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Lagging Strand Synthesis in Coordinated DNA Synthesis by Bacteriophage T7 Replication Proteins

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The proteins of bacteriophage T7 DNA replication mediate coordinated leading and lagging strand synthesis on a minicircle template. A distinguishing feature of the coordinated synthesis is the presence of a replication loop containing double and single-stranded DNA with a combined average length of 2600 nucleotides. Lagging strands consist of multiple Okazaki fragments, with an average length of 3000 nucleotides, suggesting that the replication loop dictates the frequency of initiation of Okazaki fragments. The size of Okazaki fragments is not affected by varying the components (T7 DNA polymerase, gene 4 helicase-primase, gene 2.5 single-stranded DNA binding protein, and rNTPs) of the reaction over a relatively wide range. Changes in the size of Okazaki fragments occurs only when leading and lagging strand synthesis is no longer coordinated. The synthesis of each Okazaki fragment is initiated by the synthesis of an RNA primer by the gene 4 primase at specific recognition sites. In the absence of a primase recognition site on the minicircle template no lagging strand synthesis occurs. The size of the Okazaki fragments is not affected by the number of recognition sites on the template.

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Keywords: replication; replisome; Okazaki fragment; DNA polymerase; helicase; primase

Introduction

The replication system of bacteriophage T7 is advantageous for studies on the protein-protein interactions that occur at a replication fork, since the fundamental reactions can be reconstituted with only four proteins.[1] The functional T7 DNA polymerase, whose crystal structure is known,[2] consists of a 1:1 complex of the T7 gene 5 protein encoded by the phage and a processivity factor, thioredoxin, encoded by the host, *Escherichia coli*.[3,4] The T7 hexameric gene 4 protein provides both helicase and primase activities.[5–7] The helicase activity resides within the carboxyl-terminal half of the protein and the primase activity in the amino-terminal half.[8,9] The crystal structure of the helicase domain has been established to 2.3 Å resolution.[10,11] The helicase uses the energy of hydrolysis of dTTP to unwind duplex DNA in a polar 5′ to 3′ direction relative to the strand to which it is bound.[12,13] The acidic C terminus of the helicase domain of gene 4 protein physically interacts with T7 DNA polymerase.[14] The gene 4 primase recognizes specific sequences on single-stranded DNA (ssDNA) and catalyzes the synthesis of tetraribonucleotides that function as primers for T7 DNA polymerase.[15–17] T7 gene 2.5 protein, whose structure has been determined recently,[18] is a ssDNA binding protein that physically and functionally interacts with T7 DNA polymerase and gene 4 protein.[19–21]

We have shown previously that the four proteins of the T7 replication system are sufficient to form a replisome that mediates leading and lagging strand synthesis in a coordinated manner.[22] Synthesis of the two strands is coupled in that rates of synthesis on both strands are identical and inhibition of synthesis on one strand leads to inhibition on the other. Both strands are synthesized processively, each by a distinct DNA polymerase; the lagging strand polymerase recycles from one completed Okazaki fragment to a new primer without dis-

Abbreviations used: ss, single-stranded; ds, double-stranded; EM, electron microscopy; TE, Tris-EDTA buffer.

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sociation from the complex. Most striking is the presence of a replication loop consisting of both duplex and ssDNA. Such a replication loop had been proposed in order to accommodate all of the replication proteins and the 3′ termini of both leading and lagging strands in a single complex.[23] In these studies we used a minicircle of 70 nucleotides to eliminate the problems inherent with naturally occurring large templates (see Figure 1(a)). The use of such minicircles permits a larger ratio of replication forks to replication proteins and hence alters the stochiometry of the reaction. In addition the base composition of leading and lagging strands can be dictated as well as the number of primase recognition sites.

Lagging strand synthesis is inherently more complex than leading strand synthesis. Leading strand synthesis requires only one initiation event, the addition of nucleotides by the polymerase to the preexisting primer strand. In the presence of the gene 4 helicase extensive processive leading strand synthesis can then commence.[22,24] Lagging strand synthesis on the other hand is dependent on multiple initiation events, each of which necessitates the synthesis of short RNA primers at specific recognition sites on the lagging strand. Furthermore, if lagging strand synthesis is to be processive, the lagging strand polymerase must cycle from one completed Okazaki fragment to a new primer in order to initiate synthesis of the next Okazaki fragment.

In the T7 replication system the multifunctional gene 4 protein provides the requisite primase activity.[6,7,15,25,26] The primase domain of gene 4

