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Bacteriophage SPP1 DNA replication strategies promote viral and disable host replication in vitro.

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Abstract

Complex viruses that encode their own initiation proteins and subvert the host's elongation apparatus have provided valuable insights into DNA replication. Using purified bacteriophage SPP1 and *Bacillus subtilis* proteins, we have reconstituted a rolling circle replication system that recapitulates genetically defined protein requirements. Eleven proteins are required: phage-encoded helicase (G*40*P), helicase loader (G*39*P), origin binding protein (G*38*P) and G*36*P single-stranded DNA binding protein (SSB); and host-encoded PolC and DnaE polymerases, processivity factor (β_2), clamp loader (τ-δ-δ′) and primase (DnaG). This study revealed a new role for the SPP1 origin binding protein. In the presence of phage SSB it is required for initiation on replication forks that lack origin sequences, mimicking the activity of the PriA replication restart protein in bacteria. The SPP1 replisome is supported by both host and viral SSBs, but phage SSB is unable to support *B. subtilis* replication, likely due to its inability to stimulate the PolC holoenzyme in the *B. subtilis* context. Moreover, phage SSB inhibits host replication, defining a new mechanism by which bacterial replication could be regulated by a viral factor.

Introduction

Double-stranded (ds) DNA viruses may encode most if not all the components for their own replication, as in the case of the T4, Φ29 or HSV-1 viruses (1-3), or may encode a subset of proteins, including an origin-specific initiation protein, and recruit the host DNA replication machinery to achieve efficient viral replication. The study of the replication mechanisms of the latter type of viruses has provided significant insight into cellular DNA replication processes. For example, SV40, a virus that encodes its own origin binding protein and helicase within the T antigen has provided a viral window into eukaryotic DNA replication (4,5). Its use enabled the only full reconstitution of a eukaryotic DNA replication system with purified proteins and revealed the special roles of Pol α-primase and the Pol δ holoenzyme in the process (5,6). Bacteriophage λ has provided similar insight into the replication of Gram-negative bacteria (7). λ encodes its own origin protein and a helicase loader that subverts the host $DnaB_6$ replicative helicase, leading to the acquisition of the cell's elongation apparatus. Through this system, the role of heat shock proteins in freeing the DnaB helicase from tightly bound λ O and P proteins was discovered, providing one of the initial observations of chaperone function (7,8). The mechanism used by these viruses to recruit host proteins could provide significant insight into viral and host processes.

Bacillus subtilis SPP1 is a virulent dsDNA phage whose mature genome is a linear 45.4-kb dsDNA. The ends of the packaged DNA are terminally redundant and are permuted to facilitate circularization after DNA injection into cells. SPP1 replication starts with the circle-to-circle replication mode (θ replication), but after one or a few rounds, it switches to concatemeric replication (termed σ replication or rolling circle replication) by a process driven by homologous recombination (9,10). This switch in the

mode of replication is a strategy used by many viruses to produce linear head-to-tail concatemers that are used by the packaging machinery. This late-phase DNA replication, which is believed to be independent of an origin of replication, has been reconstituted *in vitro* for viruses that encode their own polymerase [*e.g.*, HSV-1, T7, T4 and Φ29, (11-14)] and for bacteriophage λ (15).

Genetic analyses showed that SPP1 DNA replication is independent of the host origin binding protein (DnaA), the replicative DNA helicase (DnaC), primosomal proteins DnaB and PriA, and RNA polymerase. These studies also showed that SPP1 replication requires the host DnaG primase and PolC DNA polymerase (16-18).

The SPP1 phage possesses two origins of replication, *ori*L and *ori*R, which are 32.1 kb apart in a linear map of the SPP1 genome. Replication proteins are encoded by two operons. The first one, which is under the control of the early promoter P_{E2}, codes for proteins that have been shown to be required for θ replication: the G*38*P origin binding protein, the G*39*P helicase loader, and the G*40*P helicase. G*38*P, which does not belong to the AAA+ family, is widely conserved in phages (19,20). G*38*P binds with high affinity to the two origins of replication (21) and forms a complex with G*39*P (17). G*39*P does not share homology with other studied helicase loaders, but performs a similar role: it delivers G*40*P, upon interacting with G*38*P, to the origin of replication (22,23). G*40*P is a widely studied helicase that belongs to the DnaB family (24-26).

Genes required for the recombination-dependent σ replication mode are under the control of the early promoter P_{E3}. These include a recombinase, G*35*P (18,27), and a 5′ → 3’ exonuclease, G*34.1*P (28). In this operon there is also a gene (gene *36*) that encodes a single-stranded DNA binding protein (SSB), G*36*P, whose role in replication has not yet been analyzed. G*36*P is 48% identical to the essential host SSB (*B. subtilis*

SsbA, Supplementary Figure S1), and 38% identical to the competence-specific SSB [*B. subtilis* SsbB, (29)].

We have exploited the apparatus required for the σ mode of replication to establish a robust rolling circle replication system that requires four phage proteins and seven host elongation proteins. These studies revealed surprising new roles for the G*38*P origin binding protein in the initiation of DNA replication on forks that do not contain origin sequences. In addition, they show the versatility of the SPP1 replication fork, where both the viral and the host SSB may be used, in contrast to the *B. subtilis* replication fork, which uses only its own SSB (the SsbA protein). Moreover, *B. subtilis* replication is inhibited by the viral SSB (G*36*P), a mechanism that is likely exploited by the phage to shut down host DNA replication synthesis and foster its own replication.

Materials and methods

Rolling circle assays

Standard reactions consisted of 30 nM G*40*P_6, 300 nM G*39*P, 300 nM G*38*P, 8 nM DnaG, 15 nM DnaE, 20 nM PolC, 25 nM τ_4, 25 nM δ, 25 nM δ′, 24 nM β_2, 30 nM G*36*P_4, 5 nM mini-circular DNA template, 350 μM ATP, 100 μM CTP, GTP and UTP, 48 μM dNTPs (except 18 μM dCTP or dGTP for the leading and lagging strand DNA synthesis respectively), and 0.2 μCi/reaction [α-^{32}P]dCTP or [α-^{32}P]dGTP. The DNA template was a 409 nt circle containing a 396 nt tail described in (30), but prepared by an alternative procedure that included a PCR amplification step (Yuan and McHenry, in preparation). The reactions were carried out in 12.5 μl of buffer BsRC (40 mM Tris-acetate (pH 7.8), 12 mM magnesium acetate, 3 μM $ZnSO_4$, 1 mM dithiothreitol, 0.02% (w/v) Pluronic F68, (30)) that contained 500 mM potassium glutamate and 1% (w/v) polyethylene glycol (PEG-8K). The buffer also contained 4% glycerol, 19 mM NaCl and 4 mM Tris-HCl that was contributed by the addition of protein solutions. Incubations were conducted for 10 min at 37 ºC. An enzyme mix containing all protein components except SSB (G*36*P, or SsbA as indicated) was prepared in buffer BsRC. Two different substrate mixes containing template DNA, rNTPs, dNTPs, SSB (G*36*P or SsbA) and either [α-^{32}P]dCTP or [α-^{32}P]dGTP for measurement of leading and lagging strand synthesis, respectively, were prepared. Reactions were initiated by mixing the enzyme mix and a substrate mix. After incubation, reactions were stopped by addition of an equal volume of stop mix [40 mM Tris-HCl (pH 8.0), 0.2% SDS, 100 mM EDTA, and 50 μg/ml proteinase K]. Samples were treated for 20 min at 37 ºC, then applied onto Sephadex G-50 columns to eliminate non-incorporated dNTPs. The extent of DNA synthesis in leading and lagging strands was quantified by scintillation counting.

For the analysis of the size of leading and lagging strand products, samples were brought to 50 mM NaOH, 5% (v/v) glycerol and 0.05% bromphenol blue and fractionated on alkaline 0.5% agarose gels for approximately 3 h at 80 V. Alkaline agarose gel buffer consisted of 30 mM NaOH and 0.5 mM EDTA. Gels were fixed in 7% (w/v) trichloroacetic acid, dried, autoradiographed on storage phosphor screens and analyzed with Quantity One (Bio-Rad) software.

For calculating the rate of SPP1 fork progression, aliquots were removed, quenched and processed as described above. The molecular weight of the longest leading strand product at each time was extrapolated from labeled DNA size standards and plotted as a function of time. The elongation rate was determined by calculating the slope of this curve (31).

The protein concentrations in the *B. subtilis* replication system were: 15 nM DnaE, 20 nM PolC, 8 nM DnaG, 25 nM τ_4, 25 nM δ, 25 nM δ′, 24 nM β_2, 30 nM $DnaC_6$, 15 nM PriA, 50 nM $DnaD_4$, 100 nM $DnaB_4$, 40 nM $DnaI_6$ and various amounts of $SsbA_4$ and G*36*P_4.

Extension of DNA primers annealed to M13

Templates were prepared by mixing 50 pmol single-stranded $M13_{Gori}$ DNA (32) with 60 pmol synthetic DNA primer (5'AGGCTGGCTGACCTTCATCAAGAGTAATCT) in 70 µl of a buffer consisting of 40 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 1 mM EDTA, heating to 95°C, cooling to room temperature over 1 h and diluting the resulting mixture to 29 nM as circles. Holoenzyme reactions (25 µl each) contained 2.3 nM template; 2 nM PolC or 3 nM DnaE; 25 nM β_2, if present, 15 nM τ_4, 20 nM δ, 20 nM δ', and variable concentrations of *B. subtilis* $SsbA_4$ or SPP1 G*36*P_4; 48 µM dATP, dGTP and dCTP; 18 µM [^{3}H]dTTP (specific activity 113 cpm/pmol); and 250 µM ATP in buffer BsM13 (40 mM Tris-acetate (pH 7.8), 340 mM potassium glutamate, 10 mM

magnesium acetate, 4 μM $ZnSO_4$, 0.015% (w/v) Pluronic F68). Reaction mixtures were prepared on ice, initiated by incubation at 30 °C, stopped after 3 min (for PolC) or 5 min (for DnaE) with 2 drops of 0.2 M sodium pyrophosphate, and incorporated nucleotides were precipitated with 0.5 ml 10% (w/v) trichloroacetic acid. Unincorporated nucleotides were removed and reaction products were quantified as described (33).

