Architecture of the Replication Complex and DNA Loops at the Fork Generated by the Bacteriophage T4 Proteins*

Rolling circle replication has previously been reconstituted in vitro using M13 duplex circles containing preformed forks and the 10 purified T4 bacteriophage replication proteins. Leading and lagging strand synthesis in these reactions is coupled and the size of the Okazaki fragments produced is typical of those generated in T4 infections. In this study the structure of the DNAs and DNA-protein complexes engaged in these in vitro reactions has been examined by electron microscopy. Following deproteinization, circular duplex templates with linear tails as great as 100 kb are observed. The tails are fully duplex except for one to three single-stranded DNA segments close to the fork. This pattern reflects Okazaki fragments stopped at different stages in their synthesis. Examination of the DNA-protein complexes in these reactions reveals M13 duplex circles in which 64% contain a single large protein mass (replication complex) and a linear duplex tail. In 56% of the replicating molecules with a tail there is at least one fully duplex loop at the replication complex resulting from the portion of the lagging strand engaged in Okazaki fragment synthesis folding back to the replisome. The single-stranded DNA segments at the fork bound by gene 32 and 39 proteins are not extended but rather appear organized into highly compact structures (“bobbins”). These bobbins constitute a major portion of the mass of the full replication complex.

DNA replication is accomplished by a highly organized DNA-protein ensemble, operating as a molecular machine that replicates both strands in a highly coordinated manner. How the large number of protein species at a DNA replication fork, perhaps as many as 20 in Escherichia coli, and several kilobases of DNA strands, are arranged remains poorly understood. To accommodate the requirement that the two strands must be copied in the same enzymatic (5’ to 3’) but opposite physical direction, Alberts and co-workers (1–3) proposed that the polymerase synthesizing the lagging strand remains in contact with the leading strand polymerase at the fork. This would fold the lagging strand into a loop and couple leading and lagging strand synthesis. The loop would grow as the Okazaki fragment lengthens, then collapse as the replication machinery moves from the mature Okazaki fragment to the next to be completed. Because of the visual resemblance to a trombone slide, this model has been termed the “trombone model.”

Evidence for the coupling of leading and lagging strand synthesis and a lagging strand loop has come from physical and biochemical studies. The presence of two DNA polymerase III cores within one holoenzyme particle has been demonstrated in the E. coli replication system (4–7) and both polymerases within the complex can function simultaneously (4). Early evidence for the coordination of leading and lagging strand synthesis obtained in E. coli (5, 8, 9), T4 (2, 10, 11), and T7 (12) was based on gel electrophoretic demonstrations that the average length distribution of Okazaki fragments did not change upon dilution of the polymerase or the helicase/primase. More direct evidence has come from recent studies with the purified T7 proteins and a miniature 70-bp synthetic circular template in which incorporation into the leading and lagging strands could be followed because of a strand bias (13, 14). The incorporation of guanosine residues provided a direct measure of leading strand synthesis, and cytosine residues measured synthesis of the lagging strand. Using this template, it was shown that optimal synthesis in a rolling circle mode required a strict coupling of synthesis of both strands. A similar conclusion was later reached with the T4 replication proteins (15).

Using the purified T7 replication proteins the DNA-protein complexes engaged in rolling circle replication from circular templates containing preformed forks have been examined by electron microscopy (EM). Using an M13 template of 7.2 kb (16) or the synthetic 70-bp mini-circle (13, 14) these studies revealed a DNA loop at the fork associated with a replication complex. It was formally shown using these template molecules that the replication loops found at the junction originated from newly synthesized DNA, and are the result of the lagging strand folding back on itself. For both templates the loop size was similar and consistent with the duplex portion of a single Okazaki fragment (around 1500 nt) as determined by parallel gel analysis. Many questions remain, however, concerning the arrangement of the DNA strands and proteins at a replication fork. Are the loops visualized with the relatively simple T7 system specific to T7 or typical of more complex systems such as T4 and E. coli? In the T7 studies, the single-stranded DNA (ssDNA) segments at the fork were not extended as predicted in the original models but rather were in compact bodies that were termed “bobbins.” This may reflect the weaker binding of the T7 gene 2.5 ss-binding protein as contrasted to the robust binding of the T4 gene 32 or E. coli SSB proteins to ssDNA, or may represent a general feature of DNA replication machines.