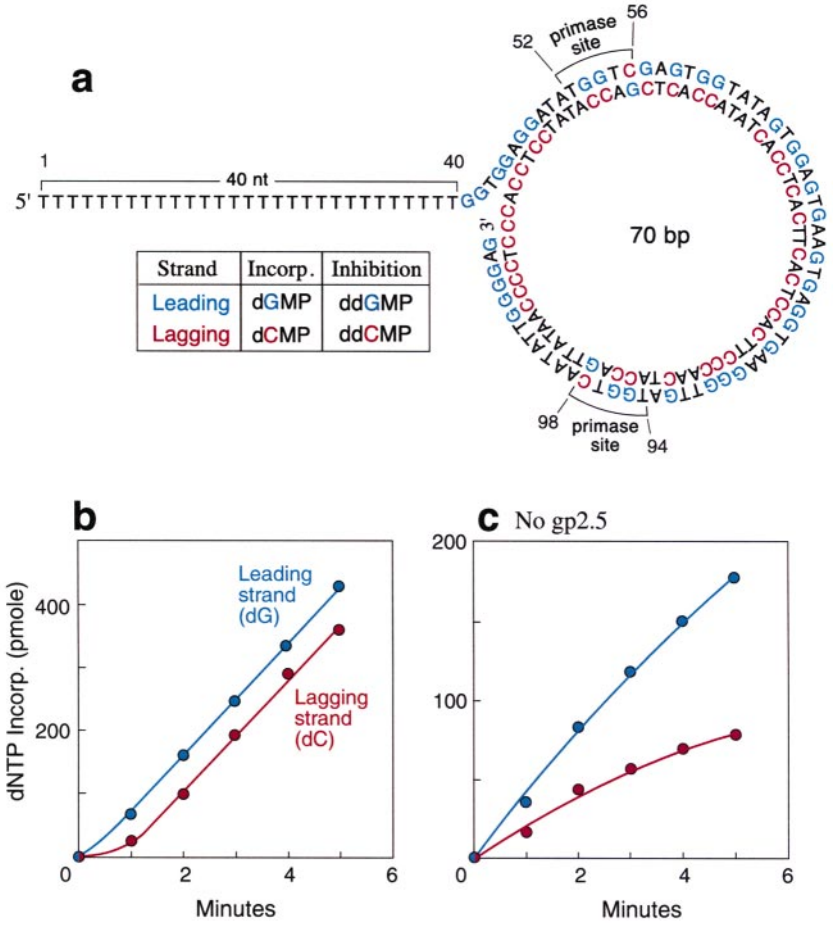

Figure 1. Coordinated synthesis on a minicircle DNA. (a) Structure and nucleotide sequences of the minicircle. The minicircle consists of a dsDNA circle (70 bp) bearing a 5′-ssDNA tail (40 nt). One strand of the circle is devoid of cytosine residues except for two residues located in the two primase recognition sites (5′-TGGTC-3′). Consequently the complementary strand has only two guanine residues. With this template, leading and lagging strand synthesis can be identified and measured by monitoring the incorporation of dGMP and dCMP, respectively. (b) Leading and lagging strand synthesis under standard conditions. DNA synthesis using the minicircle with the complete set of replication proteins (T7 DNA polymerase, gene 4 protein, and gene 2.5 protein) was carried out under the standard conditions described in Materials and Methods. To provide for a molar excess of minicircle over protein components, the ratio of minicircle:gene 4 hexamer:T7 DNA polymerase is 10:1:8. After incubation at 30 °C, aliquots (4 μl) from the reaction were assayed for the amount of radioactivity incorporated, using [^{3}H]dGMP and [^{3}H]dCMP for leading and lagging, respectively. (c) Leading and lagging strand synthesis in the absence of the gene 2.5 protein. Reactions were as described in (b) except that gene 2.5 protein was omitted from the reaction.

protein synthesizes functional tetraribonucleotide primers 5′-pppACCC-3′, 5′-pppACCA-3′, and 5′-pppACAC-3′ at primase recognition sequences 5′-GGGTC-3′, 5′-TGGTC-3′, and 5′-GTGTC-3′, respectively.[15–17,27] All of these recognition sequences contain the core recognition sequence 5′-GTC-3′.[2]erestingly, the 3′-cytidine in the primase recognition sequence is required but not copied into the primer. The utilization of the tetraribonucleotides as primers by T7 DNA polymerase requires the presence of T7 gene 4 protein; other primases will not function in this reaction.[15,17] One role of the gene 4 protein in this reaction is to stabilize the tetraribonucleotide on the template until it can be extended by the polymerase. Although the essential role of the T7 gene 2.5 ssDNA binding protein in coordination of synthesis is not known, it most certainly plays a major role in stabilizing the ssDNA located on both sides of nascent Okazaki fragments.[22]

Although our earlier studies demonstrated the coordination of leading and lagging strand synthesis in the reconstituted T7 replication system, they left a number of questions unresolved. The identification of replication loops fulfilled the prediction of several models of coupled DNA synthesis but the precise relationship between size of Okazaki fragments and the single and double-stranded regions of the loop, a relationship that is addressed by the models, was not examined in detail. How invariant is the distribution of lengths of Okazaki fragments? For example, will changes in the overall rate of DNA synthesis or in the pools of ribonucleotides affect the length of fragments? In the T4 and *E. coli* replication systems, it had been reported that the limitation of ribonucleotide concentration or the dilution of primase changes the size profile of lagging strands.[28–33] As discussed above, T7 primase has a rather stringent requirement for a primase recognition site but it is not known if this specificity is maintained during coordinated DNA synthesis. An intriguing finding was the size distribution of the Okazaki fragments that are the products of lagging strand DNA synthesis. Most of the Okazaki fragments ranged from 1000 to 6000 nucleotides with an average size of 3000 nucleotides, a rather remarkable distribution in that the protein complex repeatedly passes over a priming site in the minicircle approximately every 35 nt. Closely related to this phenomenon is the size distribution of Okazaki fragments within each individual DNA product molecule. Does the heterogeneity in lengths arise from variation in Okazaki fragments within individual molecules or to the variation in lengths among a population of molecules? Studies of the T4 system have shown that there are variations of the Okazaki fragment lengths within individual molecules.[34] Here we have examined some of these parameters to better understand the mechanisms underlying lagging strand DNA synthesis.

Results

The minicircular DNA template depicted in Figure 1(a) has two major advantages over conventional naturally occurring DNA templates. First, the relatively small size of the minicircle (70 bp) with a single replication fork allows for reactions in which there is a molar excess of replication forks over the replication proteins. In our earlier studies the ratio of minicircle:gene 4 helicase-primase:T7 DNA polymerase was 10:1:8.[22] Gene 2.5 ssDNA binding protein is usually present in amounts (4 μM) sufficient to bind any ssDNA formed as intermediates during the reaction. The second advantage of a minicircle template is the ability to select the sequence and base composition of each strand. Inspection of the sequence shown in Figure 1(a) reveals that the leading strand is essentially devoid of cytosine residues except for the two that represent the essential cryptic residue in the two primase recognition sequences (5′-TGGTC-3′); consequently, the lagging strand contains only two guanosine residues. This bias of base composition allows for a rapid and quantitative measurement of leading and lagging strand synthesis by following the incorporation of labeled dGMP and dCMP, respectively. In addition the precise locations of the two primase recognition sites are known.

