

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

Elena M. Seco¹, John C. Zinder², Carol M. Manhart², Ambra Lo Piano¹, Charles S. McHenry^{2,*} and Silvia Ayora^{1,*}

¹Departamento de Biotecnología Microbiana, Centro Nacional, Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain and ²Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309, USA

Received October 17, 2012; Revised and Accepted November 8, 2012

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 (G40P), helicase loader (G39P), origin binding protein (G38P) and G36P 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 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 owing 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, Φ 29 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 binding protein and a helicase loader that subverts the host DnaB₆ 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 Φ 29, (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.

*To whom correspondence should be addressed. Tel: +34 91585 4615; Fax: +34 91585 4506; Email: sayora@cnb.csic.es
Correspondence may also be addressed to Charles S. McHenry. Tel: +1 303 6206357; Fax: +1 303 7351347; Email: charles.mchenry@colorado.edu

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, *oriL* and *oriR*, 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 G38P origin binding protein, the G39P helicase loader and the G40P helicase. G38P, which does not belong to the AAA+ family, is widely conserved in phages (19,20). G38P binds with high affinity to the two origins of replication (21) and forms a complex with G39P (17). G39P does not share homology with other studied helicase loaders but performs a similar role: it delivers G40P, on interacting with G38P, to the origin of replication (22,23). G40P 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, G35P (18,27), and a 5' \rightarrow 3' exonuclease, G34.1P (28). In this operon, there is also a gene (gene 36) that encodes a single-stranded DNA-binding protein (SSB), G36P, whose role in replication has not yet been analysed. G36P 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 G38P 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 (G36P), 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 G40P₆, 300 nM G39P, 300 nM G38P, 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 G36P₄, 5 nM mini-circular DNA template, 350 μ M ATP, 100 μ M CTP, GTP and UTP, 48 μ M dNTPs (except 18 μ M dCTP or dGTP for the leading and lagging strand DNA synthesis, respectively) and 0.2 μ Ci/reaction [α -³²P]dCTP or [α -³²P]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 polymerase chain reaction amplification step (Yuan and McHenry, in preparation). The reactions were carried out in 12.5 μ l of buffer BsRC [40 mM Tris-acetate

(pH 7.8), 12 mM magnesium acetate, 3 μ M ZnSO₄, 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°C. An enzyme mix containing all protein components except SSB (G36P, or SsbA, as indicated) was prepared in buffer BsRC. Two different substrate mixes containing template DNA, rNTPs, dNTPs, SSB (G36P or SsbA) and either [α -³²P]dCTP or [α -³²P]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 μ g/ml proteinase K]. Samples were treated for 20 min at 37°C, 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% bromophenol blue and fractionated on alkaline 0.5% agarose gels for ~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 analysed with Quantity One (Bio-Rad) software.

For calculating the rate of SPP1 fork progression, aliquots were removed, quenched and processed as described earlier in the text. The molecular weight of the longest leading strand product at each time was extrapolated from labelled 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 as follows: 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 DnaC₆, 15 nM PriA, 50 nM DnaD₄, 100 nM DnaB₄, 40 nM DnaI₆ and various amounts of SsbA₄ and G36P₄.

Extension of DNA primers annealed to M13

Templates were prepared by mixing 50 pmol single-stranded M13_{G_{ori}} DNA (32) with 60 pmol synthetic DNA primer (5'AGGCTGGCTGACCTTCATCAAGA GTAATCT-3') in 70 μ l 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 μ l 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* SsbA₄ or SPP1 G36P₄; 48 μ M dATP, dGTP and dCTP; 18 μ M [³H]dTTP (specific activity 113 cpm/pmol); and 250 μ M ATP in buffer BsM13 [40 mM Tris-acetate (pH 7.8), 340 mM potassium glutamate, 10 mM magnesium acetate, 4 μ M ZnSO₄, 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'-tetrachloro-fluorescein (TET)-CGCGTATAGATCATTACTATAAC ATGTTAGATTTCATGATAATATAAGAGATGACGA ATATGATTTTGTGCGCTAATGTAAGAATCTTCA A-3' contained fluorescent TET at the 5' terminus. Lagging strand template 90-mer- 5'-TT(biotin)T₄₄ATAT TATCATGAATCTAACATGTTATAGTAATGATCT ATACGCG-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'-TTGAAGATTCTTACATTAGT TGACAAAATCATATT-3, when annealed to the leading strand template, created a 10-nt gap. Trap oligo 45-mer 5'-TATATTATCATGAATCTAACATGTTATAGTAAT GATCTATACGCG-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 *ssbA35* background

