDNA in the reaction traps replication proteins, in particular gp32, and that this trapping can lead to the collapse of the active replisomes.

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