Coordinated DNA synthesis

The four proteins of T7 DNA replication discussed above mediate coordinated leading and lagging strand synthesis. In our earlier study we showed that coordinated DNA synthesis was characterized by equal rates of synthesis of both strands, coupling of synthesis of one strand to the other, processivity of synthesis of both strands, and the presence of a replication loop. In our analysis of lagging strand synthesis and the parameters that affect lagging strand synthesis, it is important to know that coordinated DNA synthesis is maintained under the conditions used to modify the system. Here we use three criteria to score coordinated DNA synthesis: (i) identical rates of synthesis of both strands; (ii) a lag period before the onset of lagging strand DNA synthesis; and (iii) coupling of leading and lagging strand synthesis. In those experiments in which the rates of synthesis of the two strands are identical following a lag period for the onset of lagging strand synthesis we score the reaction as coupled. In those cases where the rates of synthesis of the two strands are identical but the lag period is not well defined we determine the effect of inhibition of lagging strand synthesis on leading strand synthesis. We have previously shown that under the condition of coupling when lagging synthesis is inhibited by the incorporation of dideoxy CMP then leading strand synthesis is inhibited to the same extent.[22]

Under the standard conditions for minicircle replication, leading and lagging strand synthesis

proceed at identical rates over the five minutes of incubation (Figure 1(b)). In this experiment leading strand synthesis was measured by following the incorporation of dGMP and lagging strand synthesis by the incorporation of dCMP. Under conditions that preclude coordinated DNA synthesis lagging strand synthesis proceeds at a slower rate. One such condition is the omission of gene 2.5 protein. As shown in Figure 1(c), in the absence of gene 2.5 protein lagging strand synthesis is not linear and the rate progressively diminishes during the incubation.

When synthesis of the two strands is coordinated there is always a lag of approximately 30 seconds prior to the onset of lagging strand synthesis, after which the rate assumes that of leading strand synthesis. Although not shown in Figure 1(b) we have measured lagging strand synthesis at times earlier than one minute. The rates of lagging strand synthesis at those earlier time points support the extrapolated values shown in Figure 1(b). For example at 15 secods of incubation lagging strand synthesis is bearly detectable (<5% of leading strand synthesis). We do not know the explanation for this observation but we speculate that it reflects the establishment of a replication loop that is essential for coordinated DNA synthesis. The presence and absence of a lag period is apparent in Figure 1(b) and (c), respectively, that illustrate coordinated and non-coordinated synthesis, respectively.

Size distribution of leading and lagging strands

The size distribution of the products of leading and lagging strand synthesis were determined by specifically labeling the leading and lagging strands with [α-^{32}P]dGMP and [α-^{32}P]dCMP, respectively, and then analyzing the distribution of radioactivity by electrophoresis through an alkaline agarose gel (Figure 2(a)). As previously shown, the majority of the products of leading strand synthesis exceed the resolving power of the agarose gel, having lengths greater than 30,000 nucleotides (lanes 3 and 4) at both two and four minutes of synthesis. In striking contrast the products of lagging strand synthesis are relatively short fragments with lengths less than 6000 nucleotides at both time points.

In order to display and analyze the distribution in lengths, we analyzed the gel with a phosphoimager (Figure 2(b)). The lagging strand products at both two and four minutes of incubation range in length from 1000 to 6000 nt; the most frequently occurring products are approximately 2400 nt. However, due to a skewing of the distribution toward the higher molecular mass range the average distribution is around 3000 nt. Considering the presence of two primase recognition sites in the minicircular template only 35 nucleotides apart, the consistency of these considerably large Okazaki fragments is rather striking.

As described in the previous section, the omission of gene 2.5 protein results in non-coordinated DNA synthesis, as judged by unequal rates of lead-

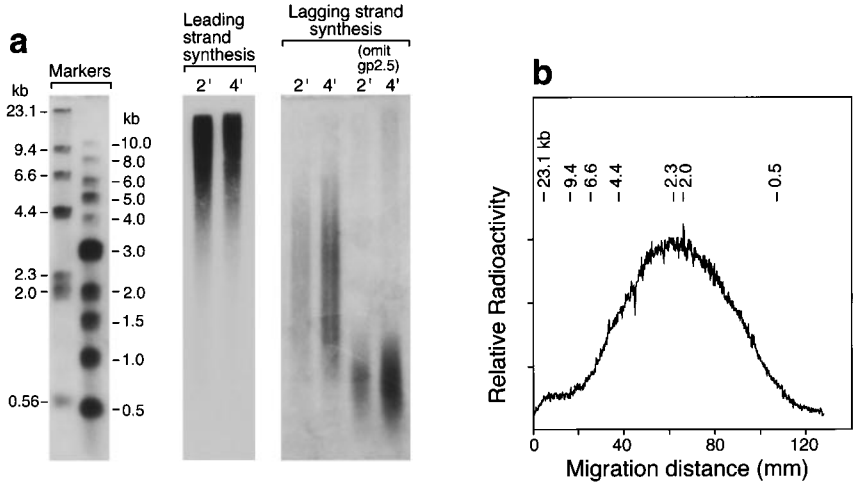

Figure 2. Size distribution of leading and lagging strand synthesis (a) Alkaline agarose gel analysis of products of leading and lagging strand synthesis. DNA synthesis was carried out in the standard reaction containing minicircle either in the presence or absence of gene 2.5 protein and with either [α-^{32}P]dGTP or [α-^{32}P]dCTP. After incubation at 30 °C for two or four minutes, the products of the reaction were denatured and analyzed by electrophoresis through a 0.8% (w/v) alkaline agarose gel. Lane 1 contains a *Hin*dIII digest of λ DNA to provide markers of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb. Lane 2 contains a 1 kb ladder purchased from New England Biolabs (10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.5, 1.0, 0.5 kb). Lanes 3 and 4 represent leading strand synthesis. Lanes 5 and 6 represent lagging strand synthesis. Lanes 7 and 8 represent lagging strand synthesis in the absence of gene 2.5 protein. (b) Phosphoimager analysis of the sizes of lagging strand fragments. The scan represents the distribution of radioactivity along lane 6 in the gel shown in (a).

ing and lagging strand synthesis and by the absence of a lag period before the onset of lagging strand synthesis (Figure 1(c)). The absence of gene 2.5 protein likewise has a dramatic affect of the size of Okazaki fragments. In the absence of gene 2.5 protein the average size of the lagging strand fragments is 500 nt (at both two and four minutes of incubation in Figures 2 and 4(a)). This marked effect of uncoupled leading and lagging strand synthesis on the size of Okazaki fragments indicates that studies on parameters affecting Okazaki fragment size must assure that coordinated leading and lagging strand synthesis is maintained. In the following sections we have examined the effect of a number of parameters on lagging strand synthesis and we have also determined whether coordinated synthesis is maintained under these conditions.