The *B. subtilis* FLB22 (*ssbA35*) 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. *ssbA35* cells are temperature

sensitive for growth above 47°C in LB medium. FLB23 is an isogenic strain encoding a wild-type SsbA protein. FLB22 and FLB23 cells were grown at 30°C in LB medium with 0.5 mM IPTG until OD₅₆₀ = 0.2 and then shifted to 50°C. 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°C. 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 G39P and G40P 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, it 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 G38P origin binding protein was also essential, even though the origin sequence was not contained within the DNA template. G36P was also required, but some synthesis was observed in the absence of G36P, owing 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 ~5 min (Figure 2A). We examined the elongation rate of reconstituted SPP1 replication forks by analysing leading

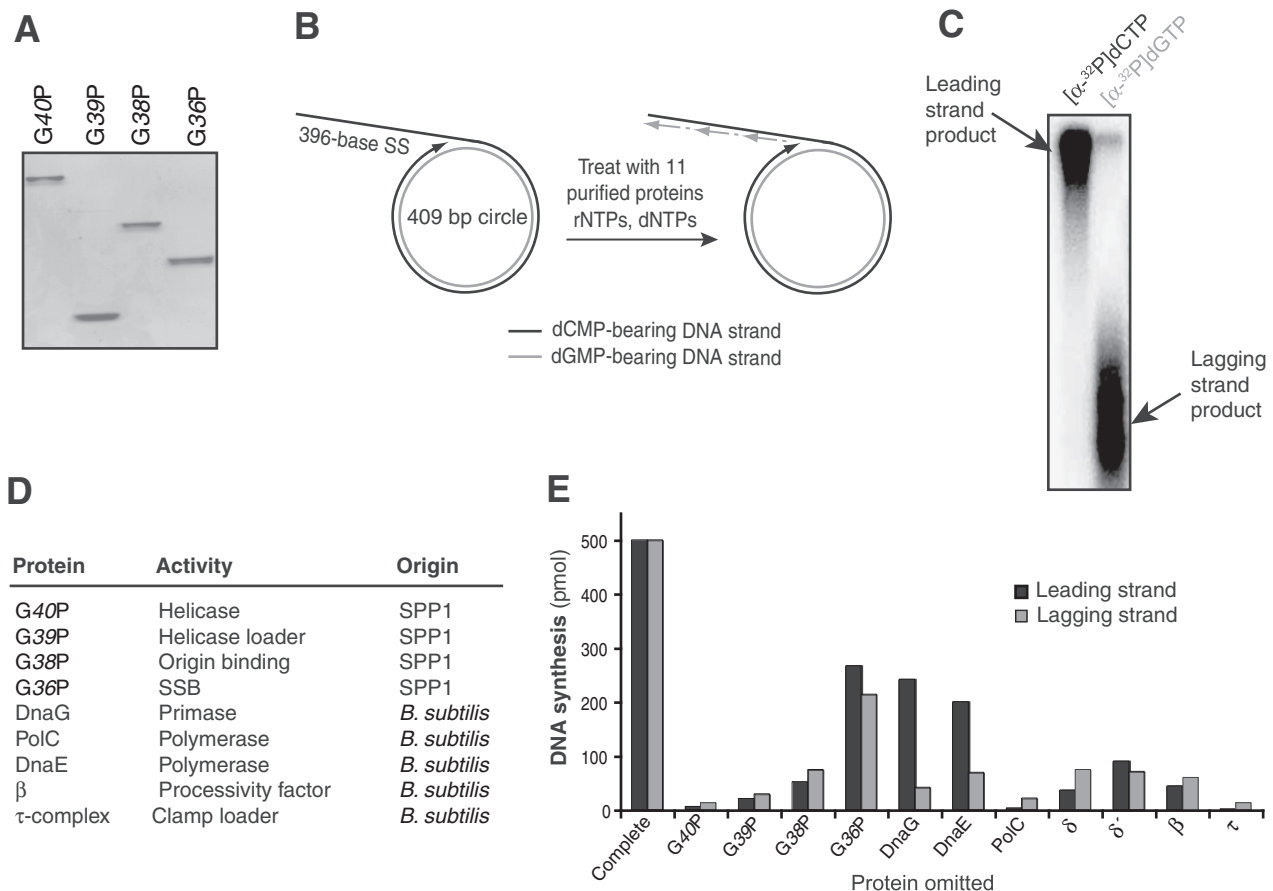


Figure 1. Reconstitution of SPP1 rolling circle DNA replication with *B. subtilis* and SPP1 purified proteins. (A) 15% SDS-PAGE gel of purified SPP1 bacteriophage proteins used to reconstitute rolling circle DNA replication: G40P (49.8 kDa), G39P (14.6 kDa), G38P (29.9 kDa) and G36P (18 kDa). The purification 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.

strand product formation after the first minute lag (Figure 2B and C). From these data, a rate of progression of 224 ± 7 nt/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 nt/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 owing 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

To see if some SPP1 components could be replaced by their *B. subtilis* counterparts, we first determined whether host SsbA could replace G36P. *B. subtilis* SsbA