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§The abbreviations used are: EM, electron microscopy; ds, double-stranded; ss, single-stranded; nt, nucleotide(s).
Bacteriophage T4 provides a natural extension with which to examine the generality of the findings made with T7. It is replicated by 10 different proteins including a sliding clamp and clamp loading system typical of E. coli and complex eukaryotic systems; each of the 10 proteins has been extensively purified and studied (reviewed in Refs. 17 and 18). Synthesis on both strands is catalyzed by T4 DNA polymerase, a relatively non-processive enzyme with an associated 3'→5' editing exonuclease. The 44/62 protein complex, a ssDNA-dependent ATPase, acts catalytically as a clamp loader to secure the trimer of 45 protein as a ring-shaped clamp that binds directly to the polymerase, greatly increasing its processivity (19–22).

Gene 32 protein, a cooperative ssDNA-binding protein, stimulates clamp loading, and also increases polymerase processivity itself by destabilizing secondary structure in the template. T4 polymerase alone is not capable of strand displacement synthesis. Efficient synthesis of both the leading and lagging strands requires the clamp, clamp-loader, 32 protein, 41 helicase, and 61 primase, in addition to polymerase. Finally, gene 59 protein likely plays several functions in replication. It binds preferentially to fork DNA (23, 24) and aids in loading 41 helicase onto the DNA (25, 26). The 59 protein also binds ssDNA but with an affinity less than that of 32 protein (27). In vitro replication is much slower in the absence of 59 protein because of the delayed loading of the helicase.

In this study we have utilized a similar approach to that employed in Park et al. (16) with the T7 replication proteins. An M13 circular template with a pre-formed fork was incubated with the highly purified T4 replication proteins, and the nature of the DNA products and DNA-protein complexes engaged in replication was examined by EM. We describe the first visualization of a trombone loop in the T4 system and the finding that the ssDNA segments involved in Okazaki fragment synthesis are organized into compact “bobbins.”

**EXPERIMENTAL PROCEDURES**

Reagents and Enzymes—Unlabeled nucleoside triphosphates and T4 DNA ligase were obtained from Amersham Biosciences, and [α-32P]dCTP and dTTP from PerkinElmer Life Sciences. The bacteriophage T4 gene 32 ssDNA-binding protein, 41 helicase, 43 DNA polymerase, 44/62 clamp-loader, 45 clamp, 59 helicase loading protein, 61 primase, and RNase H proteins were purified to homogeneity as described elsewhere (28). All proteins were free of detectable endonuclease activity. The 44/62 and 45 proteins were prepared from T4-infected cells, whereas the other proteins were the products of cloned T4 genes expressed on plasmids in E. coli.

**DNA Synthesis and Gel Analysis—**Generation of the M13mp2 circular duplex template with a pre-formed fork, buffers, and DNA synthesis conditions with the purified T4 proteins are described elsewhere (29). Reaction mixtures including M13mp2 ssDNA with an 84-nt forked primer (1.6 nt), the 44/62 clamp loader (242 nt), 45 clamp (162 nt), and the 32 (2 µm) and 59 (95 nt) proteins were incubated for 2 min at 37 °C before synthesis was begun by addition of a mixture of polymerase (30 nt), 41 helicase (328 nt monomer), 61 primase (64 nt), and RNase H (186 nt) and ligase (416 nt), unless otherwise indicated. At times from 2 to 5 min, aliquots of the reaction mixtures were mixed with an equal volume of 0.2 mM EDTA to stop the synthesis. Samples were either fixed for EM (below), analyzed by alkaline-agarose gel electrophoresis, or deproteinized by treating with SDS and proteinase K at a final concentration of 1% and 1 mg/ml, respectively, at 55 °C for 60 min, filtered through 2-ml columns of Bio-Gel A-5m (Bio-Rad) equilibrated with 10 mM Tris, pH 7.5, 1 mM EDTA, and then prepared for EM as described below.

**Electron Microscopy—**Following addition of EDTA to the reaction mixtures, the products were treated with glutaraldehyde (0.6% final concentration) for 5 min at 22 °C, and then filtered through 2-ml columns of Bio-Gel A-5m (Bio-Rad) equilibrated with 10 mM Tris, pH 7.5, 1 mM EDTA. The fixed samples were adsorbed to thin carbon foils, washed, air-dried, and rotary shadowcast with tungsten at high vac (30). Deproteinized samples were complexed with E. coli SSB protein (16) and prepared for EM as above. Samples were examined in a Phillips CM12 at 40 kV. Length measurements were made by capturing the images with a Gatan 794 CCD camera attached to the CM12 and using Digital Micrograph 3.3 (Gatan Inc., Pleasanton CA). Images for publication were captured on sheet film, scanned with a Nikon LS4500 film scanner, and the contrast optimized and panels arranged using Adobe Photoshop software.