Modifications in primase recognition site

The minicircle used in the standard assay contains the two primase recognition sites (5′-TGGTC-3′). The sequence of this site is one of three, 5′-GGGTC-3′, 5′-TGGTC-3′, 5′-GTGTC-3′, that are the most freqently used by the gene 4 primase to synthesize di-, tri- and tetranucleotides.[17,27] At the site 5′-TGGTC-3′ the products are pppAC, pppACC, and pppACCA. However, only the tetranucleotide can serve as a primer for T7 DNA polymerase.[15,16] Common to all of these recognition sites is the core sequence 5′-GTC-3′ in which the cryptic cytosine is requried for recognition by the primase but is not copied into the ribonucleotide product.

The studies on the specificity of the primase recognition sequence specificity have all been carried out in reactions that are not coupled to coordinated DNA synthesis. In order to determine if the gene 4 primase has reduced specificity under conditions of coordinated DNA synthesis we modified the primase recognition site to 5′-GGGTT-3′, thus eliminating the cryptic cytosine residue. In the absence of the cryptic cytosine residue no detectable lagging strand synthesis was observed as measured by the incorporation of [α-^{32}P]dCMP into the product (less than 5 pmol in five minutes, data not shown).

The T7 gene 4 primase can also extend preformed di- and triribonucleotides at primase recognition sites, if the appropriate nucleoside triphosphates are provided.[35] The cryptic cytosine is also essential in this reaction. We asked whether tetraribonucleotides had to be synthesized *de novo* by the gene 4 protein in order to serve as primers for lagging strand synthesis under conditions of coordinated DNA synthesis. As shown in Figure 3(a), lagging strand synthesis proceeds normally when ACC and CTP are provided to the reactions containing the minicircle with the recognition sequence 5′-GGGTC-3′ in the absence of ATP. No lagging strand synthesis is observed under these conditions where the recognition sequence has been altered to 5′-GGGTT-3′ (Figure 3(a)). As a control, leading strand synthesis in the absence of gene 2.5 protein was carried out with both minicircles (Figure 3(b)). Leading strand synthesis proceeds at similar rates on both templates.

The minicircle used in the standard assay contains two primase recognition sites. We have modified this minicircle by eliminating one of the two primase recognition sites. And the cytosine residue at the position 98 was changed to a guanosine resi-

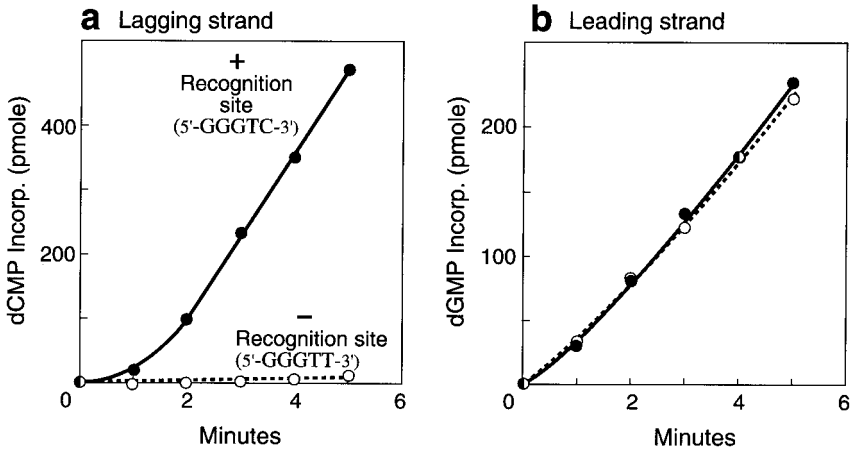

Figure 3. A minicircle lacking a primase recognition sequence. (a) Analysis of lagging strand synthesis with [^{3}H]dCTP. DNA synthesis reactions were carried out with ACC trimer and CTP in the presence of gene 2.5 protein with a minicircle in which thymine replaces the cryptic cytidine residues in the minicircle presented in Figure 1(a) (see Materials and Methods). At one minute intervals during the five minute incubation, aliquots (4 μl) were removed, the reaction stopped with EDTA, and the amount of radioactivity incorporated into DNA determined as described for Figure 1(b). (b) Leading strand synthesis in the absence of gene 2.5 protein. The DNA synthesis reaction was carried out as described in (a) except that gene 2.5 protein was omitted and [^{3}H]dGTP was used to monitor leading strand synthesis.

due. Rates of leading and lagging strand synthesis are identical with the original and modified minicircles and the profile and size distribution of the Okazaki fragments are identical (data not shown). Synthesis is coordinated on both templates.

Effect of T7 gene 2.5 protein on lagging strand synthesis

To determine the effect of varying the amount of gene 2.5 protein in the minicircle DNA replication system we examined coordination of leading and lagging strand synthesis, the size of Okazaki fragments, and the rates of lagging strand synthesis in reactions lacking gene 2.5 protein or in the presence of gene 2.5 protein at concentration up to 6 μM; under standard conditions the concentration is 4 μM. To determine if leading and lagging strand synthesis are coordinated, we compared the rates of synthesis and checked for the presence of a delay in the onset of lagging strand synthesis (as described in Figure 1(b) and (c)). As shown in the inset in Figure 4(a), coordinated synthesis was observed at concentrations of gene 2.5 protein from 2 to 6 μM. At a concentration of 1 μM or in the absence of gene 2.5 protein, synthesis was not coordinated.

The size distribution of lagging strands was determined by analyzing the [α-^{32}P]dCMP-labeled DNA product (two minutes incubation) by electrophoresis through alkaline agarose gels. In order to compare the relative sizes of Okazaki fragments synthesized at each concentration of gene 2.5 protein, equal amounts of incorporated [α-^{32}P]dCMP were applied to each lane. The gels were analyzed with a phospimager and the size distributions of the fragments are presented in Figure 4(a). In the range of 3 to 6 μM gene 2.5 protein the peaks of the fragments were between 2300 and 2500 nt. However, below 2 μM there is a striking shift of the size distribution to less than 1000 nt. At 2 μM gene 2.5 protein, a concentration between these two extremes, there is a slight shift of the profile to lower molecular mass fragments, a profile that may reflect a mixture of both short and normal size Okazaki fragments. Based on the presence of a lag period and equal rates of leading and lagging strand synthesis we scored the reaction as coordinated at 2 μM gene 2.5 protein. The size distribution of Okazaki fragments synthesized at 2 μM gene 2.5 protein is similar to that observed with coordinated synthesis, whereas the rate of lagging strand synthesis at this concentration of gene 2.5 protein more closely resembles that observed for uncoordinated synthesis at 1 μM. It is possible that at this transition point both coordinated and uncoordinated synthesis are present. It is clear that once a critical amount of gene 2.5 protein has been obtained the size of Okazaki fragments is not affected by the presence of higher concentrations of the protein.

