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Coupling DNA unwinding activity with primer synthesis in the bacteriophage T4 primosome

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Abstract

The unwinding and priming activities of the bacteriophage T4 primosome, which consists of a hexameric helicase (gp41) translocating 5′ to 3′ and an oligomeric primase (gp61) synthesizing primers 5′ to 3′, has been investigated on DNA hairpins manipulated by a magnetic trap. We find that the T4 primosome continuously unwinds the DNA duplex while allowing for primer synthesis through a primosome disassembly mechanism or a novel DNA looping mechanism. A fused gp61-gp41 primosome unwinds and primes DNA exclusively via the DNA looping mechanism. Other proteins within the replisome control the partitioning of these two mechanisms disfavoring primosome disassembly thereby increasing primase processivity. In contrast priming in bacteriophage T7 involves discrete pausing of the primosome and in *Escherichia coli* appears to be associated primarily with dissociation of the primase from the helicase. Thus nature appears to use several strategies to couple the disparate helicase and primase activities within primosomes.

A model system used to study DNA replication is the bacteriophage T4 replisome. Eight proteins, corresponding to seven different activities, have been identified that together are able to reconstitute *in vitro* leading and lagging strand DNA synthesis1. The leading and lagging strand templates are copied by two holoenzyme complexes, each composed of the polymerase (gp43) and the clamp (gp45)2. The clamp protein is loaded by the clamp loader complex (gp44/62) in an ATP-dependent fashion3,4. DNA polymerases can only synthesize nascent DNA in the 5′ to 3′ direction; therefore, the leading strand holoenzyme may

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AUTHOR CONTRIBUTIONS

M.M. contributed to experimental design, performed single-molecule experiments, and wrote the manuscript. M.M.S. contributed to experimental design, prepared the DNA substrates and proteins, performed bulk assays, and wrote the manuscript. Z.Z. constructed the fusion protein and performed bulk assays. S.J.B and V.C. wrote the manuscript and V.C. built the magnetic tweezers.

COMPETING INTERESTS STATEMENT

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synthesize DNA continuously, while the lagging strand holoenzyme must synthesize DNA in short, approximately 1 kb segments know as Okazaki fragments. The primosome is a subassembly of the replisome composed of a hexameric helicase (gp41) that unwinds dsDNA5 and an oligomeric primase (gp61) that synthesizes pentaribonucleotide primers at 5′-GTT and 5′-GCT sequences to initiate repetitive Okazaki fragment synthesis6,7. There is biochemical evidence for a gp41/gp61 complex, including EMSA8,9, protein kinase protection10, and single-molecule FRET11; although the complex has not been actually isolated. Their activities are closely associated since the processivity of gp41 is increased in the presence of gp6112 and gp41 greatly increases the overall priming rate and influences the sequence of primers made by gp616,13. In the presence of ssDNA binding protein (gp32), which coats the ssDNA produced by the helicase14, the primosome requires helicase accessory protein (gp59) for efficient loading15,16.

Three possible models have been suggested to explain how helicase is able to unwind dsDNA translocating 5′ to 3′ on the lagging strand while primase travels in the opposite direction (3′ to 5′) in order to synthesize an RNA primer (Fig. 1). In the first model (pausing), the helicase temporarily pauses or stops translocating to allow for primer synthesis and then resumes unwinding the DNA; helicase pausing would necessitate the pausing of the entire replisome while a primer is being synthesized. This behavior has been observed for the bacteriophage T7 replisome17. In the second model (disassembly), one or more primase subunits dissociate from the helicase and remain behind to synthesize a primer while the helicase and any remaining primase subunits continue to translocate along the lagging strand. In this model, leading strand synthesis would continue uninterrupted; however, new primase subunits might need to be recruited for each cycle of Okazaki fragment synthesis. This behavior has been well established for the *Escherichia coli* replisome18. Trapping experiments have shown the T4 primase to be somewhat distributive suggesting that new primase subunit(s) may be recruited with initiation of each Okazaki fragment19. In the third model (DNA looping), the primosome remains intact and the DNA that is continuously unwound by the helicase during primer synthesis forms a loop which is released once the primer is transferred to the lagging strand polymerase. Recently, the formation of a DNA priming loop was hypothesized when the T4 primase was found to be moderately processive with the processivity being dependent on the efficiency of primer transfer to the lagging strand polymerase indirectly through the clamp and clamp loader proteins20. However, this priming loop has not been directly observed.

Here we use a magnetic trap to manipulate a DNA hairpin and study the behavior of the T4 primosome during primer synthesis. Our results demonstrate that the T4 primosome is capable of simultaneous helicase and primase activity through both the primosome disassembly and DNA looping mechanisms. In a primosome complex with separate wild-type (wt) helicase and primase, the disassembly mechanism is favored over DNA looping. However, a primosome consisting of a fused primase-helicase protein successfully unwinds and primes DNA exclusively through the DNA looping mechanism. The frequency of DNA looping is increased by the presence of clamp and clamp loader proteins. Through comparison of these data with ensemble experiments20 we conclude that both the

disassembly and DNA looping mechanisms are operative; however, in a replisome the DNA looping mechanism is probably favored.

RESULTS

Experimental configuration and DNA substrate

The experimental configuration consists of a DNA hairpin specifically attached between a glass surface and a magnetic bead (Fig. 2a). The DNA is manipulated by capturing the bead in a magnetic trap generated by a pair of permanent magnets. The pulling force is controlled by varying the distance of the magnets from the sample. Video microscopy is used to track the position of the magnetic bead in three dimensions from which the extension of the DNA molecule and the strength of the stretching force is deduced21.

To investigate primase activity, we have designed two complementary DNA hairpins (Supplementary Fig. 1a–c; Supplementary Methods online). The DNA hairpins have approximately nine 5′-GCT priming sites along each strand of the hairpin; however, substrate S1 has eleven 5′-GTT priming sites on the 5′ strand accessible during DNA unwinding and substrate S2 has nine 5′-GTT priming sites on the 3′ strand accessible during ssDNA translocation and hairpin reannealing. All sites can be utilized by the primase when all four rNTPs are present; however, priming can be restricted to the 5′-GTT recognition sites by providing only CTP in addition to ATP required for helicase activity. Limiting priming to one of the two strands of the DNA hairpin simplifies the interpretation of the data. Presented results correspond to the S1 substrate unless explicitly stated otherwise.

The mechanical stability of the DNA hairpin was characterized demonstrating mechanical unzipping above 15 pN and stable folding below 12 pN (Supplementary Fig. 1d). Therefore, experiments were performed at a constant stretching force of 9 pN (or at 5 pN as stated) to ensure that changes in DNA extension were the result of enzyme activity and not mechanical manipulation.

Primer synthesis depends on rNTP concentration

In the presence of helicase and ATP, helicase activity leads to an increase in the DNA extension as the DNA is unwound followed by a decrease in the DNA extension corresponding to either the rapid rehybridization of the hairpin after helicase dissociation (data not shown) or the slower reannealing of the hairpin as helicase translocates on ssDNA until the extension of the folded hairpin is recovered (Fig. 2b)22.

We have monitored the changes in DNA extension resulting from primosome activity 1) in the absence of rNTPs with no primer synthesis, 2) in the presence of all rNTPs with primer synthesis, or 3) in the presence of CTP and ATP with primer synthesis limited to 5′-GTT priming sequences. In the absence of rNTPs, the primosome activity is indistinguishable from the activity observed with helicase alone displaying a relatively uniform increase associated with the unwinding activity followed by a decrease corresponding to ssDNA translocation activity and rezipping of the hairpin (Fig. 2b). The primosome unwinding and translocation velocities are similar to those measured with the helicase alone (Supplementary Fig. 2 online).

In contrast, two new features in the DNA extension traces are observed with the primosome and all rNTPs. Periods of constant DNA extension followed by bursts of rapid DNA rehybridization during translocation and sudden increases in DNA length during unwinding are described as blocks and jumps, respectively (Fig. 2c). The frequency of these new features is directly dependent on the rNTP concentration; in the absence of rNTPs, jumps during unwinding are never observed and blocks during hairpin reannealing are very rare (Fig. 2d). These features are indicative of priming events. In support, primer synthesis was directly observed by the primosome on the hairpin substrates in ensemble assays (Supplementary Fig. 3a online).