Helicase assays

Oligonucleotides were obtained from Biosearch Technologies. The substrate diagrammed in Figure 6A was assembled from the following HPLC-purified oligonucleotides. Leading strand template 90-mer- 5'-TET-CGCGTATAGATCATTACTATAACATGTTAGATTCATGATAATATAAGAGATG ACGAATATGATTTTGTCGGCTAATGTAAGAATCTTCAA contained fluorescent tetrachlorofluorescein (TET) at the 5' terminus. Lagging strand template 90-mer- 5'-TT(biotin)T_{44}ATATTATCATGAATCTAACATGTTATAGTAATGATCTATACGCG -BHQ-1-3' contained biotin conjugated to preceding thymidine and Black Hole Quencher-1 (BHQ-1) at 3' terminus that quenches fluorescent TET dye. Primer 35-mer-5'-TTGAAGATTCTTACATTAGTTGACAAAATCATATT, when annealed to the leading strand template, created a 10 nt gap. Trap oligo 45-mer 5'-TATATTATCATGAATCTAACATGTTATAGTAATGATCTATACGCG-3' was used to capture helicase-displaced leading strand so that it did not reanneal to the lagging strand template that contained the fluorescence quencher. The substrate was formed by annealing 1 μM leading strand template with 1 μM lagging strand template and 1 μM primer in a buffer containing 10 mM Tris (pH 7.75), 50 mM NaCl, and 1 mM EDTA in a final volume of 25 μl. The sample was heated to 95 °C for 5 min and cooled to 25 °C at 1 °C/min.

For FRET experiments, 20 nM oligonucleotide substrate was combined with 100 nM trap oligo, 200 nM streptavidin, and protein components in a buffer containing 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, 10 mM dithiothreitol, 20% (v/v) glycerol, 0.02% (v/v) Nonidet-P40 detergent, 200 µg/ml bovine serum albumin, 100 mM potassium glutamate, and 10 mM ATP in a round-bottomed black 96-well plate in a final volume of 50 µl. Samples were incubated at 30 °C for 15 min. Fluorescence emission was detected at 535 nm using an Envision plate reader with an excitation of 485 nm. Using concentrations of unannealed fluorescent leading strand template that are in the linear range of the assay, fluorescence units were converted to molarity using a standard curve.

In vivo replication of SPP1 in a *ssbΔ35* background

The *B. subtilis* FLB22 (*ssbΔ35*) and FLB23 (*ssb3+*) strains were a kind gift of P. Polard (CNRS, France). They were obtained by a single crossing-over integration procedure of pMUTIN-SPA derivatives; in those two strains, the essential *rpsR* gene, which is located immediately after *ssb*, is placed under the control of the IPTG-inducible P_{spac} promoter (34). FLB22 is a mutant strain, which expresses from its natural promoter an SsbA truncated of its last 35 amino acids. *ssbΔ35* cells are temperature-sensitive for growth above 47 ºC in LB medium. FLB23 is an isogenic strain encoding a wild-type SsbA protein. FLB22 and FLB23 cells were grown at 30 ºC in LB medium with 0.5 mM IPTG until OD_{560} = 0.2 and then shifted to 50 ºC. After 15 min incubation, the cells were infected with a multiplicity of infection of 10 with the SPP1 phage and the cultures were incubated for 120 min at 50 ºC. Infection experiments at permissive temperature were performed in parallel. After centrifugation, the supernatant that contained free phage particles was filtered through 0,45 µm filters. Titrations were carried out using *B.*

subtilis BG214 as the indicator strain.

Results

Reconstitution of a SPP1 Replication Fork

We reconstituted replication on a synthetic 409-bp circle containing a long flap that mimics a replication fork (Figure 1B). A 50:1 asymmetric G:C distribution in the synthetic template permits facile quantification of leading and lagging strand synthesis (Figure 1C). The structure of this template may mimic the intermediate that is formed in SPP1 once the D-loop, formed by recombination, is resolved to initiate concatemeric (σ mode) replication (35). SPP1 proteins were expressed and purified to homogeneity (Supplementary methods, Figure 1A) and added to various combinations of purified *B. subtilis* DNA replication proteins (30) to determine which combination was required for efficient replication. Consistent with genetic requirements, SPP1 G*39*P and G*40*P were required, as were host PolC and DnaG (Figure 1D and E). Additional components of the host replicase including the clamp loader [τ complex which consists of the τ, δ and δ' subunits] and sliding clamp processivity factor (β_2) were also found to be required. As observed in *B. subtilis*, DnaE was also necessary, and like primase, plays primarily a lagging strand role (30,36). The decreased level of leading strand synthesis in the absence of DnaE and DnaG may be due to a decreased efficiency when the replisome is incomplete—lacking these factors. To our surprise, the G*38*P origin binding protein was also essential, even though the origin sequence was not contained within the DNA template. G*36*P was also required, but some synthesis was observed in the absence of G*36*P, due to a non-physiological reaction where the helicase can 'self-load' by threading over the 5'-end of the template flap (Manhart and McHenry, in preparation).

The required proteins were individually titrated in the presence of optimal concentrations of the remaining proteins to optimize the replicative reaction (Supplementary Figure S2).

We examined the time course of DNA leading and lagging strand synthesis with the SPP1 replisome. Both leading and lagging strand synthesis exhibited a lag phase of 1 min, presumably the time required for loading of the helicase and assembly of the replication fork. After the lag, the synthesis rate remained linear for approximately 5 min (Figure 2A). We examined the elongation rate of reconstituted SPP1 replication forks by analyzing leading strand product formation after the first minute lag (Figure 2B and C). From these data, a rate of progression of 224±7 nucleotides/s was obtained. We performed as a control the same assays with a reconstituted *B. subtilis* replication fork and obtained a value of 200±6 nucleotides/s. These results show that both replisomes progress at a similar rate under the experimental conditions used.

Primase, not DnaE, regulates the length of Okazaki fragments

B. subtilis DnaG primase cycles on and off the replication fork through association with the DnaC helicase. Thus, higher concentrations of primase lead to more frequent associations with helicase and more frequent priming, resulting in shorter Okazaki fragments (30). We also observed a decrease in Okazaki fragment size with increasing DnaG primase concentration in our SPP1 system (Figure 3A). Okazaki fragment length varied from *ca.* 4 kb at 1.5 nM primase down to *ca.* 400 bp at 100 nM primase (Figure 3A). *B. subtilis* DnaE functions like eukaryotic DNA polymerase α, adding a stretch of deoxynucleotides to the RNA primer before handoff to the major replicase (30). We also investigated whether variations in DnaE concentration influence Okazaki fragment length. In the absence of DnaE, lagging strand synthesis was very low (Figure 1E and Figure 3B). Most of the synthesis observed in the absence of DnaE was due to

background incorporation of [α-^{32}P]dGTP into the leading strand product. The leading strand template contains 2% of the template C residues within our rolling circle template and the resulting incorporation yields a significant background only in the absence of lagging strand synthesis. The size of Okazaki fragments was similar over a wide range of concentrations (1.25-80 nM DnaE, Figure 3B).

SPP1 replication forks can be reconstituted with SsbA from *B. subtilis* but the helicase loaders are not interchangeable

In order to see if some SPP1 components could be replaced by their *B. subtilis* counterparts, we first determined whether host SsbA could replace G*36*P. *B. subtilis* SsbA supports an efficient reaction at both 30 nM [the optimal G*36*P concentration, that is also the amount of SSB needed to cover the 396-nt tail present in the DNA template in the 65-nt DNA binding mode (37) and at higher concentrations (Figure 4A, lanes 7 and 11). We observed a stronger dependence on G*38*P at 90 nM *B. subtilis* SsbA. The reactions were dependent on G*39*P under both conditions tested. In the absence of any SSB, efficient reactions are also observed, but dependence on G*38*P and G*39*P was lost. In the absence of SSB, helicases can self-assemble by threading over the exposed 5'-end of the flap of the forked substrates (Manhart and McHenry, in preparation). We found that G*40*P could not be assembled onto the fork by the host loading system (DnaB/DnaD/DnaI/PriA) in the presence of either SsbA or G*36*P (Figure 4A, lanes 2 and 15).

We then tested whether G*36*P can replace SsbA in support of the *B. subtilis* replisome. *B. subtilis* chromosomal replication does not occur in the presence of G*36*P either in the presence of the natural helicase loading system (PriA, DnaD, DnaB, DnaI) (Figure 4B, lane 4) or in the presence of the phage G*38*P-G*39*P helicase loading system

(Figure 4B, lane 6). Increasing G*36*P concentrations to levels found to be optimal for SsbA also did not stimulate the *B. subtilis* reaction (Supplementary Figure S3). In the presence of SsbA the bacterial helicase worked well with its own helicase loading proteins (Figure 4B, lane 1), but they could not be substituted with the viral helicase loading proteins (Figure 4B, lane 3).