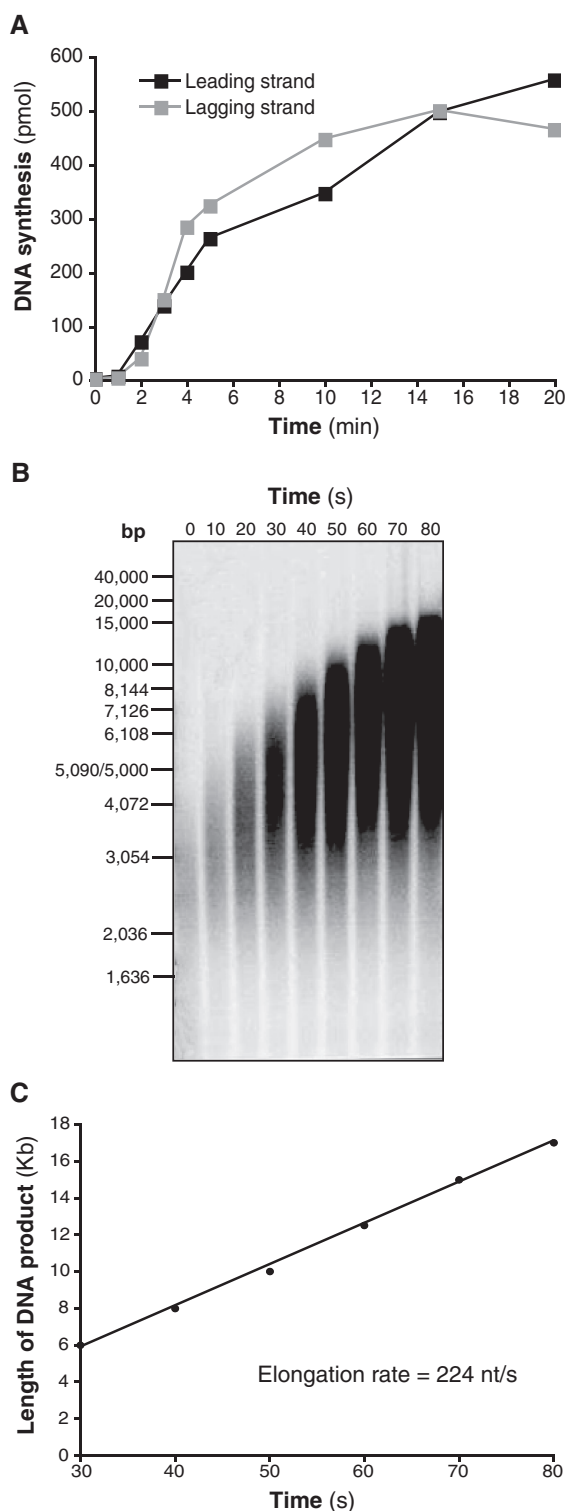


Figure 2. Progression of the reconstituted SPP1 replication fork. (A) Time course of the leading strand and lagging strand DNA synthesis. The values represented are the mean of three independent experiments. (B) Rate of SPP1 replication fork progression. Standard reactions were scaled up to a volume of 125 μ l and incubated at 37°C with [α - 32 P]dCTP to detect leading strand synthesis. After the first minute (indicated as time 0), aliquots of 12.5 μ l 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.

supports an efficient reaction at both 30 nM [the optimal G36P 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 G38P at 90 nM *B. subtilis* SsbA. The reactions were dependent on G39P under both conditions tested. In the absence of any SSB, efficient reactions are also observed, but dependence on G38P and G39P 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 G40P could not be assembled onto the fork by the host loading system (DnaB/DnaD/DnaI/PriA) in the presence of either SsbA or G36P (Figure 4A, lanes 2 and 15).

We then tested whether G36P can replace SsbA in support of the *B. subtilis* replisome. *B. subtilis* chromosomal replication does not occur in the presence of G36P 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 G38P-G39P helicase loading system (Figure 4B, lane 6). Increasing G36P 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 G38P are required to reverse inhibition of DNA replication by high concentrations of G36P