**RESULTS**

**Gel Electrophoretic Analysis of Replication Reactions Catalyzed by the T4 Proteins—**Alkaline-agarose gel electrophoresis provides a direct means of following the synthesis of new DNA fragments in a rolling circle replication reaction catalyzed by the purified T4 replication proteins. This information was used to optimize the reactions for EM analysis. RNase H and ligase were omitted to allow detection of the individual fragments by electrophoresis but were included in the reactions for EM. The template for the gel and EM studies is an M13mp2 ssDNA circle to which an 84-nt primer is annealed. Only the 3’34 nt of the primer are complementary to the ssDNA leaving a 50-nt unpaired tail. Once the ssDNA template has been copied, this short tail is sufficient to allow rapid synchronous strand displacement by the T4 replication enzymes (26, 29). To follow the synthesis of nascent DNA fragments, the primed DNA was incubated for 1, 2, or 4 min (Fig. 1) with the T4 32 ssDNA-binding protein, 44/62 clamp-loader, 45 clamp, 59 helicase loading protein, primase, 41 helicase, and 61 primase, as described under “Experimental Procedures.” In the absence of helicase (reaction 4, Fig. 1A), polymerase with the clamp, clamp-loader, and 32 protein completes synthesis of the double-stranded DNA (dsDNA) circle within the first minute, and begins slow leading strand displacement synthesis (as shown by the products longer than 7.2 kb on the alkaline-agarose gel).

There are no short lagging fragments without the helicase, even though primase is present, because both helicase and primase are required for primer synthesis. In reaction 2, helicase is present without 59 protein. In the first 4 min most of the leading strands are growing at the slow rate that does not require helicase. However, at 4 min there are some longer leading strands and detectable shorter lagging strand fragments, showing that the helicase has loaded on some molecules. In the complete reaction (reaction 1) with 59 protein present, the helicase is loaded immediately, so as soon as the ds circle is completed at 1 min, there are leading strand products too large to have been made without helicase, and there are short lagging strand fragments. In the absence of primase (reaction 3), the helicase is loaded efficiently by the 59 loader, but there are no lagging strand products. Gel analysis (not shown) revealed that synthesis was equally efficient with 2 or 6 µM 32 protein or twice the concentration of primase or twice the concentration of both primase and polymerase.

T4 RNase H removes the RNA primers and about 30 nt of the adjacent DNA from the ends of the Okazaki fragments (31). In the presence of T4 RNase H and ligase (Fig. 1B), a majority of the Okazaki lagging strand fragments are sealed together, as shown by the absence of short lagging strand fragments, and the increase in longer products. The band running below 6.7 kb is denatured closed circular dsDNA, from molecules that did not begin strand displacement synthesis.

**Electron Microscopic Analysis of Deproteinized Replication Products—**The architecture of the DNA intermediates present in rolling circle replication reactions including the structure of the template and pattern of ss and ds segments on the replicating tail can be directly visualized by EM when the ss segments of deproteinized molecules are extended and thickened by E. coli SSB protein (14, 16, 29). Examples are shown in Fig. 2 and related to successive stages of Okazaki fragment synthesis (Fig. 3).

Replication reactions with the primed M13mp2 ssDNA template and the T4 replication proteins including RNase H and ligase were carried out 4 min at 37 °C. Inspection of fields of...
EM Visualization of the T4 Replisome

Fig. 1. Alkaline-agarose gels of the products of DNA synthesis with a primed M13 single-stranded circular template by the T4 replication proteins. A, T4 59 helicase loading protein, 41 helicase, and 61 primase are required for efficient leading and lagging strand synthesis. The template is M13mp2 ssDNA annealed to an 84-nt primer whose 3' 34 nt are complementary, leaving a 50-nt 5' tail. Reactions contained T4 DNA polymerase, 45 clamp, 44/62 clamp loader, and 32 single-stranded DNA-binding protein, in addition to 59 protein, helicase, and primase as indicated. Reaction conditions are described under “Experimental Procedures.” Products were separated on a 0.6% alkaline-agarose gel. B, lagging strand fragments are sealed in reactions with T4 RNase H and DNA ligase. Reaction conditions as in A, with the addition of T4 RNase H and DNA ligase, are as indicated. The band running below 6.7 kb is denatured closed circular dsDNA, from molecules that did not begin rolling circle strand displacement synthesis.

deproteinized DNA products revealed that 75% (n = 105) of the M13mp2 ssDNA circles had been converted to fully ds circles (the remaining products consist of M13mp2 ss circles (2%) and partially ss and partially dsDNA circles (23%)). Approximately 80% of the ds circles contained tails that were often 100 kb or longer as measured by EM. The tails were fully duplex with the exception of one or more ss segments located at or near the fork that had an averaged, combined length of ~2500 nt. The long ds tails reflect highly coupled leading/lagging strand replication. When primase was omitted, very long fully ss tails were present (not shown).