In the presence of CTP and ATP priming is limited to 5′-GTT priming sites during the unwinding phase on the S1 hairpin and during the rezipping phase on the S2 hairpin. Under these conditions priming events were observed only on the S1 hairpin demonstrating the primase synthesizes a primer when the helicase is unwinding the DNA and not while it is translocating along the ssDNA during the rezipping phase (Supplementary Fig. 3b online). However, the primosome readily synthesizes primers while rapidly translocating on ssDNA or circular ssM1323,6 and as part of the active replisome24 in ensemble assays. Therefore the inability of primase to synthesize primers during the rezipping phase is most likely due to the reannealing of the hairpin behind the primosome, a situation which is not encountered during DNA replication.

T4 primosome does not pause while priming

Pausing behavior by the primosome should result in DNA unwinding traces with periods of constant DNA extension while the primer is being synthesized followed by an increase in DNA extension again when helicase activity is resumed (Fig. 1a).

Pauses in the unwinding phase were observed rarely (< 0.05 events per trace) and were independent of the rNTP concentration and the DNA hairpin indicating that these pauses were not related to priming activity (Supplementary Fig. 4a online). Moreover, these pauses occurred in GC rich regions of the hairpin substrates suggesting that they correspond to pausing of the helicase when encountering regions of high DNA stability (Supplementary Fig. 4b).

Note that long periods of constant DNA extension were frequently observed during the rezipping phase of traces on either DNA hairpin in the presence of all rNTPs. However, these events can not be interpreted as pausing by the primosome since active primer synthesis is restricted to the unwinding phase under this experimental configuration and therefore, must be the signature of another phenomenon.

T4 primosome can disassemble during primer synthesis

In the primosome disassembly model, one or more of the primase subunits dissociate from the helicase and remain behind to synthesize a primer while the helicase and any remaining primase subunits continue to travel forward unwinding the DNA. This behavior should result in DNA unwinding traces with no change in the helicase unwinding rate since the helicase continues to travel along the DNA regardless of primers being made by dissociated primase subunit(s). Depending on their stability, individual primers or primer/primase

complexes on the DNA may block the rehybridization of the DNA hairpin resulting in DNA rezipping traces with periods of constant DNA extension followed by rapid rehybridization when the primer or primer/primase complex dissociates from the DNA (Fig. 1b and 3a).

Blocks during DNA reannealing are the major type of priming event observed in traces of primosome activity (Fig. 2d). A comparison of the unwinding velocity during primer synthesis ($v_{priming}$) to the mean unwinding velocity ($v_{unwinding}$) demonstrates that there is no change in the DNA unwinding velocity of the helicase due to priming (Fig. 3b). Additionally, the position of the blocks correlates with a priming site on the 5′ strand of the DNA hairpin accessible during DNA unwinding (Fig. 3c and Supplementary Fig. 5 online). For the wt primosome in situations where only one or both priming recognition sequences could be utilized, the distances between a block in DNA rehybridization and the closest priming site on the 5′ strand of the DNA substrate are best fit to a Gaussian distribution centered at one nucleotide with a variance σ of ~10 nt (Table 1 and Fig. 3d). If the position of the blocks in DNA rehybridization were not localized to priming sites, i.e.- the primase binds randomly along the 5′ strand of the hairpin, the distribution of distances between blocks and priming sites would be 2.5 – 4 times wider depending on the substrate and the rNTP present (Table 1). The constant primosome unwinding velocity and the position of blocks in DNA rezipping identified with priming sites support the primosome disassembly model for simultaneous DNA unwinding and primer synthesis.

The nature of the blocks in DNA rehybridization was investigated. We tested the ability of ribonucleotide primers of various lengths to generate blocks in DNA hairpin reannealing (Supplementary Fig. 6 online). RNA oligonucleotides shorter than eight nucleotides were unable to generate observable blocks in the DNA rehybridization indicating that alone the pentaribonucleotide primers synthesized by the T4 primase are too short to effectively block the reannealing of the DNA hairpin.

We also investigated whether the blocks in DNA rehybridization were generated by dissociated primase subunit(s) or a primase/primer complex using an active site point mutant of primase, gp61(E234Q). This mutant primase binds and recognizes priming sites, but is unable to catalyze the ribonucleotide condensation reaction and synthesize primers19,7. Experimental results demonstrate that priming site recognition by the gp61(E234Q) primosome is sufficient to induce blocks in hairpin rezipping and jumps in DNA extension, albeit at a lower frequency of events than with the wt primosome (Supplementary Fig. 7 online). The gp61(E234Q) primosome displays a similar correlation between the position of blocks in DNA rehybridization and priming sites on the 5′ strand of the DNA hairpin as the wt primosome except the distribution of distances is shifted by 4 – 5 nt and is centered at ~6 nt (Table 1 and Fig. 3d). This is consistent with the inactive mutant primase binding the priming recognition sequence, but then being unable to translocate the five nucleotides required to synthesize a primer. Additionally, the mean lifetime of blocks generated by the wt primosome is 3 – 4 times longer than the mean lifetime of blocks generated by the gp61(E234Q) primosome (Table 2 and Fig. 3e). Since the wt and mutant primase proteins have equivalent DNA-binding affinities15, we attribute this longer lifetime to an increase in the DNA-binding affinity of a primer/primase complex over the primase protein alone. Together, these results suggest that the blocks in DNA rehybridization are generated by a

complex of primase subunit(s) and RNA primer bound to the DNA hairpin and are a further indication of actual primer synthesis by the wt primosome under these single-molecule conditions.

To confirm the operation of a primosome disassembly mechanism, we performed experiments with a fused primosome, in which the primase and helicase proteins are fused into a single polypeptide. In ensemble assays, the gp61-gp41 fusion protein has nearly wt priming and DNA unwinding activity and is capable of supporting coordinated leading and lagging strand DNA synthesis (Supplementary Fig. 8a–c online). In the absence of rNTPs, the fused primosome displayed DNA unwinding and ssDNA translocation activity similar to the wt gp41 helicase in single-molecule assays (Supplementary Fig. 8d). Since the primase is fused to the helicase, the primase can no longer dissociate from the helicase in order to synthesize a primer. As expected, blocking events were not observed in DNA extension traces of the fused primosome in the presence of CTP (Fig. 3f). However, frequent jumps in the DNA extension during unwinding were observed with the fused primosome similar to those observed with the wt primosome indicating another possible mechanism for coupled DNA unwinding activity and primer synthesis.

T4 primosome can form a DNA loop during primer synthesis

In the DNA looping model, the primosome remains intact and the DNA that is being continuously unwound forms a loop that is released once the primer is transferred to the lagging strand polymerase. In our experimental configuration, the DNA extension is increased by a total of two nucleotides (one nucleotide in each strand of the hairpin) for each DNA base-pair unwound by the helicase. However, if a loop in one strand of the DNA forms, then only one of the two strands of unwound DNA would contribute to the elongation of the molecule and an apparent decrease in the unwinding velocity would be measured during DNA loop formation. A sudden increase or jump in the DNA extension would be observed upon release of the DNA loop. Thus, a primosome operating by the DNA looping model should display traces of DNA extension with a decrease in the unwinding rate followed by a jump and unaffected hairpin reannealing since a pentaribonucleotide primer is not sufficient to block the DNA rehybridization and the primase subunits remain with the helicase in this model (Fig. 1c and 4a).