Elevated levels of G*38*P are required to reverse inhibition of DNA replication by high concentrations of G*36*P

The above experiments showed that G*36*P and SsbA could work similarly on SPP1 replication forks. In titration experiments that we performed to optimize protein concentrations, we noted that increasing concentrations of G*36*P significantly reduced lagging strand DNA synthesis, whereas this effect was not observed with increasing SsbA (Figure 5A and B). We were concerned that the leading strand synthesis observed at elevated G*36*P may have been an inauthentic reaction, resulting from helicase independent strand displacement by the Pol C holoenzyme. Such a reaction is catalyzed by the *E. coli* Pol III holoenzyme at high SSB concentrations (31). However, dropout experiments where one protein was deleted from the reaction at a time confirmed that leading strand synthesis in the presence of elevated G*36*P concentrations retained a dependency on all of the leading strand replication proteins, including helicase (Supplementary Figure S4).

We suspected the inhibition by high G*36*P may have been caused by sequestering a component in a binary complex in solution, preventing its participation in the replicative reaction. In a search for proteins that reversed the G36P inhibitory effect at high concentrations, we found that G*38*P elicited this effect (Figure 5C). We also tested the other viral protein that participates in helicase loading, G*39*P but observed no effect (data not shown).

We also performed an independent fluorescence-based assay that detects helicase loading and ensuing strand separation (see Figure 6A for a description of the substrate). This permits analysis of the SSB effect on helicase activity independent of its influence on priming and polymerase activity. The 5'-end of the lagging strand template was blocked by streptavidin attachment, preventing helicase self-assembly by threading over the 5'-flap in the absence of SSB (Manhart and McHenry, in preparation). With the G*40*P helicase we also observed in these assays greater efficacy of G*36*P relative to SsbA at low concentrations (Supplementary Figure S5D). Furthermore, we observed an absolute requirement for G*38*P (Supplementary Figure S5A), consistent with a role for this protein at the helicase assembly step.

SPP1 does not require SsbA *in vivo*

The previous assays showed that the *in vitro* SPP1 replication system can use both viral G*36*P and host SsbA similarly. SPP1 rolling circle reaction requires an SSB with a C-terminal tail (Supplementary Figure S6) that is the site of interaction for almost all proteins that bind SSB (38). To test if the SPP1 phage requires the SsbA protein *in vivo*, we analyzed the levels of amplification of the SPP1 phage in a *B. subtilis* mutant strain, FLB22, which expresses from its natural promoter SsbA truncated of its last 35 amino acids. *ssbΔ35* cells and an isogenic control were infected at 30ºC, an after two hours of infection the phage titer was determined. *ssbΔ35* cells are temperature-sensitive for growth above 47 ºC in LB medium (34). Cultures were also grown at a permissive temperature until OD_{560nm}=0.2 and then shifted to 50ºC. After 15 min incubation, the cells were infected with SPP1 and the cultures were incubated for 2 h at 50ºC. Titrations of three independent experiments yielded a mean of $3x10^9$ phage/ml and $1x10^9$ phage/ml for infections of *ssbΔ35* cells at permissive and non-permissive temperature, respectively. $3x10^9$ and $4x10^9$ phage/ml titers were obtained when the isogenic strain

(*ssb3*+) was infected at the permissive and non-permissive temperature, respectively.

The DnaC helicase unwinds DNA in the presence of G*36*P

The preceding results show that G*36*P is not able to support replication in the full *B. subtilis* replication system. In an attempt to identify the defective step, we tested whether the DnaC helicase can be assembled onto DNA and unwind DNA efficiently in the presence of G*36*P. We exploited the fluorescence-based assay (Figure 6A). The results showed that SPP1 G*36*P and SsbA are interchangeable in the helicase assays (Figure 6B). So, the defect in the *B. subtilis* assay in the presence of G*36*P occurs after helicase loading and DNA unwinding.

G*36*P stimulates synthesis by DnaE, but not by the PolC holoenzyme

Continuing our search for the defect in *B. subtilis* chromosomal replication in the presence of G*36*P, we tested for its ability to stimulate reactions catalyzed by the DnaE and PolC holoenzymes. We observed that SsbA and G*36*P stimulate the DnaE holoenzyme with similar efficiency (Figure 7A). However, G*36*P failed to stimulate the PolC holoenzyme (Figure 7B). The same defect was observed in RNA primer extension reactions conducted with DnaE in the presence of PolC, which mimicked the reactions occurring at the lagging strand of the replication fork (Supplementary Figure S7). This suggests that the defect in the G*36*P-supported *B. subtilis* reaction likely resides in its inability to stimulate the PolC holoenzyme.

G*36*P blocks host DNA replication

The observation that G*36*P does not support *B. subtilis* replication led us to investigate if this could also occur in the physiological context, when both host and phage SSBs are present in the same reaction. *B. subtilis* replication forks were assembled in the presence

of saturating concentrations of $SsbA_4$ and increasing concentrations of G*36*P_4. Both leading and lagging strand synthesis were significantly inhibited even at the lowest G*36*P concentration tested (30 nM) (Figure 8).

Discussion

We have reconstituted an efficient rolling circle SPP1 reaction that recapitulates the concatemeric phase of SPP1 DNA replication. The reaction requires all proteins defined by genetic studies including the phage helicase (G*40*P), its loading protein (G*39*P) and the host DnaG primase and PolC DNA polymerase. We also observed a requirement for the G*36*P protein. Neither the *B. subtilis* helicase loading proteins nor the helicase are required, but all of the polymerase elongation components are necessary, including β_2, the τ complex and a second DNA polymerase III, DnaE. The rate of elongation is similar to the rate of *B. subtilis* replication under the conditions employed.

In the well characterized *E. coli* system, DnaG primase binds to the $DnaB_6$ helicase before the synthesis of each primer and then dissociates (39). Because of this equilibrium, high concentrations of primase lead to more frequent priming and shorter Okazaki fragments. A similar observation has been made in a reconstituted *B. subtilis* DNA replication system (30) and in the SPP1 system, presumably for the same reasons. DnaE has been previously shown to be required to extend RNA primers a short distance before handing them off to PolC, analogous to the process in eukaryotes where Pol α and the Pol δ holoenzyme are required (5,6,30). Thus, it is possible that DnaE could also influence primer synthesis due to an interaction with primase. We tested this possibility and found it not to be the case. Okazaki fragment length did not vary over a wide range of DnaE concentrations in the SPP1 system, as may also occur in the bacterial systems.

These studies identified a requirement for the G*38*P origin binding protein for a replicative reaction initiating on forks that mimic the concatemeric stage of SPP1 replication. In SPP1 this type of replication initiates after recombinational events [after

the processing of the D-loop formed by fork stalling, (10)]. Therefore, such reactions might mimic the replication restart reactions that occur on stalled forks in chromosomal systems that require PriA for their initiation (40). PriA was an essential component of the *in vitro* rolling circle replication with the *B. subtilis* system (30), but SPP1 replication does not require PriA, consistent with genetic data (18). Thus, it appears that G*38*P plays a dual role as an origin-specific and forked structure-specific initiation protein. Future studies directed at common features of PriA- and G*38*P-supported replication initiation may provide insight regarding the mechanism used by these proteins to drive the replication restart reaction in cellular systems. In *B. subtilis,* the loading of the replicative helicase DnaC at oriC relies on the ordered associations of DnaA, DnaB, DnaD and DnaI proteins (41) and outside of the origin relies on the PriA, DnaB, DnaD and DnaI proteins, also through a cascade of protein interactions that are crucial for the loading of the replicative helicase on SSB-coated ssDNA (42). The results of the current study show that in SPP1 this reaction is restricted to two proteins (G*38*P and G*39*P).

We found that both SPP1 G*36*P and *B. subtilis* SsbA supported the SPP1 replicative reaction, but with interesting differences. Under standard reaction conditions, approximately 180 nM $SsbA_4$ was required to achieve an optimal rate of leading and lagging strand synthesis. However, with G*36*P_4, an optimum rate is achieved at a 6-fold lower concentration. Further increases of G*36*P concentration result in marked inhibition of lagging strand synthesis. The most straightforward explanation for this observation is that G*36*P makes specific protein-protein interactions that sequester some viral component in inactive binary complexes. Since elevated levels of G*38*P reverse the lagging strand inhibition induced by elevated G*36*P, it is the most likely candidate as the sequestration target. Future work needs to be done to unravel the

cause of these differences. It is interesting that G*38*P is involved in both loading the replicative helicase and reversing the inhibition by high G*36*P concentrations specifically for the lagging strand. This dual activity suggests an additional function that is required for ongoing lagging strand replication after helicase loading.

In contrast to the above observations, the phage-encoded SSB, G*36*P, will not support a full host replisome-dependent replicative reaction. Dissection of the individual reaction steps shows that G*36*P is fully competent to support PriA/DnaD/DnaB/DnaI-dependent helicase loading and ensuing advancement to separate DNA strands. G*36*P is also able to stimulate a DnaE holoenzyme activity as well as the host SSB. The only defect detected was a failure of G*36*P to stimulate the major Gram-positive replicase, the PolC holoenzyme. Yet, the PolC holoenzyme supports efficient replication in the fully reconstituted SPP1 protein-dependent reaction. This suggests the presence of additional or stronger PolC holoenzyme-phage protein interactions that either stimulate PolC, protect it from inhibition, or bypass its requirement for lagging strand replication.

The lack of support by G*36*P of the *B. subtilis* replication fork and the efficient use in the SPP1 replication fork of the two SSBs (the viral G*36*P and the host SsbA) could be used *in vivo* as a strategy of the phage to amplify its own DNA while inhibiting host chromosomal replication. This hypothesis is supported by our observation that even at saturating amounts of SsbA, the *B. subtilis* replication system was inhibited by low concentrations of G*36*P. Examples of viral inhibitors that block host proliferation by binding to host proteins have been reported (43-45). But, a strategy based on inhibition by a viral encoded SSB has not been reported to date. It is interesting to note that many viruses encode SSB proteins (46). These include *Staphylococcus aureus* phage 80α, which transfers pathogenicity islands between staphylococci. This phage also encodes a G*38*P-like protein (47).