The pattern of ss segments proximal to the fork can be related to the distinct stages of Okazaki fragment synthesis at the point synthesis was stopped. Molecules consisting of a ds circle with one ss segment separating the template circle from a long fully ds tail correspond to ones that had finished replicating the last Okazaki fragment and had not initiated synthesis of a new fragment (Fig. 2A, and Fig. 3, IV), or had a new fragment too short to be seen (Fig. 3, I and II). The ss portion represents DNA that was unwound by the helicase after the last lagging strand fragment was initiated. Scoring 494 ds circles with tails, 43% fell into this class (Table I). A similar fraction (31%) consisted of molecules with two ss segments along the tail: one attaching the tail to the template circle and the second downstream on the tail separated from the first by a dsDNA segment (Table I, Fig. 2B, Fig. 3, III). These molecules represent ones in which the ssDNA closest to the fork is being generated by helicase action, whereas the distal ssDNA is being converted to dsDNA by the synthesis of a nascent Okazaki fragment. No products were observed in which a ds tail was joined directly to the template circle, rather the junction was always single-stranded. Finally (Fig. 2C, Table I), 17% of the replication products contained 3 ss segments and 9% contained 4 or more near the fork. These forms are not expected if only one Okazaki fragment is being synthesized at a time and their significance is discussed below. The same analysis was carried out for reactions in which the concentration of primase was increased 2-fold or decreased 8-fold, and in which the concentration of both primase and polymerase was increased 2-fold above the standard conditions (Table I). Scoring >150 replicating molecules for each experiment, the percentage of molecules with 1 to 4 or more ss segments on the replicating tail was relatively unchanged by these alterations in replication conditions.

If the lagging strand is folded into a trombone loop, then the number and lengths of the ds and ss segments at the fork in deproteinized molecules (in which the trombone loop would be lost) should correspond to the size of putative loop(s) that would be visualized at the fork when the proteins are fixed in place (Fig. 4). In anticipation of this analysis below, these measurements were made for DNA products from 4-min replication reactions under the standard concentration of replication proteins used here (Fig. 4B). For molecules containing only one ss segment, the range in lengths of the ssDNA fell into the range expected for a full-length Okazaki fragment (Fig. 4A, top). The average measured length (3 kb) was somewhat longer than the average gel fragment length (2 kb), probably because there were RNA primers or ssDNA, too short to be seen, within some of the longer ssDNA segments. For molecules with two ss segments separated by one ds segment, the sum of the ds segment plus the ss segment farthest from the fork also corresponded to the size of putative loop(s) that would be visualized at the fork when the proteins are fixed in place (Fig. 4). In anticipation of this analysis below, these measurements were made for DNA products from 4-min replication reactions under the standard concentration of replication proteins used here (Fig. 4B). For molecules containing only one ss segment, the range in lengths of the ssDNA fell into the range expected for a full-length Okazaki fragment (Fig. 4A, top). The average measured length (3 kb) was somewhat longer than the average gel fragment length (2 kb), probably because there were RNA primers or ssDNA, too short to be seen, within some of the longer ssDNA segments. For molecules with two ss segments separated by one ds segment, the sum of the ds segment plus the ss segment farthest from the fork also corresponded to the average Okazaki fragment size observed on the gels (Fig. 4A, middle). In molecules with 3 or 4 ss segments (Fig. 4A, bottom) (not expected based on the simplest trombone model, e.g. Fig. 3), the ds segment closest to the fork and adjacent ss segment were almost always shorter than the downstream ds and adjacent ss segments. This would be expected if the ds/ss pair closest to the fork represents an Okazaki fragment that began before the downstream ds/ss pair was completed.
EM Visualization of the T4 Replisome

Fig. 2. Visualization of deproteinated rolling circle replication intermediates. Intermediates of rolling circle replication were generated in a 4-min reaction as described in the legend to Fig. 1. The reactions were stopped and the DNA deproteinized and purified by gel filtration. *E. coli* SSB protein was added to thicken and extend the ssDNA regions, followed by preparation for EM including mounting on thin carbon foils, washing, and rotary shadowcasting with tungsten. Micrographs of molecules containing 1, 2, and 4 ss regions are in A–C, respectively. White arrows point to the filled-in template circle. Shown in reverse contrast, bar equals a length of dsDNA equivalent to 1000 bp.