Priming events with this characteristic signature for DNA loop formation and release account for approximately five percent of the total priming events observed with the wt primosome, but for all of the priming events observed with the fused primosome (Fig. 3f). In either case, the apparent DNA unwinding velocity during primer synthesis ($v_{priming}$) is approximately one-half the mean DNA unwinding velocity ($v_{unwinding}$) indicating that the primosome continues to unwind the DNA at a constant rate regardless of whether it is synthesizing a primer and consequently forming a DNA loop (Fig. 4b). The length of the sudden jump in DNA extension is a measure of the loop size that is formed during priming. Mean loop sizes of 170 ± 20 nt and 210 ± 20 nt were obtained from distributions of loop sizes measured for the wt and fused primosomes, respectively (Fig. 4c). Considering a mean DNA unwinding velocity of 230 bp/s, the average duration of loop formation and release, indicative of the time required to synthesize a primer, is 1 s. This value is consistent with the

maximum priming rate of 1 primer per second per replisome measured in bulk experiments25.

Force does not hamper loop formation

When a pulling force is applied to stretch DNA, the primosome must work against it to form a DNA loop during primer synthesis. To investigate whether the applied force might prevent loop formation thereby favoring primosome disassembly, we performed priming experiments at high and low applied force. At low applied force the signal to noise ratio is lower because the extension of ssDNA is short while the fluctuations in extension are large. Therefore, 5 pN is the lowest applied force we can work at to retain the resolution necessary to observe priming events. Surprisingly, the force does not significantly affect the frequency of total priming events or the ratio of priming by the primosome disassembly or DNA looping mechanisms (Fig. 4d) indicating that the frequent primosome disassembly and low primase processivity we observed are unlikely to be artifacts of our experimental approach, but are more likely to be intrinsic properties of the T4 wt primosome. However, we are unable to rule out the possibility that below 5 pN of applied force, the ratio of priming by the primosome disassembly or DNA looping mechanism may shift to favor the DNA looping mechanism.

Occasionally DNA looping events are detected when the primosome synthesizes a primer as it translocates along the ssDNA tails of the hairpin. These are marked by a decrease in the DNA extension as the ssDNA forms a loop and then a rapid increase in extension as the loop collapses (Supplementary Fig. 9 online). The ssDNA translocation velocity of the primosome during these events was also force independent.

Additional replisome proteins favor DNA looping mechanism

Recently we demonstrated that the primase processivity was dramatically increased in the presence of either ssDNA binding protein or clamp and clamp loader20. These data are consistent with the signaling model for Okazaki fragment initiation where the newly synthesized primer is likely transferred to the clamp and clamp loader before ultimately being transferred to the lagging strand polymerase. Attempts to assess the effect of gp32 on the distribution of priming mechanism utilized by the primosome in single-molecule experiments were hindered by its binding to exposed ssDNA regions, which affected the DNA extension as well as prevented hairpin reannealing, thus inhibiting the detection of both loop formation and blocks.

Addition of moderate levels of clamp and clamp loader to the wt primosome in our experiments increases the frequency of jumps in DNA extension by a factor of three, whereas the frequency of blocks in hairpin reannealing remained unaltered (Fig. 5a). These two proteins added in the absence of primase have no effect on the helicase activity or produce blocks in DNA rehybridization (data not shown). In contrast to priming via DNA loop formation observed in the absence of clamp and clamp loader, blocks in DNA rehybridization are now observed following a primer synthesized by the DNA looping mechanism when clamp and clamp loader are present (Fig. 5b). Additionally, the mean lifetime of blocks in DNA rehybridization is three times longer in the presence of clamp and

clamp loader than with the wt primosome alone (Fig. 5c). Note that only blocks longer than 5 s have been used to compute the histogram of lifetimes for experiments in the presence clamp and clamp loader; without this cutoff the distribution does not fit well to a single exponential function suggesting that at least two populations of events with different mean blocking lifetimes are observed under these conditions (i.e. – a population with a short mean blocking lifetime due to a primer/primase subunit(s) complex and a population with a long mean blocking lifetime due to a primer/clamp/clamp loader complex). Together, these data suggest that the primer has been transferred from the primase to a clamp/clamp loader complex blocking the reannealing of the hairpin and are consistent with the accessory proteins favoring the DNA looping pathway for primer synthesis by increasing the primer handoff efficiency and primase processivity.

DISCUSSION

Using single-molecule techniques, this paper addresses the functioning of the T4 primosome complex to simultaneously unwind dsDNA to advance the DNA replication fork and synthesize RNA primers necessary to initiate Okazaki fragments in the opposing direction. The three models of primosome behavior could be distinguished in this study because of our experimental configuration and DNA substrates. The hairpin design with separate DNA extension signals for DNA unwinding and ssDNA translocation (rezipping) allows for the detection of primosome pausing, dissociation of primase subunit(s), and DNA loop formation. Single-molecule studies on other DNA replication systems have relied on DNA synthesis by the leading or lagging strand holoenzymes to report indirectly on the priming activity of the primosome17,26. By working at 5 – 9 pN applied force, we also have greater spatial resolution than previous studies, which allows us to observe the formation of small DNA loops during primer synthesis and localize the binding site of primase subunit(s) corresponding to a priming recognition sequence or a site shifted by five nucleotides. The fact that primase is able to recognize a priming site and synthesize a primer only during the unwinding phase simplifies our analysis.

Primer synthesis and utilization is a stochastic process meaning that not every priming recognition sequence is used to synthesize a primer and not all synthesized primers are used to initiate an Okazaki fragment27. Our results are consistent with this fact. We observed approximately one priming event indicated by a block in DNA rehybridization or formation of a DNA loop per trace by the wt primosome despite the presence of ~20 possible priming sites. The large majority of such priming events correspond to the synthesis of a full pentaribonucleotide primer indicated by the comparison of results obtained with wt primase and an inactive gp61(E234Q) primase. First, the frequency of priming events with the wt primosome was twice the frequency with the mutant primosome. Secondly, analysis of limited priming on the S1 hairpin revealed that the majority of observed blocks were primarily localized to two priming sites where a complete pentaribonucleotide primer could be synthesized with only CTP and ATP. Third, the mean lifetime of the blocks was 3 – 4 times longer with the wt primosome than with the mutant primosome suggesting that blocks observed with the wt primosome were generated by a primase/primer complex. Lastly, the distribution of the blocking position with the mutant primosome was shifted by five

nucleotides compared with the wt primosome consistent with the wt primosome synthesizing a pentaribonucleotide primer.

We have determined that the T4 primosome does not pause in order to accommodate helicase and primase activities. Instead the T4 primosome can function through primosome disassembly, identified by blocks in DNA reannealing, or a DNA looping mechanism, identified by jumps in DNA unwinding. The disassembly mechanism has been previously proposed for T419 and bacterial18,28 replication to account for the distributive nature of the primase, but this is the first single-molecule study reporting evidence to supporting this model. The DNA looping mechanism is a novel priming mechanism that prevents primase dissociation through the formation of a DNA loop either between the helicase and primase proteins or after the primase if the active site were to face the outside of the primase ring as suggested for the T7 system29. This DNA looping mechanism is also consistent with the moderate processivity of primase discovered recently20. The priming mechanism employed by the primosome is determined stochastically subject to variations in the environment of the replisome each time a primer is synthesized.

In the absence of other replisome proteins, the T4 primosome under single-molecule conditions strongly favors the primosome disassembly model for priming over the DNA looping model. The ratio between these two mechanisms was independent of the applied force suggesting that the moderate processive nature of the primase is an intrinsic property of the T4 primosome. Recently, we have shown that the primase processivity was dependent on the presence of gp32 and the efficiency of indirect primer handoff leading to an additional model of the T4 replisome in which primase remains bound to the DNA replication fork during several cycles of lagging strand synthesis accommodating dsDNA unwinding by the helicase and primer synthesis with the formation of a DNA loop20. In agreement with this model we find that the number of priming events involving DNA loop formation increase in the presence of accessory proteins, clamp and clamp loader, which are likely to be involved in transferring the primer from primase to polymerase; however, at the moderate levels of accessory proteins used in our single-molecule experiments, primosome disassembly remains the predominant pathway for primer synthesis. The presence of gp32 and/or higher concentrations of clamp and clamp loader proteins may shift the ratio between the two priming mechanisms towards the DNA looping pathway20 making the DNA looping mechanism the relevant mechanism for repetitive lagging strand synthesis in the context of a complete replisome (Fig. 6). In agreement with this scenario, we find that a fused helicase/primase protein, which primes exclusively via the DNA looping mechanism, supports both leading and lagging strand synthesis at nearly wild-type levels.