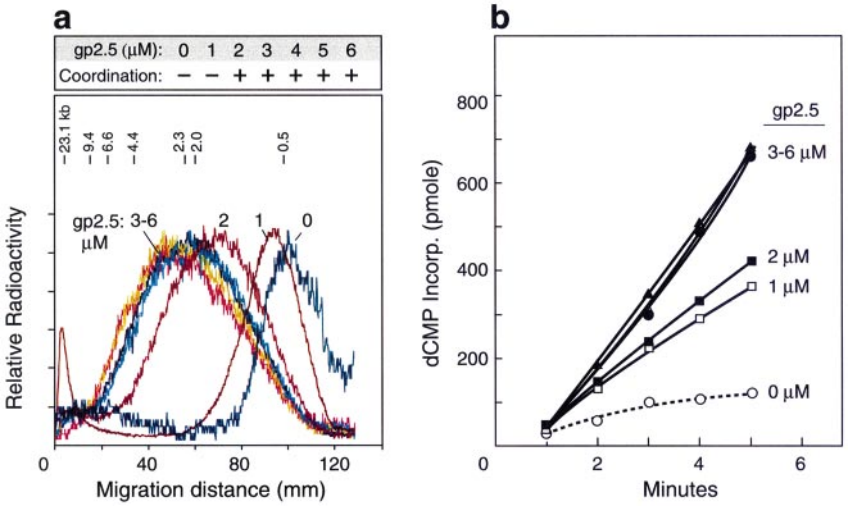

Figure 4. Effect of T7 gene 2.5 protein on DNA synthesis. (a) Size distribution of Okazaki fragments. DNA synthesis was carried out in the standard minicircle DNA synthesis with [α-^{32}P]dCTP and with different concentration of gene 2.5 protein (0, 1, 2, 3, 4, 5, and 6 μM). The products of the reaction at two minutes of incubation were analyzed by electrophoresis through an alkaline agarose gel. Approximately equal amounts of radioactivity were applied to the gel. After electrophoresis, the gel was dried and exposed on a phosphoimager screen. Each scan represents the distribution of lagging strands with the indicated concentration of gene 2.5 protein. The coordination of leading and lagging strand synthesis was determined at each concentration of gene 2.5 protein, using the criteria described in Materials and Methods, and is indicated in the inset Table. (b) Rates of lagging strand synthesis. The standard reaction was carried out in the presence of [^{3}H]dCTP with the indicated concentrations of gene 2.5 protein (0, 1, 2, 3, 4, 5, and 6 μM). Aliquots of 4 μl were taken at one minute intervals over a five minute period and the reaction stopped with EDTA. For the quantitation of DNA synthesis, samples were spotted onto DE81 filters and after washing and drying, the radioactivity was determined by scintillation counting.

The rates of lagging strand synthesis are linear at concentrations of gene 2.5 protein 2 μM or above. But at 1 μM or below a significant deviation from linearity is observed. In the absence of gene 2.5 protein lagging strand synthesis essentially reaches a plateau by three minutes after the start of synthesis (Figure 4(b)). Again it is apparent that higher concentrations of gene 2.5 protein above a critical concentration of 3 μM do not significantly affect the rate of synthesis. It appears possibly that the critical concentration of gene 2.5 protein we have documented represents the minimal quantity necessary to coat the ssDNA intermediates (see Discussion). Below that amount leading and lagging strand synthesis are no longer coordinated and the size of Okazaki fragments is greatly diminished.

Effect of T7 gene 4 helicase/primase concentration on lagging strand synthesis

In the standard minicircle replication system the T7 gene 4 helicase/primase is the limiting component, being present at 0.1 mol gene 4 hexamer per mol of replication fork. Using the same criteria as for analysis of gene 2.5 protein we examined the effect of varying the concentration of monomers of gene 4 protein from 15 to 480 nM; the concentration in the standard assay is 60 nM. Coordinated synthesis was observed from 15 to 120 nM gene 4 protein but not at the highest concentrations of 240 and 480 nM (Figure 5(a), inset). At all concentrations of gene 4 protein there was little change in the size distribution of Okazaki fragments, except for a slight skewing of the distribution to lower molecular mass for the lowest and highest concentrations tested (Figure 5(a)). Not unexpectedly by given that gene 4 protein is limiting in the standard assay, we observed a progressive increase in the rate of DNA synthesis as the amount of gene 4 protein is increased (Figure 5(b)). Most likely the number of replisomes is determined by the amount of gene 4 protein present in the reaction until the concentration exceeds 60 nM, at which point some other component of the system becomes limiting. It is clear, however, that increasing the rate of DNA synthesis by severalfold does not alter the size distribution of the Okazaki fragments. Since the primase activity resides within the gene 4 helicase/primase we can conclude that increasing primase activity does not increase the frequency of initiation events for synthesis of Okazaki fragment within the range examined.