Fig. 3. Model for trombone loop formation during lagging strand synthesis. I, an RNA primer is made on the lagging strand by the coordinated activity of the primase-helicase. (Numbers denote the T4 gene encoding the protein.) II, a replication loop is formed when the lagging strand DNA polymerase binds to and begins elongation of the RNA primer. The replication loop has both ssDNA (A) and dsDNA (B) elements. III, as more lagging strand synthesis occurs, the length of the dsDNA portion of the loop (B) increases, whereas the length of the ssDNA template ahead (C) decreases. The ssDNA in the loop (A) continues to increase in length as the helicase at the fork unwinds the duplex IV, the lagging strand polymerase completes the current fragment when it reaches the end of the previous fragment (D), and then dissociates, thereby dissolving the trombone loop as shown in I. It is currently not known whether the primer for the next fragment is made before or after the loop is dissolved. This model is adapted from the trombone model originally proposed by Alberts coworkers (1–3).

Visualization of Loops and a Replication Complex at the T4 Fork—Whereas deproteinized intermediates from in vitro replication reactions with the T4 proteins have been studied by EM (29, 32), the DNA-protein complexes prepared by fixing the proteins in place prior to preparation by EM have not been systematically examined. To do so, replication reactions were carried out for 4 min using the concentrations of proteins optimized above, followed by fixation, gel filtration to remove free proteins, and preparation for EM including rotary tungsten shadowcasting (Fig. 5). Examination of fields of replicating molecules revealed several different DNA forms, most with a dense mass (replication complex). At 4 min, ~30% of the molecules scored (*n* = 105) consisted of M13mp2 circles that were in the process of being converted into ds templates, and 6% were fully duplex but without a rolling circle replication tail. Most of the DNAs (~64%) consisted of ds M13mp2 circles with a long, fully ds tail exiting a single dense mass bound along the circle (Fig. 5). These ds tails measured up to 100 kb in length.

In a larger sample (*n* = 321) of template circles exhibiting a replication complex and tail, 56% contained one or more DNA loops (Fig. 5, A and C–E) exiting and entering the replication complex at the fork. The loops were fully duplex and did not contain segments consisting of ssDNA extended by 32 protein segments (see below). There was no loop associated with the replication complex in the remaining molecules (Fig. 5B). The frequency of the different species was not altered significantly by decreasing the amount of primase 8-fold (not shown).

**Replication Loops on the Lagging Strand Correspond to Okazaki Fragment Synthesis**—In the trombone model, the replication fork loop is generated by a fold-back of the lagging strand. Because a loop could be created by the template circle flopping over onto itself, we required that the combined length of the template and the loop be greater than the template alone for a molecule to be scored as containing a replication loop. A comparison of the deproteinized and protein-fixed molecules (Fig. 4) showed a good correlation between the frequency of ds template circles with a replication complex and tail but no loop, and in the deproteinized molecules, examples appearing as ds circles with a ds tail attached by a single ss segment (Fig. 4, A and B, top). There was also a good correlation between the fraction of molecules that had a single ds loop at the fork and molecules which, following deproteinization, contained a ds segment flanked by two ss segments (Fig. 4, A and B, middle). A histogram of 50 loop lengths for molecules containing only
Intermediates in rolling circle replication reactions were generated as in Fig. 1 for 4 min using the standard amount of polymerase and primase (“Experimental Procedures”), 1/4th the amount of primase, 2 times the amount of primase, or 2 times the amount of polymerase and primase. The samples were deproteinized and the ss segments stained with SSB followed by preparation for EM (see Fig. 2). Molecules were scored and categorized by the number of ssDNA gaps between the template and the fully duplexed DNA tail.

| % Molecules scored | None | One | Two | Three | Four or more | n = |
|-------------------|------|-----|-----|------|-------------|-----|
| Standard*         | 0    | 43  | 31  | 17   | 9           | 494 |
| 0.125× Primase    | 0    | 36  | 31  | 17   | 16          | 370 |
| 2× Primase        | 1    | 43  | 28  | 16   | 12          | 422 |
| 2× Primase, 2× polymerase | 1  | 41  | 29  | 16   | 13          | 170 |

* See “Experimental Procedures.”
FIG. 5. Visualization of rolling circle replication intermediates generated using the full complement of T4 replication proteins. A–E, intermediates of rolling circle replication were prepared as described in the legend to Fig. 1 for 4 min followed by preparation for EM as described in the legend to Fig. 2. Intermediates were present with 0 (B), 1 (A, C, and D), or 2 (E) or more replication loops. D, to visually compare the size of the replisome to a known size standard, apoferritin (443 kDa) was added to an aliquot of the reaction mixture prior to preparing the samples for EM. White arrows denote the location of apoferritin in D, and the location of replication loops in E. Shown in reverse contrast, bar equals a length of dsDNA equivalent to 1000 bp.
at the fork, 8% (n the loops arise from a folding of the lagging strand.