In contrast to the gp61-gp41 fusion protein, the T7 primosome consisting of a single protein with helicase and primase activities was found to operate as a brake causing the entire replisome to pause during primer synthesis17. In the *E. coli* system, pausing of the replisome during primer synthesis was not observed; however, the experimental configuration in this case did not allow for the determination of a primosome disassembly or DNA looping model as an alternate mechanism for helicase and primase coupling26. Ensemble studies indicate that with SSB (the ssDNA-binding protein in *E. coli*), two primase subunits dissociate and protect the primer upon completion of primer

synthesis18,28 suggesting that the *E. coli* system uses the primosome disassembly mechanism.

Thus nature appears to have evolved at least three different strategies to solve the conundrum of disparate helicase and primase activities within a single protein or primosome protein complex for the T7, T4, and *E. coli* systems. The necessity for different strategies may be related to the size of the genome being replicated and the ability of the replisome to retain and utilize the synthesized primers for repetitive lagging strand synthesis. In the case of the T7 system, pausing the entire replisome during primer synthesis appears to be an acceptable trade-off to rapid DNA replication due to the relatively small size of the T7 genome (40 kbp), whereas the T4 and *E. coli* systems cannot afford to pause the leading strand holoenzyme during primer synthesis and still complete DNA replication in a timely manner due to the larger size of their genomes, 170 kbp and 4,600 kbp, respectively. Instead, the T4 and *E. coli* replisomes accommodate continuous helicase unwinding activity and leading strand DNA synthesis with the necessity for primer synthesis using either the DNA looping or primosome disassembly mechanisms with the determining factor most likely being the nature of the clamp loader present in each system. In both systems, the primer can be transferred indirectly from the primase to the lagging strand polymerase by the clamp and clamp loader proteins. In the case of the *E. coli* system, the multisubunit clamp loader remains as part of the replisome interacting with the DnaB helicase and both leading and lagging strand polymerases functioning to load clamps and as a general organizer of the replisome. The DnaG primase binds tightly to the newly synthesized primer requiring SSB and χ (a subunit of the *E. coli* clamp loader) to displace the primase subunits and allow for loading of the β clamp18. Thus the DnaG primase and primer dissociate from the DnaB helicase and yet remain associated with the replisome through the clamp loader for efficient initiation of Okazaki fragment synthesis. On the other hand, the T4 clamp loader (gp44/62) does not remain a part of the replisome instead being recruited from solution each time a clamp needs to be loaded onto a primer for repetitive lagging strand DNA synthesis. Therefore, efficient primer handoff and initiation of Okazaki fragment synthesis might necessitate the retention of the gp61 primase and primer within the replisome by maintaining an interaction with the gp41 helicase utilizing the DNA looping mechanism for primer synthesis.

METHODS

Proteins

The helicase, wt and mutant (E234Q) primase, clamp and clamp loader proteins were prepared as previously described30,25,19,31. The gp61-gp41 fusion was constructed by fusing the C-terminus of gp61 to the N-terminus of gp41 with a flexible 24 amino acid linker (Supplementary Methods online). The fusion protein was cloned into the IMPACT system and purified using chitin-based affinity chromatography and a self-cleaving intein25. Standard assays were used to characterize the unwinding and priming activity of the fusion protein as well as its ability to support coordinated leading and lagging strand synthesis (Supplementary Methods; Supplementary Fig. 8).

DNA Hairpin Substrates

The two DNA hairpin substrates were made from the same 1.1 kbp insert of plasmid pNo_GTT (Supplementary Methods) sequentially digested with restriction enzymes ApaI and NotI generating a DNA fragment containing 5′-GTT priming sites on only one strand and two different compatible ends. The fork structure was formed by two partially annealed oligos of which the “flap” oligo was 5′-biotinylated to allow for attachment to the magnetic bead. This fork structure and a short hairpin oligo were annealed and ligated to either end of the 1.1 kbp fragment based on their compatible ends. Substrate 1, which has 5′-GTT priming sites on the 5′ strand accessible during DNA unwinding, and substrate 2, which has 5′-GTT priming sites on the 3′ strand accessible during ssDNA translocation, were created by ligating the fork structure and hairpin oligo on opposite ends. The hairpins were purified from excess oligos and concatemers by agarose gel. The digoxigenin label was incorporated by annealing a primer to the template strand and filling in the overhang with T4 polymerase in the presence of dATP, dCTP, and dUTP-digoxigenin. The primer was not extended due to the absence of dGTP. The completed hairpin substrates were again purified from excess primer, nucleotides, and polymerase by agarose gel. (Complete oligo sequences are giving in Supplementary Methods.)

Ensemble Priming Assays

Priming reactions were carried out in replication buffer containing 25 nM DNA hairpin substrate, 2 mM ATP, 100 μM each CTP, GTP, and UTP, 20 μCi of [α-^{32}P]CTP, and 500 nM each gp41 and gp61 (monomeric concentrations) in a reaction volume of 20 μL. The reactions were carried out at 37 °C, and aliquots were withdrawn at the indicated times and quenched with an equal volume of 250 mM EDTA and loading buffer (formamide, 1 μg/mL bromophenol blue, 1 μg/mL xylene cyanol FF). Priming products were separated by denaturing 20% PAGE and analyzed using a PhosphorImager.

Single-Molecule Assay

The glass surface was treated with anti-digoxigenin antibody and passivated with BSA. The magnetic beads (Dynal) were ~1 μm in diameter and coated with streptavidin. Bead images were acquired at 60 Hz using a PicoTwist prototype inverted microscope (www.picotwist.com) and the DNA extension was measured by tracking the bead position in real time21. The mechanical stability of the DNA hairpins was characterized by measuring the extension of the substrate as a function of the pulling force along a force-cycle in which the force is first increased and then relaxed. A calibration curve for force versus magnet position was used to exert forces of 5 or 9 pN with 10% error on the DNA molecules. All experiments were performed at 29 °C in 25 mM Tris-Ac (pH 7.5), 150 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, and 5 mM ATP. Protein concentrations were 50 nM gp41, 200 nM gp61, 30 nM gp61-41 fusion, 90 nM gp45 and 30 nM gp44/62 (all monomeric concentrations). An excess of primase with respect to helicase was used to maximize the formation of the primosome complex. The excess primase protein does not interfere since primase alone has insignificant priming activity6,13. No blocks in hairpin reannealing were observed when the hairpin was repeatedly mechanically opened and allowed to reanneal in the presence of 200 nM primase and rNTPs. Moreover, we find that under equimolar

conditions, 50 nM primase and helicase, the frequency of total priming events (blocks and jumps) is only 10% lower than with excess primase (data not shown).