Effect of T7 DNA polymerase concentration on lagging strand synthesis

The concentration (80 nM) of T7 DNA polymerase in the standard minicircle replication system provides an eightfold excess of polymerase over gene 4 hexamer. However, on the assumption that two DNA polymerases must function simultaneously at the replication fork this would be only a fourfold excess. As shown in the inset for Figure 6(a), concentrations higher than 80 nM do not provide for coordinated synthesis. The lack of coordinated synthesis was based on the observation that no lag period proceeded the onset of lagging strand synthesis, a conclusion that was

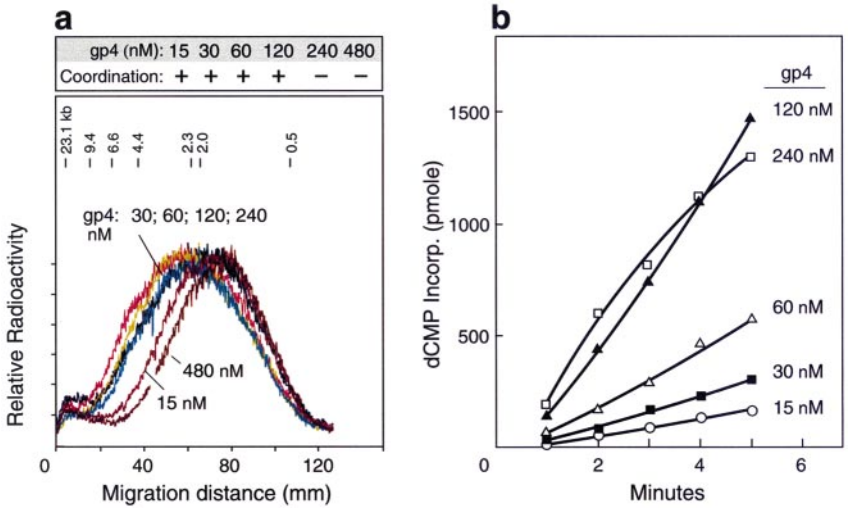

Figure 5. Effect of gene 4 helicase/primase on DNA synthesis. (a) Size distribution of Okazaki fragments. DNA synthesis was carried out in the standard reaction in the presence of [α-^{32}P]dCTP with the indicated concentrations of the 63 kDa gene 4 protein (15, 30, 60, 120, 240, and 480 nM). The products of the reaction at two minutes of incubation were analyzed by electrophoresis through 0.8% alkaline agarose gel as described in the legend of Figure 4(a). Each scan represents the distribution of lagging strands with a particular concentration of gene 4 proteins. In the standard assay, the concentration of gene 4 helicase/primase is 60 nM (10 nM as hexamer). (b) Rates of lagging strand synthesis. The standard reaction was carried out in the presence of [^{3}H]dCTP with the indicated concentrations of gene 4 protein (15, 30, 60, 120, and 240 nM). Aliquots were analyzed as described for Figure 4(b).

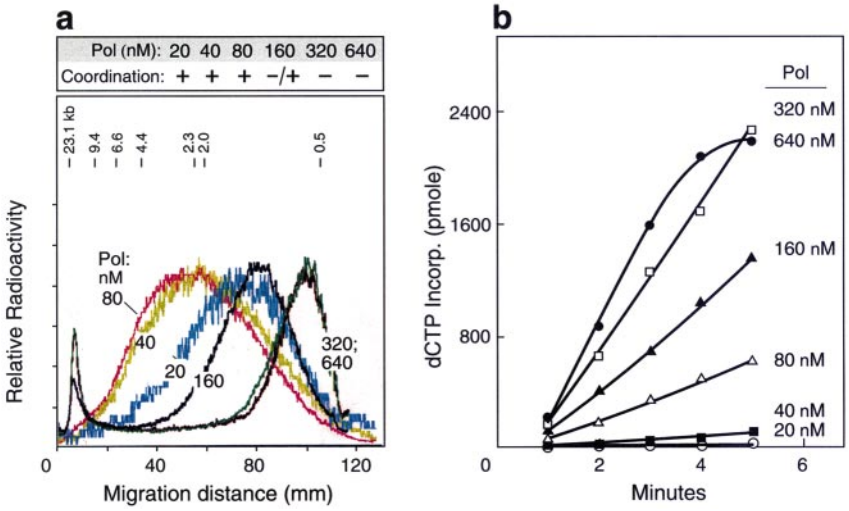

Figure 6. Effect of T7 DNA polymerase on DNA synthesis. (a) Size distribution of Okazaki fragments. DNA synthesis was carried out in the standard reaction in the presence of [α-^{32}P]dCTP with the indicated concentrations of T7 DNA polymerase (20, 40, 80, 160, 320, and 640 nM). The products of the reaction at two minutes of incubation were analyzed by electrophoresis through a 0.8% alkaline agarose gel as described in the legend to Figure 4(a). Each scan represents the distribution of lagging strands with a particular concentration of DNA polymerase. In the standard assay, the concentration of T7 DNA polymerase is 80 nM. (b) Rates of lagging strand synthesis. The standard reaction was carried out in the presence of [^{3}H]dCTP with the indicated concentrations of DNA polymerase (20, 40, 80, 160, 320, and 640 nM). Aliquots were analyzed as described for Figure 4(b).

supported by the lack of inhibition of leading synthesis upon addition of ddCTP.[22] A significant amount of synthesis scored as lagging strand synthesis in the presence of high concentration of T7 DNA polymerase probably represents the elimination of gaps in DNA molecules that arise as a result of abortive attempts to establish coordination.

At 40 nM and 80 nM DNA polymerase the Okazaki fragment size is centered around 3000 nt, with a size distribution similar to that observed under the condition for coordinated synthesis presented above (Figure 6(a)). At the higher concentration of polymerase (320 and 640 nM) the Okazaki fragments are quite short, only 500 nucleotides in length; at 160 nM there is an intermediate length with a peak at 1500 nt (Figure 6(a)). At the limited DNA polymerase concentration (20 nM), the Okazaki fragments are also approximate 1500 nt in length. The rates of lagging strand synthesis over the same range of polymerase concentrations are shown in Figure 6(a). Here the rate of DNA synthesis increases with increasing polymerase concentration.

Like the reactions described for gene 4 protein, the number of replisomes is determined by the amount of polymerase present in the reaction until the concentration exceeds around 80 nM, at which point some other component of the system is limiting. Within the range of the coordination, increasing the rate of DNA synthesis does not alter the size distribution of the Okazaki fragments.

Effect of CTP concentration on lagging strand synthesis

In the T4 and *E. coli* replication systems, an alteration of the ribonucleotide concentration had a dramatic effect on the size profile of lagging strands.[28–33] The tetranucleotides synthesized by the gene 4 primase at the primase recognition sites in the minicircular DNA template have the sequence pppACCA. Therefore, we have varied the concentration of CTP in the reaction and analyzed the effect on lagging strand synthesis. Lagging strand synthesis in the T7 replication system was examined with concentrations of CTP ranging from 3 to 300 μM; the ATP concentration was held constant at 300 μM (Figure 7). Only at the lowest concentration of CTP (3 μM) were leading and lagging strand synthesis not coordinated (Figure 7(a), inset). At all other concentrations the distribution of Okazaki fragments was identical with a peak at approximately 2400 nt. At 3 μM CTP, where synthesis is no longer coordinated, the size of Okazaki fragments was significantly increased with a peak at 7000 to 8000 nt.