ment lengths in deproteinized replication intermediates. Rolling circle replication intermediates were prepared as described in the legends to Figs. 1 and 5, including preparation for EM. Aliquots of the samples were deproteinized, and the DNA was purified by gel filtration followed by staining the ss segments with SSB protein and preparation for EM. In the samples with the replication proteins fixed in place, the length of the dsDNA loop in molecules containing one loop was measured (black bars) and compared with the length of the dsDNA segment closest to the template circle in the deproteinized intermediates containing one ds segment near the fork flanked by 2 ss segments (gray bars). To generate the histogram, the dsDNA loop or dsDNA segment lengths were scored as having a dsDNA length between 1 and 425 bp, between 425 to 850, etc., and then the total number scored for each size range was divided by the total number of loop or segment lengths analyzed to determine the percentage of DNA molecules measured that fell within that length range.

the loops arise from a folding of the lagging strand.

Whereas 43% of the replicating molecules contained one loop at the fork, 8% (n = 321) contained two, and 5% contained more than two (Fig. 4, B, bottom, and E). The trombone models do not predict the presence of multiple loops nor do they account for molecules with 3 or more ss segments along the replicating tail following deproteinization. Because the frequencies of these species are similar, it is possible that molecules with multiple loops may be related to molecules with multiple ss segments on the rolling circle tail. Several possible explanations for these multiple loops and ss segments are described below (“Discussion”).

The Replication Complex Contains ssDNA Organized into Compact Bobbins—The trombone models were based on the T4 system, and as originally drawn, the lagging strand loop should consist of a ds and ss segment, with the latter being extended by the binding of 32 protein (see Fig. 3). A second 32 protein-bound ss segment, corresponding to the template for the nascent fragment, was supposed to be present along the rolling circle tail. Counter to this expectation, the loops and tails were fully duplex and no instances were observed in which there were extended ss segments, typical of what is seen when ssDNA is bound by saturating amounts of 32 protein (33). Because deproteinized molecules from the same reactions had the expected pattern of ss and ds segments, the ss segments must reside within the large replication complex at the fork. This assumption is reasonable because the replication complexes appeared much larger than what would be expected for a single complex of the T4 replication proteins alone. Whereas the replication complexes had a variety of shapes, in nearly all cases (>90%) they were highly compact.

The core replication complex would be expected to contain two copies each of the monomeric T4 DNA polymerase and the trimeric 45 clamp, with a total molecular mass of 354 kDa. At present it is not known whether the clamp-loader (165 kDa) remains with the complex. The primosome would contain a hexamer of 41 helicase, and one or more copies of the 61 primase and the 59 helicase loader. Assuming six copies of each protein, the primosome would be 714 kDa, giving a combined size for the core complex a maximum of 1068 kDa. The calculated mass of a 2000-nt segment of ssDNA covered by a maximum of 286 molecules of 32 protein would be 10,280 kDa, giving the largest full replication complex an estimated mass of 11,350 kDa. To provide a rough mass estimate for these complexes, apoferritin (443 kDa), which is easily distinguished by EM, was mixed with the replication complexes and micrographs taken in which apoferritin molecules lay adjacent to replication complexes. On the micrographs the projected areas of the apoferritin molecules and replication complexes were measured and compared as described in Griffith et al. (34) (Fig. 5D). The average projected area of the replication complex was 11 times that of apoferritin, roughly equivalent to a replication complex containing the full complement of replication proteins and ~740 nt of ssDNA covered with 32 protein.

To demonstrate the presence of ssDNA in the replication complexes, we used a brief treatment with higher salt. In vitro replication with the T4 proteins was carried out with 25 mM Hepes buffer, 63 mM potassium acetate, and 6 mM magnesium acetate. Because 32 protein remains bound to ssDNA in 1 x salt it should not be released by a severalfold increase in salt concentration in the replication reaction. Following a 2-min incubation, EDTA was added to 20 mM, and Hepes buffer and potassium acetate were added to 100 and 252 mM, respectively. The samples were left for 30 s to 5 min and then fixed and prepared for EM. Replicating DNAs were frequently observed in which the replication complex had opened significantly and loops or segments of ssDNA bound, it appeared, by 32 protein and likely 59 protein (see below) were observed separated from the rest of the replication complex (Fig. 7A). These images demonstrate that the ss segments are indeed present in compact structures tightly associated with the fork and replication proteins under conditions of optimal synthesis.