G*36*P is 48% identical to *B. subtilis* SsbA. Identity in the C-terminus, which is important for most SSB-protein interactions, is even higher (~75%). The most critical residues required for SSB-protein interactions are the Pro-Phe pair found at the extreme C-terminus (38). The C-terminal Phe of the *E. coli* homolog fits into a hydrophobic pocket of the χ subunit of the *E. coli* DNA polymerase III holoenzyme and the carboxyl group of this residue forms a salt bridge (48). Slightly more internal within SSB are three conserved acidic residues that form ionic bonds with positively charged residues in SSB binding sites (49). We note that the C-terminus of G*36*P contains two of these three acidic residues. In other systems, mutation of only one of the C-terminal three acidic residues of SSB results in only a modest decrease in binding to interacting proteins (38,49). Thus, explanation of the differential effects observed between G*36*P and SsbA on PolC holoenzyme stimulation and the specific inhibition of SPP1 replication at high G*36*P levels may require additional interactions outside of the prototypical C-terminal SSB interaction sequences.

Viruses that encode their own initiation machinery but depend on the host for elongation functions have provided significant insight into cellular processes (4,5,7,50). With the simple single-stranded DNA coli-phages, the only viral protein that is required for replication is a nicking/religation activity that functions after duplex formation to shift SS -> RF to duplex DNA replication (51). The bacteriophage λ encodes its own origin binding protein and helicase loader, and subverts the host helicase (7). Thus, it differs from SPP1 in that it does not encode a helicase or an SSB. However, important similarities are also noted. λ, like SPP1, encodes an origin-specific initiation protein (O protein) that is not a DnaA homolog. The O protein, like SPP1 G*38*P, can serve to initiate DNA synthesis on artificial replication forks that lack origin sequences (15). SV40, like SPP1, encodes its own origin binding protein and helicase, both contained

within the same polypeptide chain as the T-antigen (52). This system has been invaluable in revealing the mechanisms of eukaryotic DNA replication (4,5).

The best biochemically defined Gram-positive bacteriophage is Φ29 (53). It encodes its own replication machinery and has provided a wealth of tools and insight, varying from basic models for viral replication that extend to eukaryotic adenoviruses (54) to tools for biotechnology (55). Before this work, an *in vitro* replication system for a Gram-positive phage that is dependent on host proteins had not been established.

Thus, the present study provides a new viral window into Gram-positive replication processes. The availability of a hybrid assay that exploits the efficiency and simplicity of initiation by phage-encoded proteins and a requirement for host proteins for elongation provides a powerful tool to support studies of fork dynamics, macromolecular interactions and regulation or replicative processes in Gram-positive organisms. Having two SSBs that can be used interchangeably with differential effects should help unravel the importance of SSB interactions in replication fork processes. And, having the G*38*P protein that supports initiation on both defined duplex origins and replication forks may provide an opportunity to learn more about the mechanistic features common to these two important processes.

Supplementary data

Supplementary Data are available at NAR online: Supplementary Materials and Methods, Supplementary Reference [56], and Supplementary Figures S1-S7.

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FIGURE LEGENDS

Figure 1. Reconstitution of SPP1 rolling circle DNA replication with *B. subtilis* and SPP1 purified proteins. **(A)** 15% SDS-PAGE gel of purified SPP1 bacteriophage proteins used to reconstitute rolling circle DNA replication: G*40*P (49.8 kDa), G*39*P (14.6 kDa), G*38*P (29.9 kDa) and G*36*P (18 kDa). The purification of these proteins is described under *Supplementary Materials and Methods*. **(B)** Diagram of the DNA template used. It has an asymmetric G:C ratio (50:1) between the two strands, permitting quantification of leading and lagging strand synthesis by measuring radioactive dCMP and dGMP incorporation. **(C)** Addition of [α-^{32}P]dCTP or [α-^{32}P]dGTP allows the detection of products corresponding to leading and lagging strands synthesis, respectively, resolved on an alkaline agarose gel. **(D)** Activity and source of proteins involved in SPP1 rolling circle replication. **(E)** Protein requirements for SPP1 rolling circle DNA replication. The values represented are the mean of three independent experiments.

Figure 2. Progression of the reconstituted SPP1 replication fork. **(A)** Time course of the leading strand and lagging strand DNA synthesis. The values represented are the mean of three independent experiments. **(B)** Rate of SPP1 replication fork progression. Standard reactions were scaled up to a volume of 125 µl and incubated at 37 ºC with [α-^{32}P]dCTP to detect leading strand synthesis. After the first minute (indicated as time 0), aliquots of 12.5 µl were removed and quenched every 10 s. **(C)** Quantification of the rate of SPP1 fork movement. Plot of the largest DNA fragment present *versus* time to determine replication fork rate.

Figure 3. Effect of increasing DnaG and DnaE on the size of Okazaki fragments. **(A)** Size of Okazaki fragments varies inversely with primase concentration. Rolling circle reactions were performed in the presence of [α-^{32}P]dGTP, and were stopped after 10

min. The reactions were run on an alkaline agarose gel. Primase concentrations assayed varied in a 2-fold dilution series from 100 nM down to 0.4 nM. **(B)** Variations in DnaE concentrations do not alter the size of the Okazaki fragments. Reactions were performed changing the DnaE concentration in a 2-fold dilution series from 80 nM to 1.25 nM.

Figure 4. Interchangeable components of the replication machinery of *B. subtilis* and SPP1. **(A)** The SPP1 replication fork can use SsbA or G*36*P. *In vitro* replication of reconstituted SPP1 replication forks assembled in the presence of 30 nM G*36*P_4 (green box), 30 nM or 90 nM $SsbA_4$ (grey boxes), and in the absence of an SSB (white box). The presence of G*39*P (helicase loader) and G*38*P (origin binding) or the *B. subtilis* loading system (formed by DnaB-DnaD-DnaI-PriA) is indicated. **(B)** The *B. subtilis* replication fork strictly requires SsbA and it own helicase loading system. *In vitro* replication of reconstituted *B. subtilis* replication forks assembled in the presence of 30 nM G*36*P_4 (green box) or 90nM $SsbA_4$ (grey boxes) and the indicated components. The data shown are the mean of at least three independent experiments.

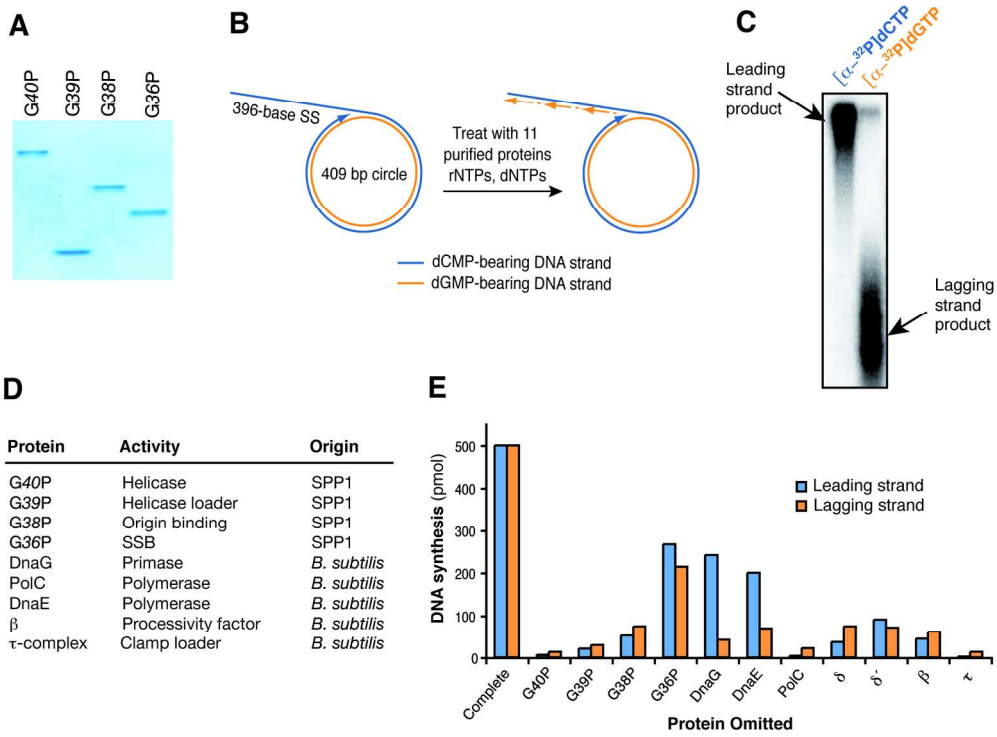
Figure 5. Increasing concentrations of G*36*P protein inhibit lagging strand DNA synthesis; synthesis can be restored by increasing G*38*P. **(A)** G*36*P_4 titration in SPP1 replication fork reconstitution. Standard reactions to measure DNA synthesis at leading and lagging strands were performed at the indicated G*36*P_4 concentrations. **(B)** High $SsbA_4$ concentrations do not inhibit lagging strand synthesis. **(C)** Effect on lagging strand synthesis of increasing G*38*P at high G*36*P_4 (180 nM). The values represented are the mean of three independent experiments.