Concentrations of CTP from 10 μM to 300 μM have essentially no effect on the rate of leading or lagging strand DNA synthesis (Figure 7(b)). However, at 3 μM, where synthesis is not coordinated, there is a twofold decrease in the rate of lagging strand synthesis. Leading strand synthesis is unaffected (data not shown). If we consider the fact that the K_M value for CTP in the primase reaction is 0.85 mM,[36] primer synthesis for lagging strands in the replisome is much more efficient than in the reaction with primase alone.

A replication loop

The assembly of the replication proteins into a replisome dictates the looping of the lagging strand[23] such that the 3′ terminus of a completed Okazaki fragment is juxtaposed to the next primer site. Such replication loops have been observed with the T7 replication system[22,37] and the T4 system (Chastian *et al.*, unpublished data). In our

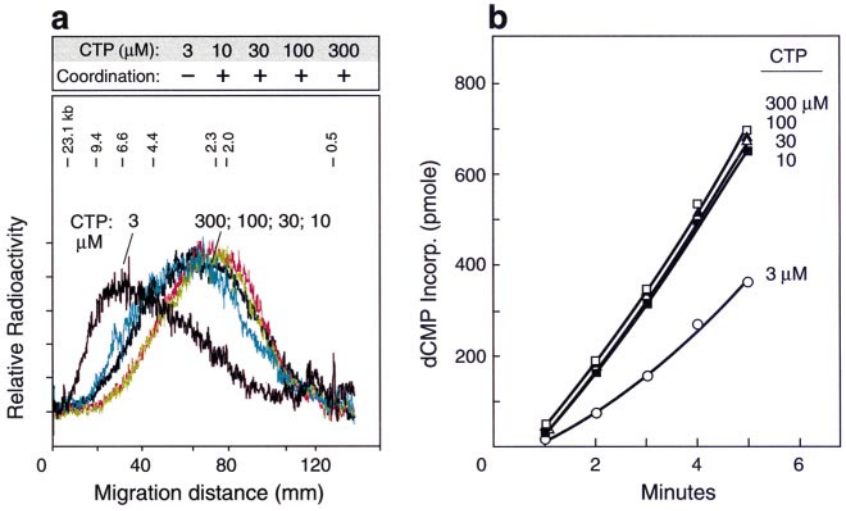

Figure 7. Effect of CTP concentration on lagging strand synthesis. (a) Size distribution of Okazaki fragments. DNA synthesis was carried out in the standard minicircle DNA system with [α-^{32}P]dCTP and different concentrations of CTP (3, 10, 30, 100, and 300 μM). The products of the reaction at two minutes of incubation were analyzed by electrophoresis through alkaline agarose gels as described in the legend to Figure 4(a). (b) Rates of lagging strand synthesis. The standard reaction was carried out in the presence of [^{3}H]dCTP with the indicated concentrations of CTP. Aliquots were analyzed as described for Figure 4(b).

earlier studies with the minicircle T7 replication system approximately 50 % of the replicating molecules had replication loops.[22] Although these replication loops appear as dsDNA in the electron micrographs of the protein-DNA complexes, the replication loops should consist of ssDNA and dsDNA segments (see Figure 9(c), below). As described below, these inherent intermediates in lagging strand synthesis provide information on the size of nascent Okazaki fragments and the relative rates of leading and lagging strand synthesis. In the present study we have examined the replication loops for the presence of single and duplex regions and have measured the length of each to compare the size of Okazaki fragments with the relevant regions in the loop.

To visualize the DNA-protein replicative intermediates, aliquots of the reaction mixtures were treated with glutaraldehyde to stabilize the complexes. As shown in Figure 8, most of the replicative intermediates have an amorphous mass of electron-dense material, most likely a complex of the replication proteins and ssDNA. These DNA-protein complexes contain not only the polymerase and gene 4 helicase-primase molecules but numerous molecules of gene 2.5 protein bound to single-stranded regions present in the replication loop. We show below that deproteinization of the replicative intermediates reveals the single-stranded regions not seen prior to deproteinization. The minicircle is too small to be visualized by these electron microscopy methods.

Using the presence of a DNA-protein complex as an indication of a replication intermediates we identified several different intermediates. These intermediates consisted of DNA-protein complexes with (i) a dsDNA loop (25 %), (ii) a dsDNA loop and a long linear dsDNA tail (27 %), and (iii) a dsDNA linear molecule (48 %). Examples of the latter two species are depicted in Figure 8(a) and (b), respectively. The length of the dsDNA loops varied from 0.1 to 2.1 kb with an average and median length of 0.8 and 0.6 kb, respectively (Figure 8(c)). The lengths of the linear dsDNA tails were in excess of 2 kb by two minutes incubation with some molecules having dsDNA tails in excess of 19 kb. We do not know the origin of the replication loops lacking dsDNA tails. We speculate that they represent molecules in which synthesis of the first Okazaki fragment was aborted or the replication loop was not resolved. The resulting ssDNA tail would not be visible in these elctron micrographs but their presence is supported by the relatively high percentage of deproteinized short dsDNA molecules with ssDNA tails.