Generation of the Bobbins Requires the Complete, Active Replisome—In our T4 replication reactions, the primed template was incubated for 2 min at 37 °C with the 45 clamp, the 44/62 clamp loader, and 32 and 59 proteins, before addition of a mixture of polymerase, helicase, primase, RNase H, and ligase (see “Experimental Procedures”). A clue into the nature of the compact bobbins at the fork arose from examining the molecules just prior to the addition of these other proteins (Fig. 7D). These ss circles appeared open and extended in structure, not unlike what is seen when just the 32 or the 32 and 59 proteins are incubated with ssDNA in the presence of magnesium under these conditions (data not shown). This observation argues that the compact bobbins are not a simple product of the binding of 32 and 59 proteins to ssDNA under replication conditions. It should be noted that Lefebvre et al. (27) did observe compact structures when 59 and 32 proteins were added together to M13 ssDNA. However, they used a much higher concentration of 59 protein (2.25 μM versus 95 nM) that would severely inhibit replication under our conditions.

To further investigate the requirements for bobbin formation, full replication reactions were carried out for 2 to 15 min in the absence of 59 protein. Whereas many of the ss circles did not initiate rolling circle replication because of the failure to load helicase (see Fig. 1), some did and in those (Fig. 7B) the ssDNA in the replication complex appeared more open and extended than when 59 was present. An extreme case was observed when the full replication reactions were carried out for 4 min in the absence of primase. Here, very large masses of what appeared to be partially extended ssDNA bound by 32
...likely some 59 protein was seen associated with circular ds templates (Fig. 7C). These observations point to a model in which the generation of the compact bobbins requires the presence of the full T4 replication machinery engaged in synthesis. The details of the generation of this structure will be described in a future publication.
DISCUSSION

In this paper we utilized the highly purified T4 replication proteins to probe the architecture of the DNA strands and protein complex at a rolling circle replication fork. A 7.3-kb M13mp2 dsDNA circle with a pre-formed fork was used as the template. As shown by electrophoretic analysis, this resulted in highly coordinated/coupled synthesis and generation of Okazaki fragments of a size (average 2 kb) observed in previous studies. EM revealed that 64% of the forked ssDNA templates were able to initiate and undergo rolling circle replication as evidenced by the presence of a fully duplex template, a large replication complex at a single site and a long rolling circle tail that was fully duplex except for short single-stranded segments near the fork. A single DNA loop arising from the lagging strand folding back was associated with the replication complex in ~40% of the replicating molecules. Two major observations reported here are the presence of these loops at the fork, predicted originally for the T4 system but heretofore not demonstrated and second, the highly compact nature of the ssDNA segments at the fork.

This study extends a number of previous studies of in vitro DNA replication using the T4, and T7 systems. In an EM study of T4 replication, Selik et al. (32) visualized deproteinized intermediates from reactions catalyzed by some but not all of the T4 replication proteins employed here. In particular, the role of 59 protein was not appreciated at that time and was not included. They described rolling circle replication intermediates containing one or two ss segments along otherwise long duplex tails and the lengths of the ss and ds segments are in the same general range as reported here and in Chastain et al. (29). Selik et al. (32) observed three times more molecules with one than two ss segments, leading them to conclude that elongation of lagging strand fragments is more rapid than elongation of the leading strand. In contrast, we found approximately equal numbers of molecules with one or two ss segments in deproteinized samples, consistent with our observation of similar numbers of no-loop and one-loop molecules in protein-DNA fixed samples. This difference may reflect more rapid leading strand synthesis and more efficient priming of the next Okazaki fragment in the presence of 59 protein.

These studies also extend the work that we described in Chastain et al. (29). Using the same set of proteins and M13mp2 template to analyze the spacing of adjacent Okazaki fragments along the rolling circle tails, an exonuclease-defective mutant of T4 polymerase was employed to help mark the junctions between Okazaki fragments, but otherwise the mutant protein did not affect the overall rate or extent of Okazaki fragment synthesis. We found that the length distribution of fragments along individual molecules was as great as the fragment length distribution in the population of molecules. The mean size of the fragments measured in the present study is the same as we reported there and thus the two reports complement each other very well.

The original proposal that the lagging strand at a fork loops back to engage the moving replication complex was based on consideration of the mechanical constraints of replication and the specifics of the T4 system (1–3). Hence it was particularly pleasing to visualize these loops for the first time. Most of the loops at the replication fork fell into a size range of 700 to 1500 nt, which also corresponded to the major fraction of the lengths of the duplex segment of the nascent Okazaki fragments in these reactions (Fig. 4). The variation in loop size is expected from a “moving trombone slide” in which the loop grows in length until the completion of the previous Okazaki fragment and the initiation of a new one. Single loops were present in 43% of the replicative intermediates (circular dsDNAs containing a replication complex and tail). Because we likely missed loops that were less than 150 bp in length, the percentage of replication intermediates with a loop may be higher. The percentage of looped intermediates is a rough gauge of the time spent in the active synthesis of Okazaki fragments, as contrasted to the time spent in pausing at the end of a cycle and synthesis of the next primer (molecules with a replisome and long duplex tail, but no loop). Thus our analysis indicates that much of the replication cycle is spent in active lagging strand synthesis.