Figure 6. Either SsbA or G*36*P will support *B. subtilis* helicase loading and unwinding. **(A)** DNA substrate used in unwinding reactions. The fluorescence of tetrachlorofluorescein (TET) on the 5' terminus increases upon being separated from a quencher (BHQ-1) on the opposing strand. The 5'-end of the lagging strand template is

blocked by attachment of streptavidin to an incorporated biotinylated thymidine to inhibit helicase self-loading by threading over a free 5'-end. **(B)** *B. subtilis* helicase activity in the presence of SsbA or G*36*P. DNA substrate was combined with varying amounts of either SsbA or G*36*P, and 150 nM PriA, 75 nM DnaD, 75 nM DnaB, 12 nM $DnaC_6$, and 50 nM DnaI.

Figure 7. SPP1 G*36*P stimulates DnaE holoenzyme but not PolC holoenzyme. **(A)** Extension of a DNA primer by the DnaE holoenzyme using either SsbA or G*36*P. SsbA or G*36*P were titrated in a DNA-primed $M13_{Gori}$ reaction containing 3 nM DnaE in the presence (+) or absence (-) of β_2 as indicated. **(B)** Extension of a DNA primer by the PolC holoenzyme (2 nM) using either SsbA or G*36*P. Total DNA synthesis was analyzed by quantification of [^{3}H]dTTP.

Figure 8. G*36*P inhibits *B. subtilis* rolling circle DNA replication even when SsbA is present at saturating concentrations. *In vitro* replication of reconstituted *B. subtilis* replication forks assembled in the presence of increasing concentrations of $G36P_4$ (30, 60, 90, 180 and 270 nM tetramer) and 90nM $SsbA_4$. Both SSBs were added simultaneously to the substrate mix. The data shown are the mean of at least three independent experiments.



Protein	Activity	Origin
G*40*P	Helicase	SPP1
G*39*P	Helicase loader	SPP1
G*38*P	Origin binding	SPP1
G*36*P	SSB	SPP1
DnaG	Primase	*B. subtilis*
PolC	Polymerase	*B. subtilis*
DnaE	Polymerase	*B. subtilis*
β	Processivity factor	*B. subtilis*
τ-complex	Clamp loader	*B. subtilis*

Figure 1
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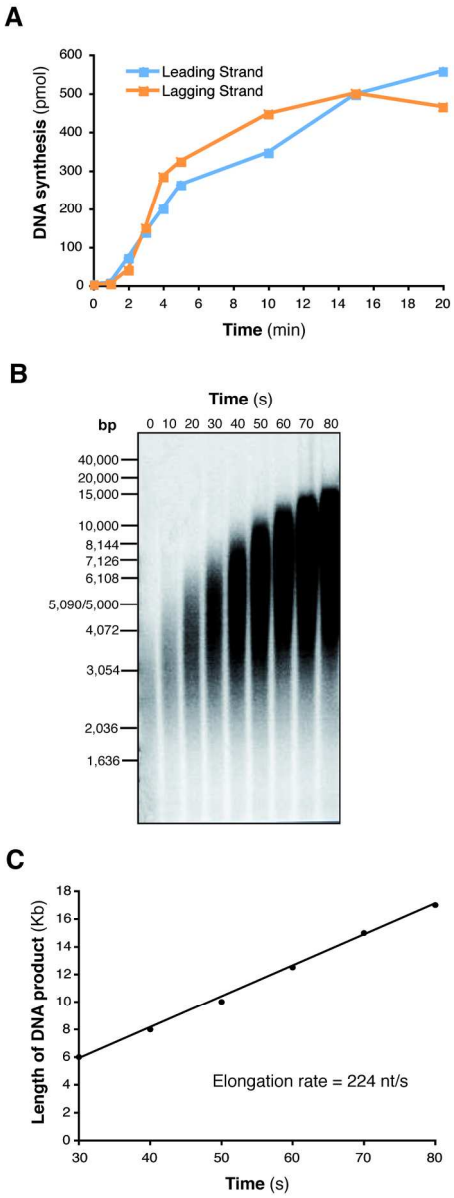

Figure 2
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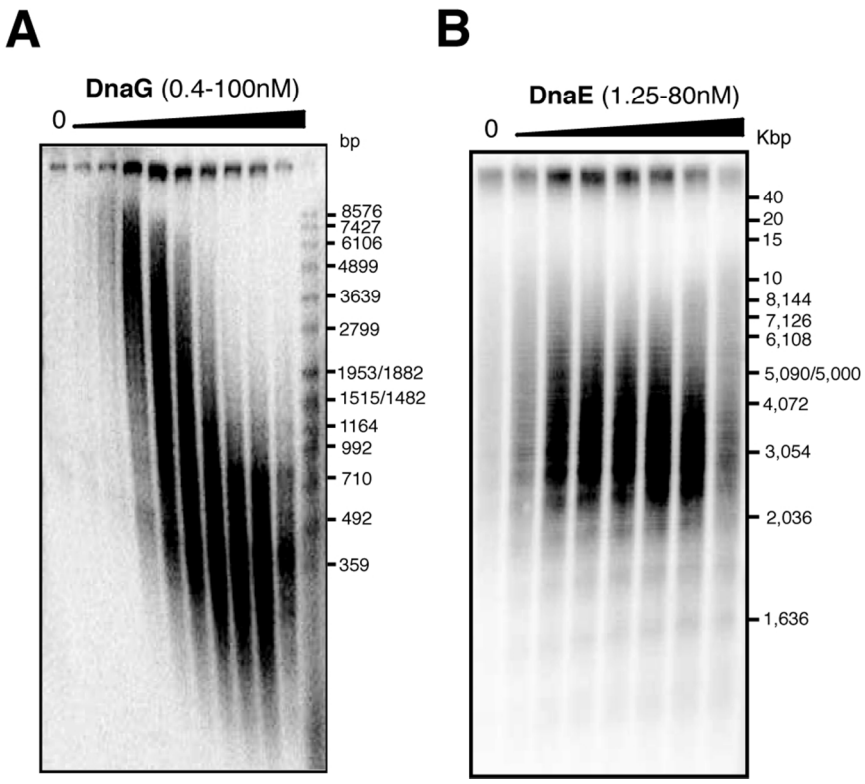

Figure 3
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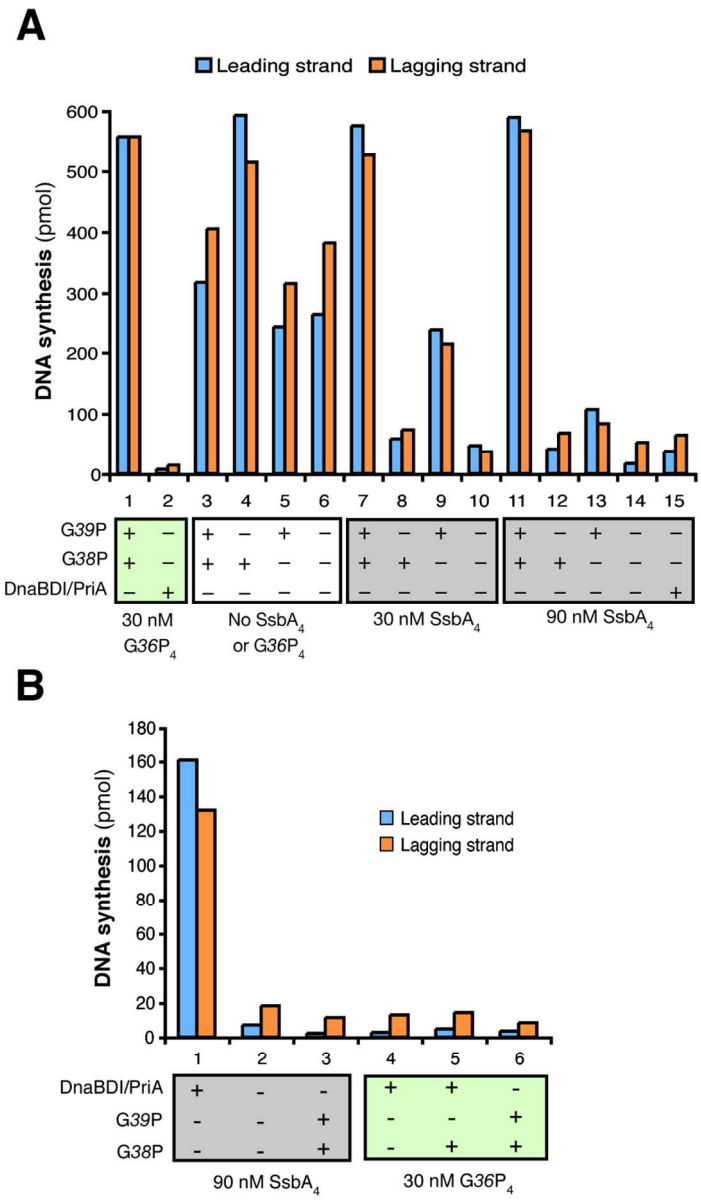

Figure 4
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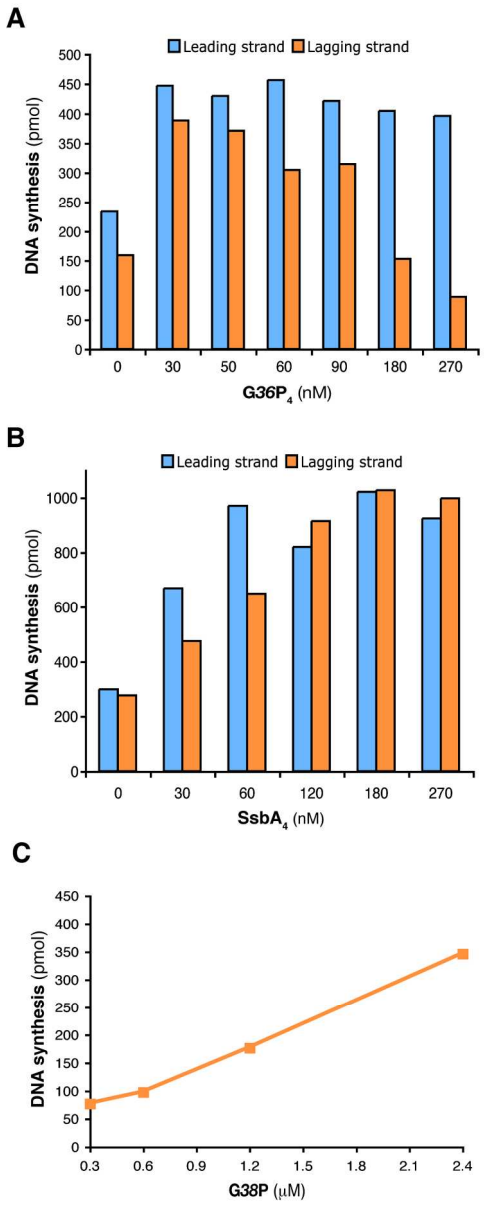