Single-Molecule Data Analysis

Raw data, corresponding to the real-time evolution of the DNA extension $x(t)$ in μm, was converted into the number of base-pairs unwound $n(t)$ as a function of time using either the equation $x_{max} - x_{min} = 604$ bp unwound or 1208 nt for the S1 hairpin or the equation $x_{max} - x_{min} = 605$ bp unwound or 1210 nt for the S2 hairpin. The unwinding or rezipping velocity ν at time t was computed as the slope of the best linear fit to a 30 point (corresponding to 0.5 s) segment $S(n(\tau), \tau\varepsilon \{t - 15\Delta t, t + 15\Delta t\})$, where Δt is 1/60 s, the time interval between two data points (Supplementary Fig. 2a online). The mean unwinding rate was calculated from segments of traces that do not display a priming signature, while the unwinding rate during primer synthesis was measured from that segment of the unwinding trace corresponding to right before a block in hairpin rehybridization or a jump in extension. Segments where $\nu = 0$ were identified as blocks, while points that satisfied $n(t + 2\Delta t) - n(t) > 70$ bp unwound or 140 nt were identified as jumps in DNA extension. The minimum cutoff for loop size was chosen to be well above the noise of our system (~10 bp); however, this stringent criterion may lead to the underestimation of the frequency of looping events. The frequency of priming events was measured from multiple molecules where multiple traces were observed and recorded for specific substrate, protein, and nucleotide conditions; the error bars represent the standard error of the mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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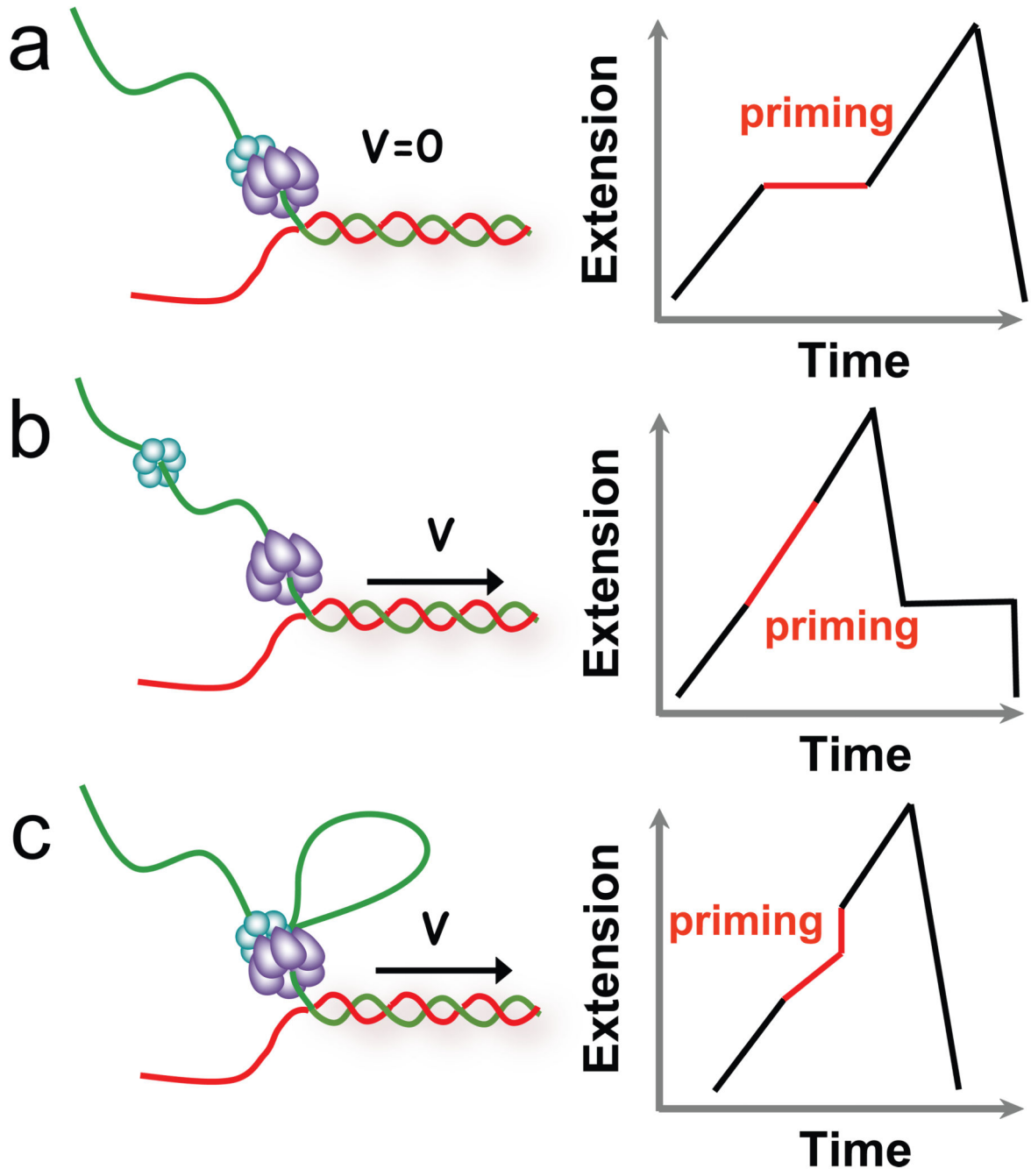

Figure 1.

Models of primosome behavior during primer synthesis. Schematic representation of three possible models for helicase and primase interaction during primer synthesis (left) and the real-time DNA extension traces expected for each model (right). (**a**) In the pausing model the helicase and primase temporarily stop translocating during priming. (**b**) In the disassembly model the primase dissociates from the helicase to synthesize a primer while the helicase continues unwinding DNA. (**c**) In the DNA looping model the primosome remains intact and DNA unwound during priming forms a loop.

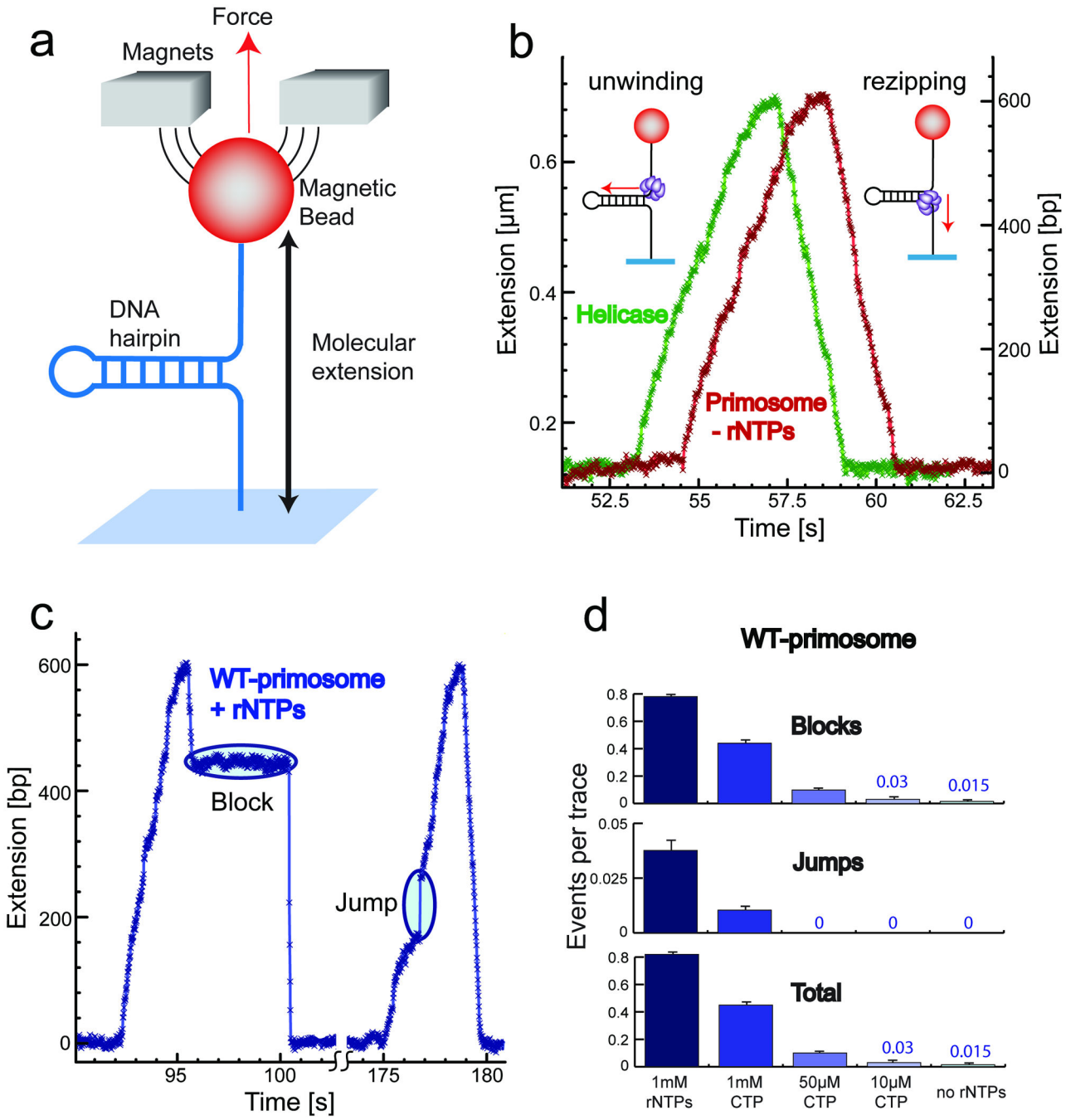

Figure 2.