The general features of the T4 loops are very similar to what we observed in parallel studies (13, 14, 16) employing full-length M13 or 70-bp circular templates with T7 replication proteins. In the T7 studies a lower fraction of the M13 template circles were engaged in rolling circle replication (10–25%) and of these, only 27% showed loops at the replication fork. The distribution of loop size, however, for both templates was very similar for both templates and similar to the distributions presented here. The lower fraction of active replicative intermediates in the T7 study more likely reflects the lack of a true clamp and clamp loader than any difference in the robustness of the T7 and T4 polymerases.

In our scoring of replication intermediates we found some deproteinized molecules that had more than the expected one or two ssDNA segments, and a similar fraction (13%) of intact replication molecules with more than one loop (Figs. 2–4). The most likely explanation for these molecules is that a second Okazaki fragment was initiated before the first fragment was completed. Our finding that the distribution of potential lengths of the fragment closest to the fork (dsDNA plus ssDNA template ahead) is shorter than that of the distal fragment, and that the distribution of the distal fragment lengths is similar to that in molecules with a single fragment (Fig. 3) is consistent with this idea. Either the polymerase has moved from the distal fragment to a new primer, aborting the synthesis of the older fragment, or another polymerase has been recruited from solution to elongate the new primer. Our reactions were carried out under conditions where polymerase and primase concentrations are not limiting, and the fraction of molecules with more than one nascent fragment did not change with changes in polymerase or primase concentration (Table I). The alternative hypothesis that the extra ssDNA segments are the result of DNA degradation by the 5’ to 3’ nuclease of T4 RNase H is unlikely because this nuclease removes only 30–50 nt from each fragment under these replication conditions (37). Moreover, degradation by nuclease would not be expected to generate additional loops. It is also unlikely that 3’ to 5’ digestion by the polymerase exonuclease accounts for the extra ssDNA segments because they were observed with similar frequency when the exonuclease defective (D219A) polymerase replaced the wild type enzyme (29).

The factors that control the beginning of a new fragment in the T4 system have not been established, and indeed we have shown that there is a wide variation in fragment size along individual molecules (29). We have previously suggested that the compact structure of the lagging strand template may limit access of the primase and the clamp-loader. At present it is not clear whether the molecules with more than a single loop are a result of the in vitro replication conditions, or are a feature of normal replication.

In the trombone models there are two ssDNA segments at the fork. One lies within the trombone loop and is the result of helicase action ahead of the fork. The second represents the remaining ssDNA template for the Okazaki fragment that is being synthesized (see Fig. 3). During the trombone cycle, as the first segment grows in length, the second will diminish in
size as the fragment nears completion. In the original models both ss segments were shown as extended ssDNA-gene 32 protein filaments based on EM studies of 32 protein bound to ssDNA in vitro (33, 35). Our results show that the ss segments are not extended but rather exist in compact structures at the fork. These have been termed bobbins based on the previous T7 studies (16). Rough mass analysis by EM confirmed that the total mass of the DNA-protein complex at the fork was consistent with a structure much too large to contain just the core replication proteins, and indeed was consistent with the replication proteins together with about 740 nt of ssDNA saturated with 32 protein (Fig. 5D). A similar observation was made in our T7 studies. The T7 2.5 ss-binding protein, however, is far less robust in binding than 32 protein or E. coli SSB, and in the presence of the magnesium gene, 2.5-ssDNA complexes are compact (36) as contrasted to 32 protein-ssDNA filaments that remain extended (this work). Hence it was unclear whether the ss segments in the T4 intermediates would be arranged into similar bobbins or not. The observation of T4 bobbins points to these structures as being central elements of the machine that replicates DNA, and indeed may be the core structure around which the other proteins are organized. Consistent with this view was our finding that the unreplicated M13 ssDNA circles in the T4 reactions remained in a relatively extended conformation (bound by 32 protein and likely 59 protein as well). Furthermore, elimination of either 59 protein or primase from the reactions led to rolling circle intermediates with ss segments that were also relatively open, suggesting that the generation of the compact rolling circle intermediates with ss segments that were also relatively open, suggesting that the generation of the compact

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