Figure 5
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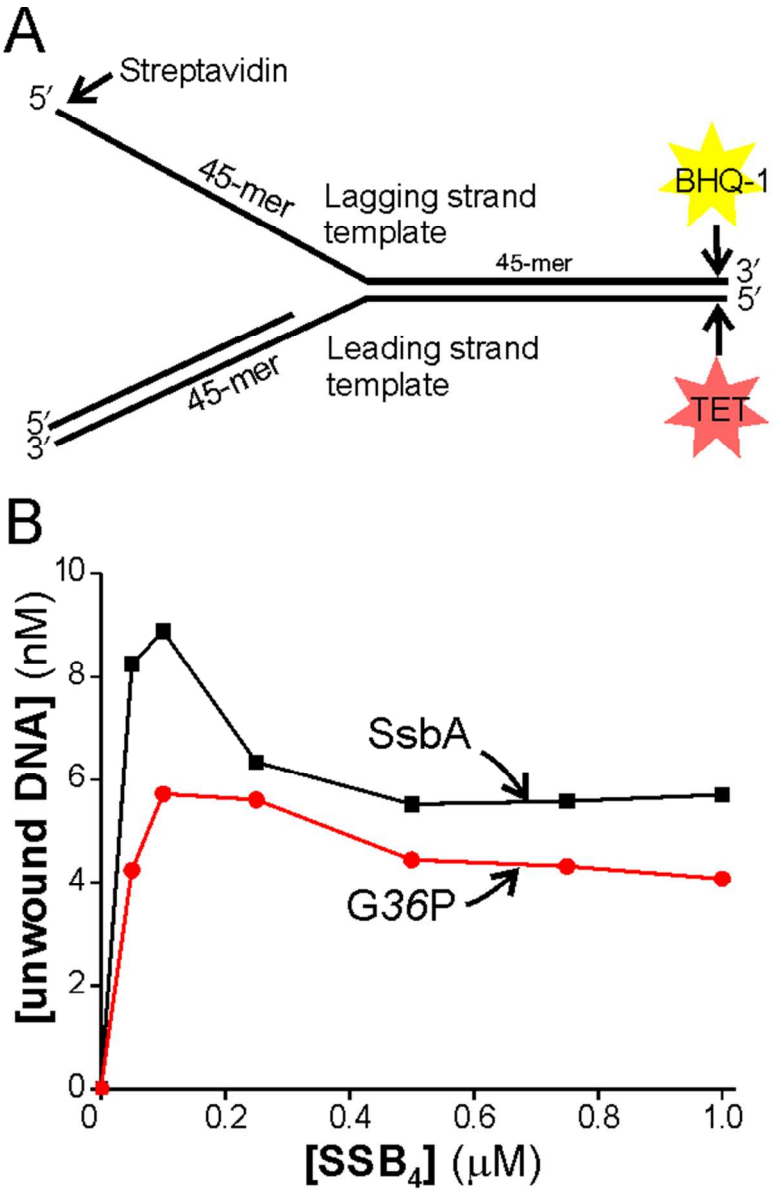
A
Streptavidin
5′
45-mer
Lagging strand template
BHQ-1
45-mer
3′
5′
45-mer
Leading strand template
5′
3′
TET
B
[unwound DNA] (nM)
10
8
6
4
2
0
SsbA
G36P
0
0.2
0.4
0.6
0.8
1.0
[SSB4] (μM)

Figure 6

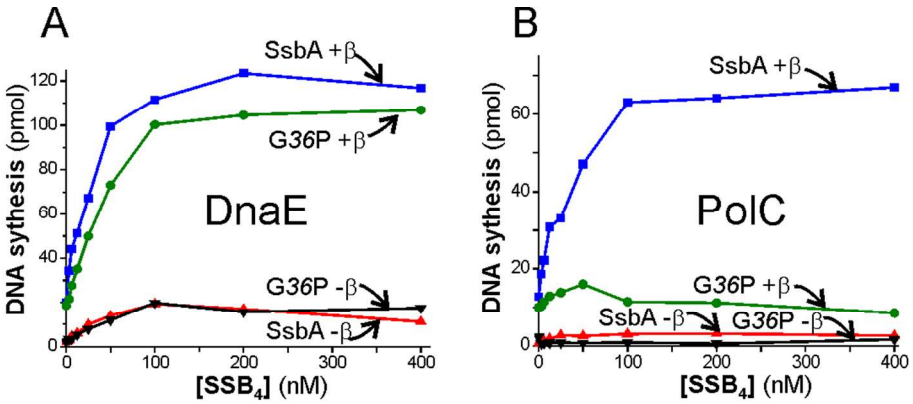

Figure 7

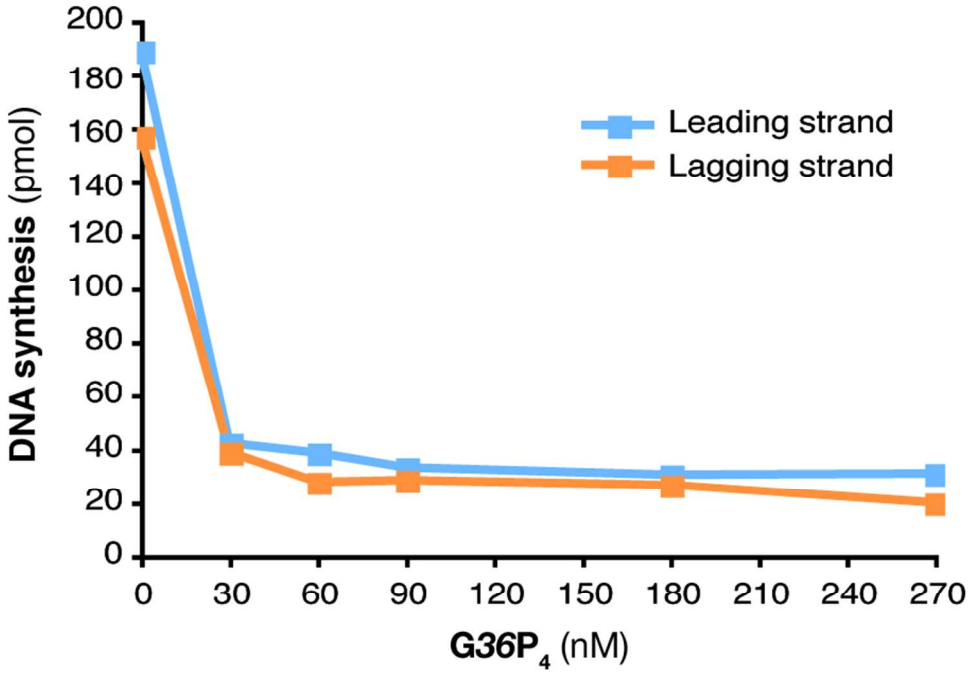

Figure 8
79x58mm (300 x 300 DPI)

Supplementary Information

Bacteriophage SPP1 DNA replication strategies promote viral and disable host replication

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Supplementary Materials and Methods

Purification of SPP1 DNA Replication Proteins

All proteins were expressed in *E. coli* overexpression vectors in which the encoding genes are the native genes without added tags. *B. subtilis* PriA, DnaD, DnaB, DnaI, DnaC helicase, DnaG primase, DnaE, PolC, τ, δ, δ', β and SSB were purified as described (30). G*40*P was purified using pCB367 plasmid (25). pCB367 is a pQE-11 (Qiagen) derivative containing wild-type gene *40* and the gene *39* carrying a hexa-histidine tag on its N-terminus, which allows the affinity purification of the native G*40*P. His_6-G*39*P stabilizes G*40*P and forms a complex with it only in the presence of ATP. Hence, in the presence of ATP, the co-expressed His_6-G*39*P-G*40*P complex is retained in a Ni-NTA column. Cells were grown at 37 ºC to an OD_{560} = 0.8, induced with 2 mM IPTG for 2 h, and harvested and stored at -20 ºC. Five g of cells were suspended in 25 ml buffer A [50 mM sodium phosphate (pH 7.5), 10% (v/v) glycerol, 1