Primer synthesis by primosome depends on rNTP concentration. (**a**) Schematic representation of the experimental configuration. (**b**) Experimental traces corresponding to the gp41 helicase activity (green) and the wt primosome activity (red) in the absence of rNTPs. (**c**) Examples of two new features, blocks in hairpin reannealing and jumps in extension during unwinding, observed in experimental traces from wt primosome activity in the presence of 1 mM rNTPs. (**d**) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower

panel) measured in experiments with the wt primosome at the indicated rNTP concentration. The frequency is calculated as the number of events per enzymatic trace, where an enzymatic trace is defined as a trace demonstrating complete unwinding and rezipping. The values for low frequency measurements are explicitly given and error bars are the SEM. The number of molecules (N_{mol}) analyzed for each condition is 29, 21, 10, 6 and 12 in order, resulting in 447, 612, 136, 102 and 226 number of enzymatic traces (N), respectively.

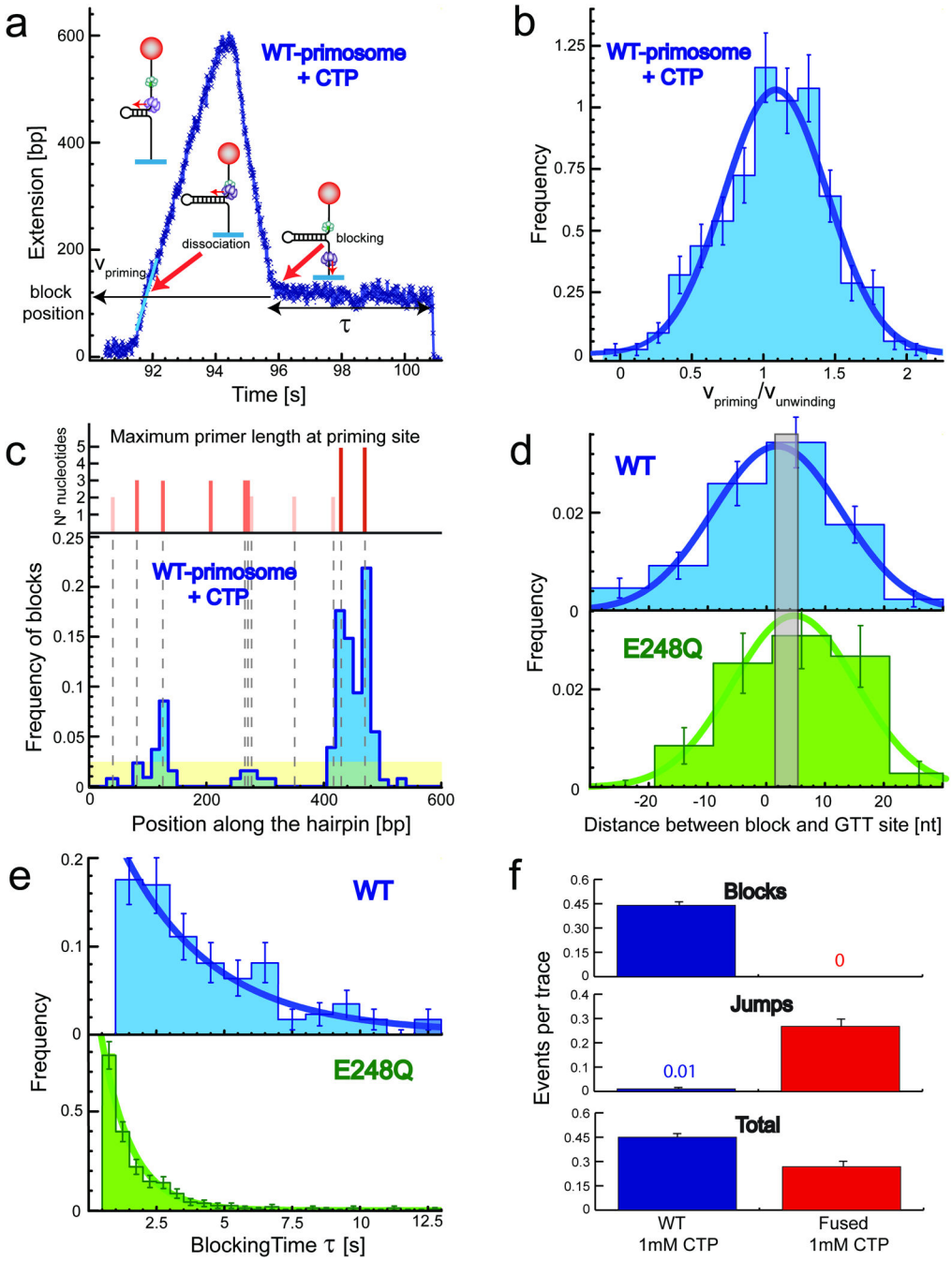

Figure 3.

Primosome disassembly model for primer synthesis. (**a**) Experimental trace displaying characteristics (unwinding velocity during priming, position and lifetime of the block) for the primosome disassembly model. (**b**) Distribution of $v_{priming}/v_{unwinding}$ for the wt primosome, where $v_{priming}$ is the unwinding velocity during primer synthesis and $v_{unwinding}$ is the mean unwinding velocity. A Gaussian fit yields $\langle v_{priming} \rangle = (1.08 \pm 0.02)\ v_{unwinding}$ (N_{mol}=21, N=421). (**c**) Distribution of blocks plotted against the corresponding position along the substrate for the wt primosome in the presence of ATP and CTP (N_{mol}=21,

N=345) compared to a uniform distribution of primase binding randomly along the DNA (yellow). Priming sites are indicated by dashed gray lines; the maximum primer length synthesized with ATP and CTP is shown in the upper panel. (**d**) Distribution of the distances between the position of the blocks and the nearest priming site for wt (blue; N_{mol}=21, *N*=345) and gp61(E234Q) (green; N_{mol}=13, *N*=101) primosome. Gaussian fits yield a variance of ~10 nt and a mean distance of 1 or 5 nt, respectively (gray bar emphasizes shift). (**e**) Distribution of lifetimes of blocks generated by wt (blue; N_{mol}=21, *N*=476) and gp61(E234Q) (green; N_{mol}=13, *N*=214) primosome. Exponential fits yield a mean blocking time $<\tau>$ of 3.5 ± 0.3 s and 0.9 ± 0.2 s, respectively. (**f**) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower panel) measured in experiments with wt (blue; N_{mol}=21, *N*=447) and fused (red; N_{mol}=25, *N*=169) primosome.