To visualize the anticipated ssDNA portion of the replication loop the samples were gently deproteinized and then incubated with *E. coli* SSB protein to complex any ssDNA segments. The resulting thick SSB-ssDNA segments are easily distinguished from duplex DNA by EM and are 1.9-fold shorter than the length of equivalent dsDNA.[37]

As shown in Figure 9(a), the majority of the molecules observed after deproteinization were short (<3000 nt) ssDNA molecules. These molecules must arise from limited DNA synthesis on the minicircle (not visible) and probably represent abortive DNA synthesis in which lagging strand synthesis was never initiated. The next most frequently observed intermediates were molecules containing a short ssDNA region attached to a dsDNA segment of variable length. Molecules of this type would be expected to arise upon the completion of an Okazaki fragment, leaving only a newly initiated Okazaki fragment. Present in almost equal amounts were molecules such as that depicted in Figure 9(b) in which the two ssDNA segments, separated by a short duplex regions, are located at the end of a relatively long double segment of DNA. Our interpretation of the result is shown in Figure 9(b) in which we indicate the minicircle located at one end of the long product molecule. For purpose of discussion we have labeled the different segments I, II, III, and IV

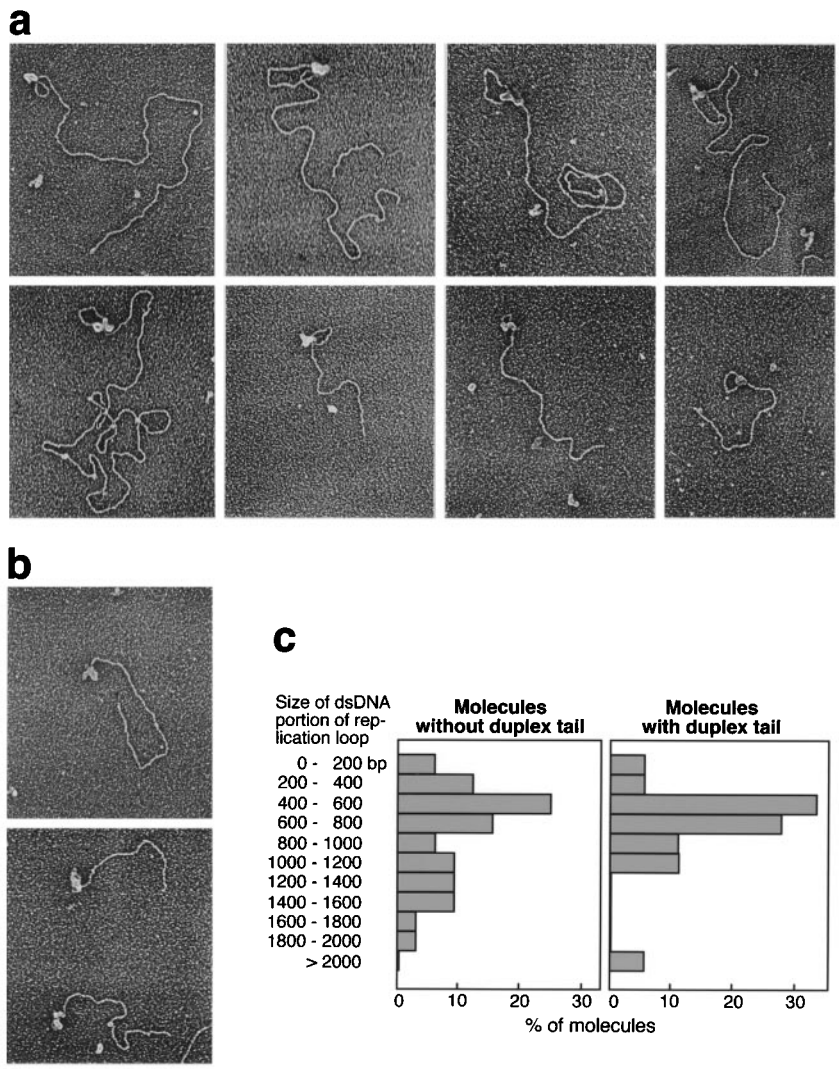

Figure 8. Visualization of minicircle replication products. The standard reaction for DNA synthesis using the minicircle and T7 polymerase, gene 4 protein, and gene 2.5 protein was carried out and the samples were incubated at 30 °C for one minute. The products were treated with 0.6 % glutaraldehyde and then filtered through columns of Bio-Gel A-5 M equilibrated with 10 mM TE (pH 7.5). The fixed samples were adsorbed to thin carbon foils, washed, air-dried, and rotary shadowcast with tungsten at high vacuum as described in Materials and Methods. (a) Electron micrographs of replication intermediates with a dsDNA loop. (b) Electron micrographs of replication intermediates without a dsDNA loop. (c) Distribution of the sizes of the dsDNA loops found in the coupled, coordinated replication intermediates reactions. The range of replication loop sizes from molecules that contained a replication loop, a DNA-protein complex, but did not contain a long dsDNA tail and the range of replication loops from molecules that contained a replication loop, a DNA-protein complex, and a long dsDNA tail are presented. Length measurements were made as described in Materials and Methods.

(Figure 9(b)). Other DNA products were also observed and these are summarized in Figure 9(a).

The patterns formed by the presence of single-stranded and double-stranded segments allow a determination of the stage of the replication process at which the reactions were interupted. Replicative intermediates consisting of solely ssDNA arise from leading strand synthesis alone. Those molecules with a ssDNA-dsDNA-ssDNA pattern are in the process of synthesizing their first Okazaki fragments. Molecules with a ssDNA region adjacent to a duplex tail have completed an Okazaki fragment and are just starting to synthesize the next. Of particular interest is the pattern presented in Figure 9(b). These molecules most likely arise from those depicted in Figure 8(a) where a replication loop is present along with a nascent Okazaki fragment.

We decided to focus on the molecules of the type depicted in Figure 9(b) and measured the lengths of the nascent Okazaki fragments (segment II) as well as the constituents of the replication loop envisioned by Alberts[23] (segments I plus II). In addition we measured the other ssDNA segment (segment III), since the sum of the nascent Okazaki fragment (segment II) and segment III should provide the length of the potential completed Okazaki fragment. We calculate the size of a completed Okazaki fragment (segment II plus III) to be 2.9 kb, based on the measurement of 61 molecules. The average size of the potential replication loop (segment I plus 1I) is 2.6 kb. In Figure 9(d) we have calculated the ratio of segment I to segment II. The ssDNA comprising segment I arises as a result of leading s strand DNA synthesis, whereas the length of the duplex segment II arises from lagging strand synthesis. If the two are equal at a micromolecular level then the ratio should approximate 1. The peaks of molecules have the ratio from 0.75 to 1.25 although molecules with more extreme ratios were observed. The sizes of the completed Okazaki fragments ranged from 0.7 to 7.4 kb and the replication loop from 0.4 to 10.1 kb (Figure 9(e)).