mM $MgCl_2$, 0.1 mM ATP, 1 mM b-mercaptoethanol, 300 mM NaCl], lysed by sonication and centrifuged at 18,000 rpm in an SS-34 rotor for 45 min. The supernatant carrying G*40*P and G*39*P was brought to a concentration of 20 mM imidazole and loaded onto a Ni-NTA column (2 ml) equilibrated in buffer A carrying 300 mM NaCl and 20 mM imidazole. A gradient of fifty column volumes of buffer A containing 20–500 mM imidazole and in the absence of ATP was applied and the last fractions, which contained G*40*P enriched with respect to G*39*P, were pooled and dialyzed against buffer B [50 mM Tris HCl (pH7.5), 10% (v/v) glycerol, 1mM $MgCl_2$, 50mM NaCl, 1mM b-mercaptoethanol] to be then loaded onto a Q-sepharose column. A linear gradient from 50 mM to 500 mM NaCl was applied and fractions enriched in G*40*P protein were pooled and loaded again onto a new Ni-NTA column (0.5 ml) in which the rest of the G*39*P forming a complex with G*40*P was retained. The flow-through containing pure G*40*P was dialyzed against buffer B and loaded onto a Q-sepharose column (0.5 ml) to elute 1 mg of G*40*P in the storage buffer [50 mM Tris HCl (pH 7.5), 50% (v/v) glycerol, 1 mM $MgCl_2$, 500 mM NaCl, 1 mM β-mercaptoethanol]. His_6-G*39*P was discarded because the His-tag affected the activity of the protein. To purify untagged G*39*P, plasmid pBT318 (17) was transformed into *E. coli* BL21(DE3)/pLysS, and cells were induced with 2 mM IPTG for 2 h, harvested and stored at -20 ºC. A total of 7.5 g of cells were suspended in 37.5 ml buffer C [50 mM Tris HCl (pH 7.5), 10% (v/v) glycerol, 1 mM dithiothreitol] containing 250 mM NaCl, lysed by sonication and centrifuged at 18,000 rpm in an SS-34 rotor for 30 min. Polyethylenimine (10% v/v, pH 7.5) was slowly added to the supernatant containing G*39*P to a final concentration of 0.25% (A_{260} = 120). The DNA and certain contaminating proteins were pelleted by centrifugation (12,000 rpm in an SS-34 rotor, 10 min) and the pellet was discarded. Then proteins of the supernatant were precipitated by addition of solid ammonium

sulfate to a final concentration of 80% saturation. The pellet containing G*39*P was resuspended in buffer C without salt and dialyzed against buffer C with 10 mM NaCl to be then loaded onto a Q-Sepharose column (2 ml). The protein eluted from the column in the 25 mM and 50 mM washes. These fractions were pooled and brought to 1.2 M ammonium sulfate to be then loaded onto a phenyl-Sepharose column. Serial washings of ten column volumes with buffer C and decreasing concentrations of ammonium sulfate from 1.2 to 0 were applied (1.2, 1, 0.8, 0.6, 0.4, 0.2 and 0 M ammonium sulfate) and the pure protein eluted in the 0.4 M and 0.2 M washing steps. Both fractions containing G*39*P were pooled and dialyzed against buffer B with 10 mM NaCl. A small Q-sepharose column (0.5 ml) was used to concentrate and elute 3 mg of G*39*P in the storage buffer (50 mM Tris HCl (pH 7.5), 50% (v/v) glycerol, 150 mM NaCl, 1 mM dithiothreitol).

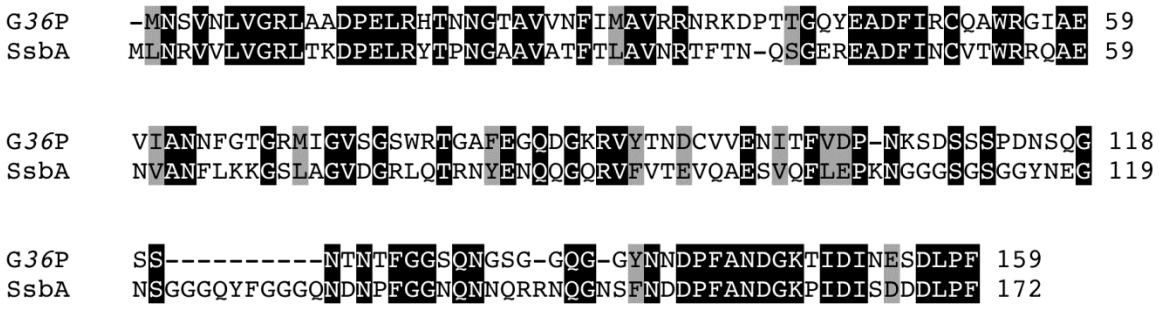
G*38*P was purified from *E. coli* BL21(DE3)/pLysS cells carrying the plasmid pBT320 as described (17). G*36*P was purified from *E. coli* BL21(DE3)/pLysS cells carrying the pCB596 plasmid. Plasmid pCB596 is a pET-3a (Novagen) derivative where gene *36* was cloned into the NdeI and BamHI restriction sites. Cells were grown to an OD_{560} = 0.8 at 37 ºC, 2 mM IPTG was added, and after 120 min cells were harvested by centrifugation and stored at -20 ºC. Five g of cells were resuspended in 25 ml buffer D [50 mM Tris HCl (pH 7.5), 1 mM dithiothreitol, 15% (v/v) glycerol] containing 150 mM NaCl. Lysis was accomplished by sonication and cell debris was removed by centrifugation (18,000 rpm in an SS-34 rotor, 30 min). DNA and G*36*P were precipitated from the supernatant by addition of polyethylenimine (final concentration 0.25% v/v with A_{260} = 120) and centrifugation at 12,000 rpm in an SS-34 rotor for 10 min at 4 ºC. The protein was solubilized from the pellet in 25 ml buffer D containing 300 mM NaCl, and then precipitated by addition of ammonium sulfate to

30% saturation. Protein was redissolved in 25 ml of buffer D containing 300 mM NaCl and reprecipitated with 30% ammonium sulfate, rendering the protein almost pure. The pellet was resuspended in 25 ml buffer D containing 50 mM NaCl and extensively dialyzed. After dialysis, the sample was loaded onto a 1 ml Q-Sepharose column equilibrated with the same buffer. Serial washings of increasing concentrations of NaCl in ten column volumes were applied to the column and the pure protein eluted in the 150 and 200 mM NaCl washings steps. The pure protein was concentrated in another Q-Sepharose column and eluted in a single step with buffer D containing 500 mM NaCl. The G*36*P protein was dialyzed against storage buffer [50 mM Tris HCl (pH 7.5), 1 mM dithiothreitol, 50% (v/v) glycerol, 300 mM NaCl] and stored at -20 °C. The identity of the protein and the absence of *E. coli* SSB in the preparation were confirmed by MALDI-TOF. Plasmid pCB596 was used as a template to generate G*36*P variants lacking the last 9, 15 or 21 C-terminal residues that were purified following a protocol similar to the used for the wild type protein. The protein concentration was determined in all cases as previously described by measuring the absorbance of the peptide bond (56)**.**

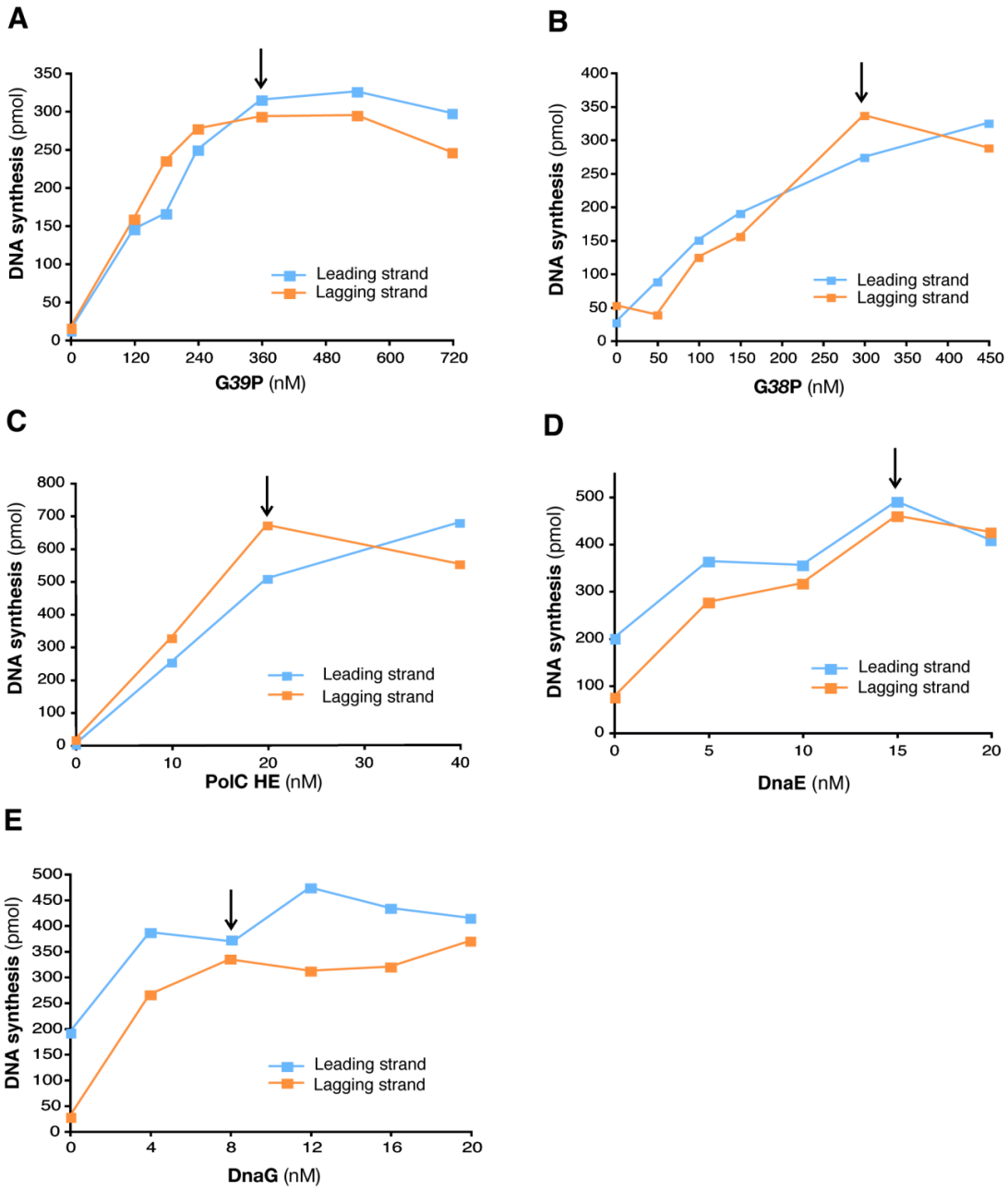
Supplementary Reference

56. Wolf, P. (1983) A critical reappraisal of Waddell's technique for ultraviolet spectrophotometric protein estimation. *Anal. Biochem.*, **129**, 145-155.