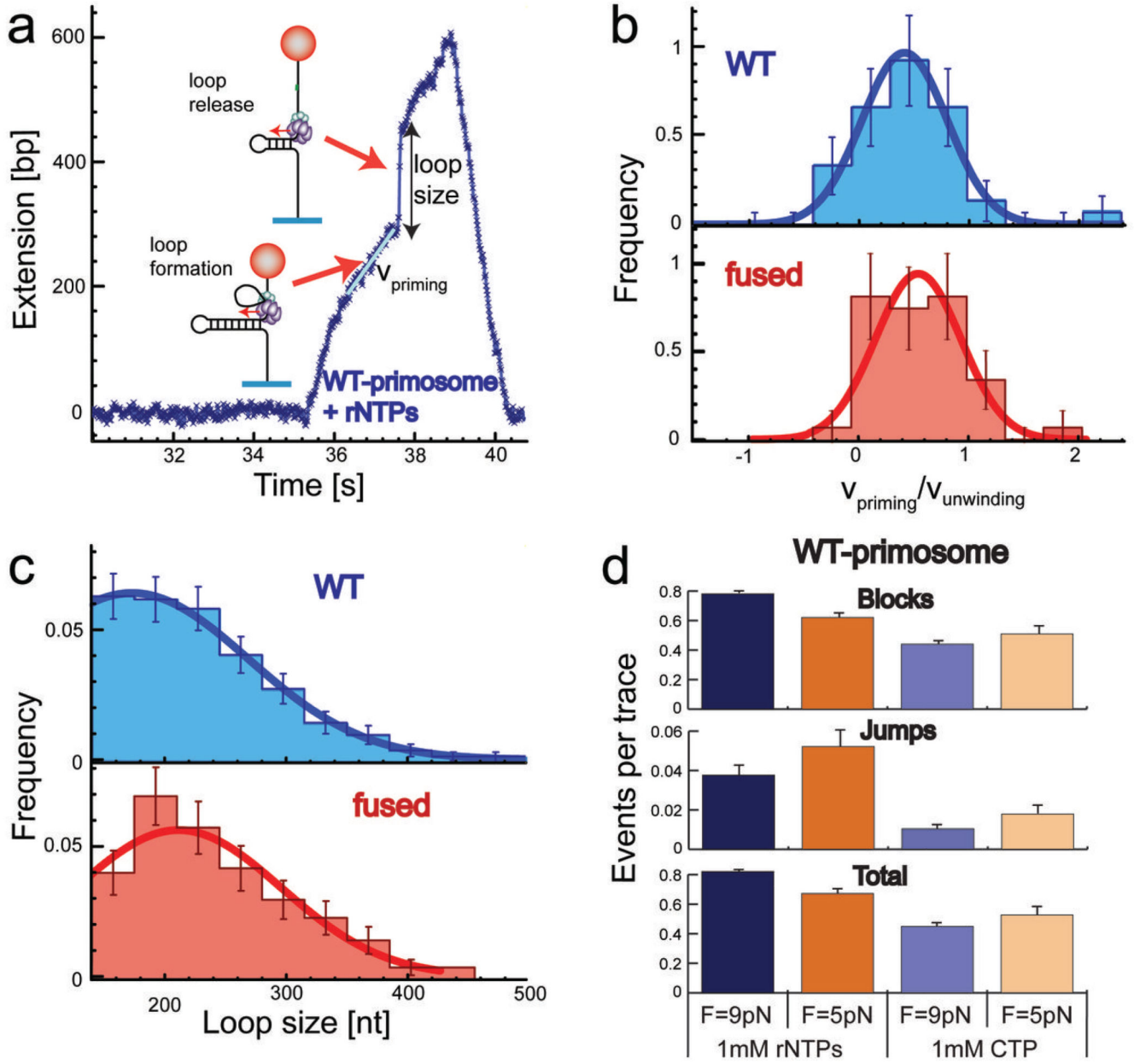

Figure 4.

DNA looping mechanism for primer synthesis. (**a**) Experimental trace displaying characteristics (unwinding velocity during priming and the loop size) for the DNA looping model. (**b**) Distribution of $v_{priming}/v_{unwinding}$, where $v_{priming}$ is the unwinding velocity during primer synthesis and $v_{unwinding}$ is the mean unwinding velocity, for wt (blue; N_{mol}=35, N=71) and fused (red; N_{mol}=21, N=42) primosome. Gaussian fits yield $\langle v_{priming} \rangle$ = (0.51 ± 0.08) $v_{unwinding}$ and (0.44 ± 0.08) $v_{unwinding}$, respectively. (**c**) Distribution of the DNA loop sizes for wt (blue; N_{mol}=35, N=71) and fused (red; N_{mol}=21, N=42) primosome; a minimum loop size of 140 nucleotides was used as a cutoff. Gaussian fits yield a mean DNA loop size of 170 ± 20 nt and 210 ± 20 nt, respectively. (**d**) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and

total priming events (lower panel) measured in experiments with wt primosome at the indicated rNTP concentration and two different applied forces, 9 pN (blue) or 5 pN (orange). The number of molecules (N_{mol}) analyzed for each condition is 29, 11, 21 and 14 in order, resulting in 447, 314, 612, and 268 number of enzymatic traces (N), respectively.

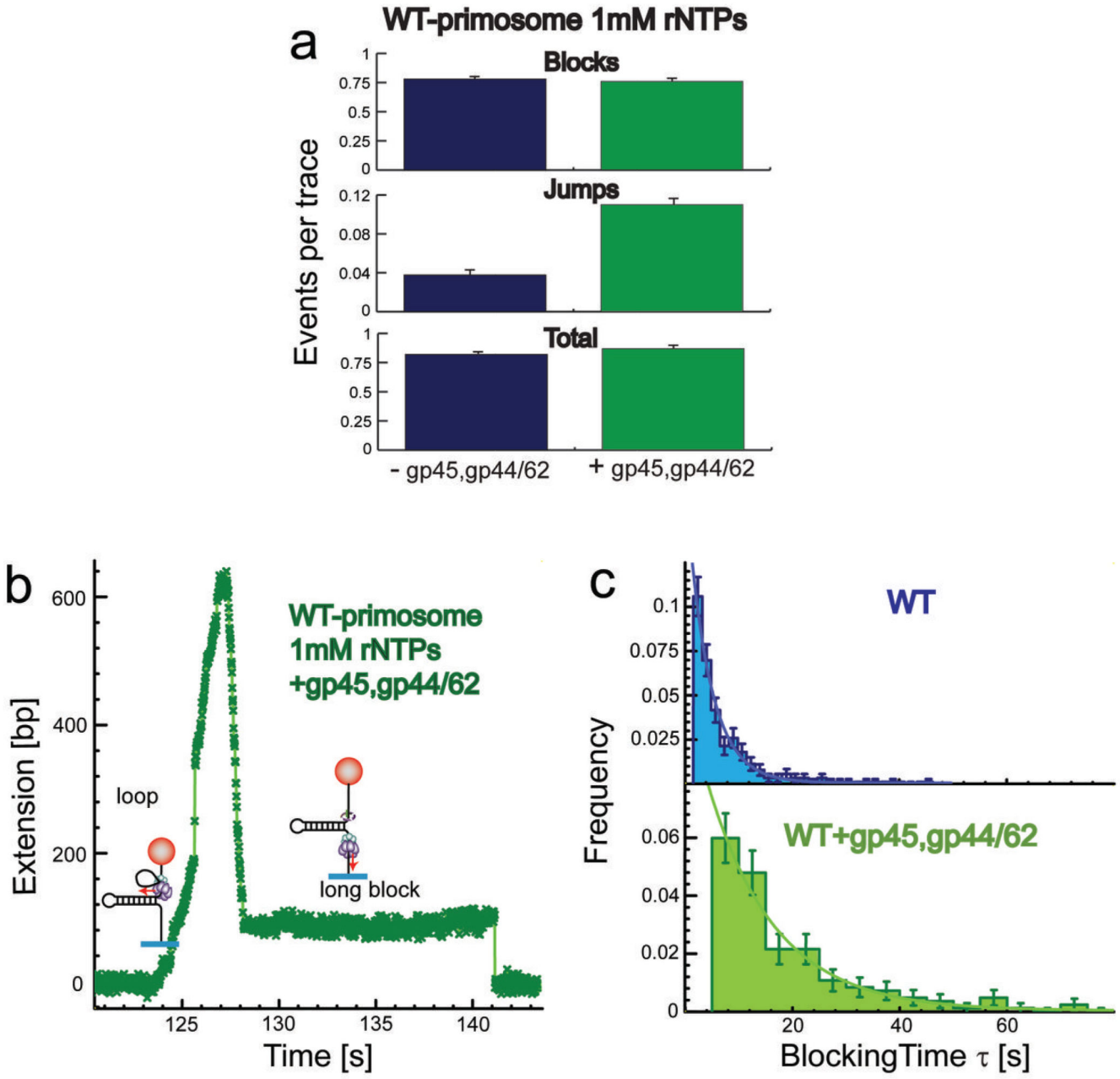

Figure 5.