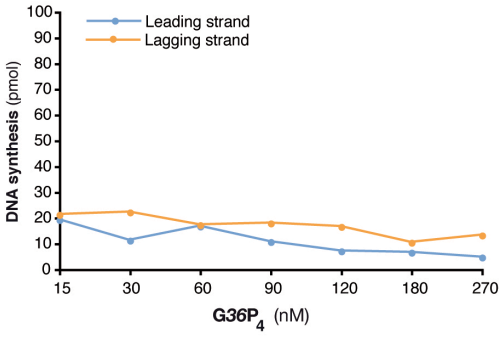
Supplementary Figures



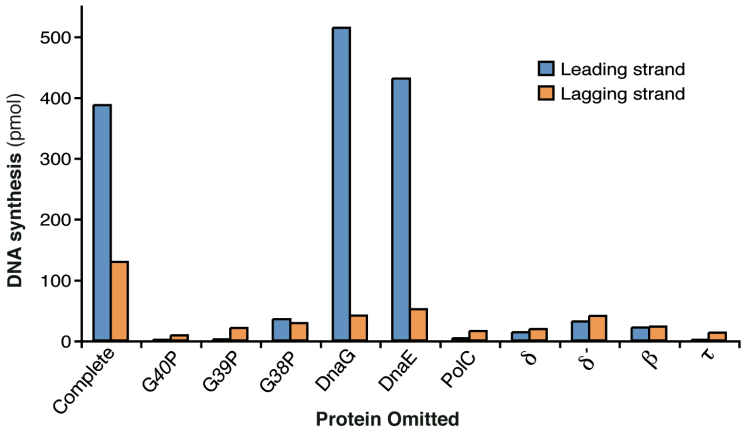
Supplementary Figure S1. Alignment of the G*36*P and SsbA proteins. Identical residues have a black background and conserved residues gray.



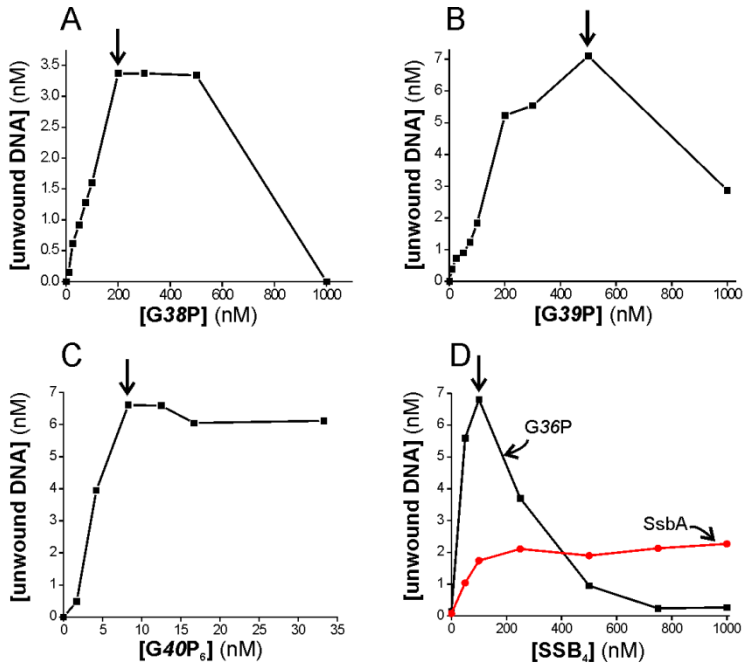
Supplementary Figure S2. Optimization of SPP1 rolling circle replication. The reactions and the measurement of DNA synthesis on both leading and lagging strands were performed as described in Experimental Procedures. All the titrations were carried out in the presence of saturating levels of the other assay components and are the mean of at least two independent experiments. Titrations of **(A)** G*39*P, **(B)** G*38*P, **(C)** PolC, **(D)** DnaE, **(E)** and DnaG. An arrow indicates the concentration of protein chosen for subsequent experiments.



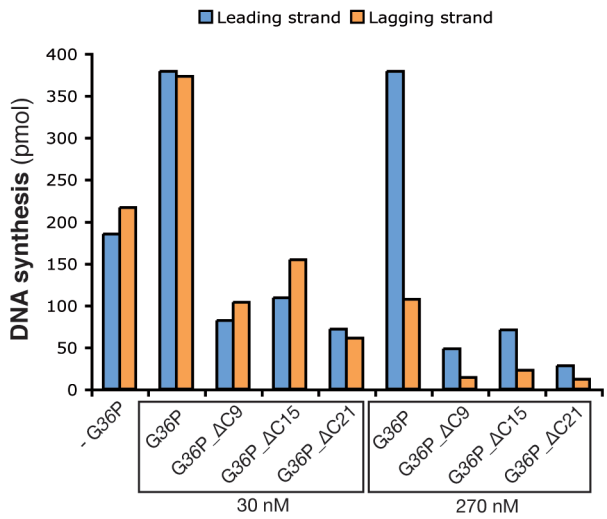
Supplementary Figure S3. The *Bacillus subtilis* replisome can not be reconstituted in the presence of G*36*P. *B. subtilis* replication reactions were assembled with all host components except that $SsbA_4$ was replaced by increasing concentrations of G*36*P_4.



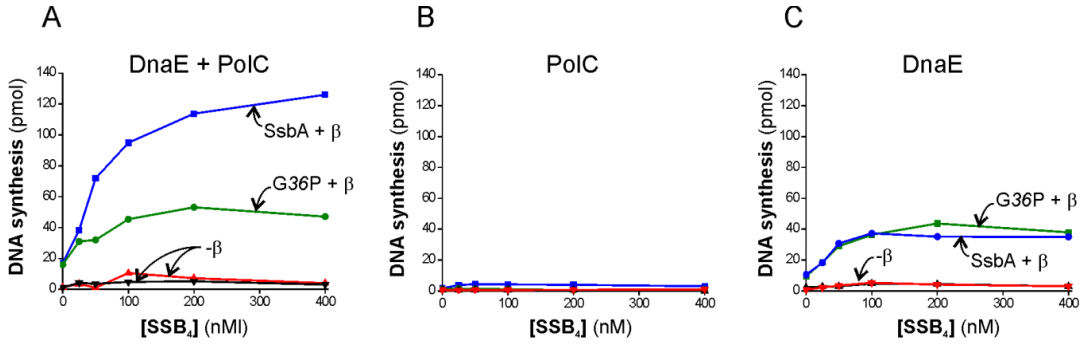
Supplementary Figure S4. Protein requirements at high (180 nM) concentrations of G*36*P_4. Leading and lagging strand synthesis were quantified by [α-^{32}P]dCTP or [α-^{32}P]dGTP incorporation, respectively. The values represented are the mean of three independent experiments.



Supplementary Figure S5. Optimization of SPP1 helicase assay. The substrate (see Figure 6A for diagram) was combined at 20 nM with SPP1 helicase and helicase loading proteins using the conditions described under *Materials and Methods* for reactions with *B. subtilis* proteins. Helicase assembly proteins were titrated one at a time to optimize unwinding. **(A)** G*38*P titrated using 120 nM G*39*P, 15 nM G*40*P_6, and 100 nM G*36*P_4. **(B)** G*39*P titrated using 200 nM G*38*P, 15 nM G*40*P_6, and 100 nM G*36*P_4. **(C)** G*40*P titrated using 200 nM G*38*P, 500 nM G*39*P, and 100 nM G*36*P_4. **(D)** G*40*P requires an SSB to unwind forked substrates. G*36*P or SsbA titrated using 200 nM G*38*P, 500 nM G*39*P, and 10 nM G*40*P_6. The arrows indicate the concentration of protein chosen for subsequent experiments.



Supplementary Figure S6. The C-terminal tail of G*36*P is required for efficient *in vitro* replication. Leading and lagging strand synthesis were quantified by [α-^{32}P]dCTP and [α-^{32}P]dGTP incorporation, respectively, in the presence of the serial C-terminal deletion mutants at low (30 nM) or high (270 nM) SSB_4 concentrations. The values represented are the mean of three independent experiments.



Supplementary Figure S7. SsbA, but not G*36*P, stimulates RNA primer extension under conditions that require a handoff of the extended primer from DnaE to PolC in a reaction containing only *B. subtilis* proteins. Reactions were conducted as in Fig. 7 but contained RNA primers that cannot be elongated by PolC alone. **(A)** Extension of an RNA primer by both DnaE and PolC replicases using either SsbA or G*36*P. SsbA (blue and red lines) or G36P (green and black lines) was titrated in a reaction containing 0.5 nM DnaE and 2.5 nM PolC in the presence or absence of β_2 as indicated. Reactions were incubated at 30 ºC for 3 min. **(B)** Extension of an RNA primer by the PolC holoenzyme using either SsbA or G36P. Reactions were conducted as in (A) except DnaE was omitted from the reaction. **(C)** Extension of an RNA primer by the DnaE holoenzyme using either SsbA or G36P. Reactions were conducted as in (A) except PolC was omitted from the reaction.