Polymerase accessory proteins increase DNA looping. (**a**) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower panel) measured in experiments with wt primosome in the absence (N_{mol}=29, N=447) and presence (N_{mol}=18, N=232) of gp45 clamp and gp44/62 clamp loader. (**b**) Experimental trace corresponding to the wt primosome activity in the presence of gp45 clamp and gp44/62 clamp loader. (**c**) Distribution of lifetimes of blocks generated by wt primosome in the absence (blue; N_{mol}=29, N=296) and presence (green; N_{mol}=18, N=167,) of gp45 clamp and gp44/62 clamp loader. Exponential fits yield a mean blocking time $<\tau>$ of 4.2 ± 0.3 s and 12.5 ± 1.5 s, respectively.

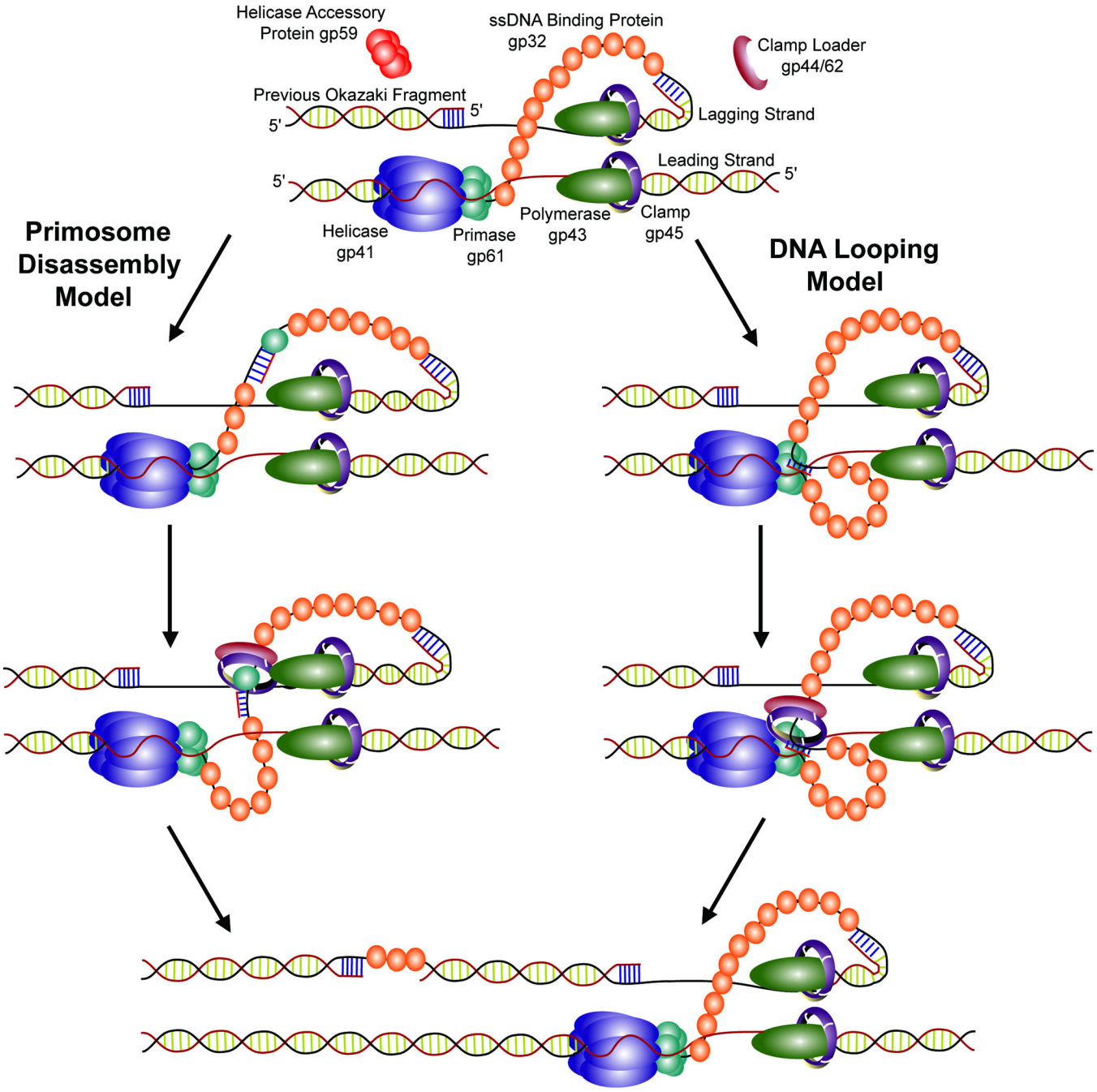

Figure 6.

Model for the primosome activity and initiation of lagging strand DNA synthesis. The T4 replication complex is composed of eight proteins that interact to synthesize DNA. Helicase (gp41) and primase (gp61) form stacked rings that encircle the lagging DNA strand. The helicase unwinds duplex DNA ahead of the leading strand polymerase (gp43) while the primase synthesizes pentaribonucleotide primers for use by the lagging strand polymerase. Initiation of lagging strand synthesis begins with the synthesis of a primer by the primase while the helicase continues to unwind the DNA duplex. To accommodate primer synthesis

in the opposing direction of helicase unwinding one or more primase subunits may dissociate from the primosome complex with the newly synthesized primer (primosome disassembly model) or the primase may remain with the replisome creating a second loop of DNA from the excess DNA unwound by the helicase during primer synthesis (DNA looping model). In either pathway, the clamp loader then loads a clamp onto the RNA primer causing the lagging strand polymerase to release the lagging strand template and recycle to the new clamp-loaded RNA primer. Okazaki fragments are derived from operation of both the collision and signaling mechanisms for lagging strand polymerase recycling.

Table 1

Analysis of the position of rehybridization blocks compared to the position of priming sites.

				Experimental Distribution[d]			Random Distribution[e]
Protein[a]	**Nucleotides[b]**	**Hairpin**	**Priming Sites[c]**	**μ (nt)**	**σ (nt)**	***N* events**	**σ (nt)**
wt	CTP	S1	GTT	1 ± 1	11 ± 1	345	40
E234Q	CTP	S1	GTT	5 ± 1	10 ± 1	101	40
wt	rNTP	S1	GTT, GCT	1 ± 1	10 ± 1	343	26
E234Q	rNTP	S1	GTT, GCT	6 ± 1	8 ± 1	271	26
wt	rNTP	S2	GCT	2 ± 1	11 ± 1	312	29
E234Q	rNTP	S2	GCT	6 ± 1	7 ± 1	418	29

[a] Experiments contained wt gp41 helicase and either wt gp61 or mutant gp61(E234Q) primase.

[b] Experiments contained either 1 mM CTP or 1 mM all rNTPs.

[c] Usable priming recognition sequences based on DNA hairpin and ribonucleotides provided.

[d] Mean (μ) and variance (σ) obtained from a Gaussian fit of the experimental distribution of the distances between a block in DNA rehybridization and the closest priming site on the 5′-strand of the DNA substrate for *N* number of blocking events.

[e] Variance (σ) calculated for the distribution of distances between blocks and nearest priming sites if primase binds randomly.

Table 2

Analysis of the mean lifetime of blocks in DNA rehybridization.

Protein[a]	Nucleotides[b]	Hairpin	Force (pN)	τ (s)[c]	*N* events
wt	CTP	S1	9	3.5 ± 0.3	476
	rNTP	S1	9	3.5 ± 0.5	233
	rNTP	S2	9	3.6 ± 0.5	327
	rNTP	S1	5	3.0 ± 0.4	162
	rNTP	S2	5	4 ± 1	208
E234Q	CTP	S1	9	0.9 ± 0.2	214
	rNTP	S1	9	1.0 ± 0.1	220
	rNTP	S2	9	1.1 ± 0.1	221

[a] Experiments contained wt gp41 helicase and either wt gp61 or mutant gp61(E234Q) primase.

[b] Experiments contained either 1 mM CTP or 1 mM all rNTPs.

[c] Mean lifetime (τ) of primase subunit(s) binding during DNA hairpin reannealing obtained from an exponential fit of the experimental distribution.