The above experiments showed that G36P 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 G36P 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 G36P may have been an inauthentic reaction, resulting from helicase independent strand displacement by the PolC holoenzyme. Such a reaction is catalysed 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 G36P concentrations retained a dependency on all of the leading strand replication proteins, including helicase (Supplementary Figure S4).

We suspected the inhibition by high G36P 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 G38P elicited this effect (Figure 5C). We also tested the other viral protein that participates in helicase loading, G39P, but observed no effect (data not shown).

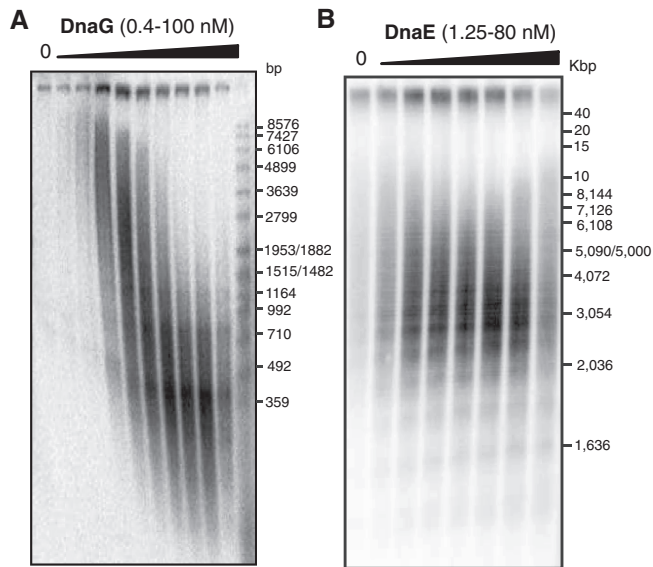


Figure 3. Effect of increasing DnaG and DnaE on the size of Okazaki fragments. (A) Size of Okazaki fragments varies inversely with primase concentration. Rolling circle reactions were performed in the presence of [α - 32 P]dGTP and were stopped after 10 min. The reactions were run on an alkaline agarose 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.

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 G40P helicase, we also observed in these assays greater efficacy of G36P relative to SsbA at low concentrations (Supplementary Figure S5D). Furthermore, we observed an absolute requirement for G38P (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 G36P 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 whether the SPP1 phage requires the SsbA protein *in vivo*, we analysed 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. *ssbA35* cells and an isogenic control were infected at 30°C, an after 2 h of infection the phage titer was determined. *ssbA35* cells are temperature sensitive for growth above 47°C in LB medium (34). Cultures were also grown at a permissive temperature until OD_{560nm} = 0.2 and then shifted to 50°C. After

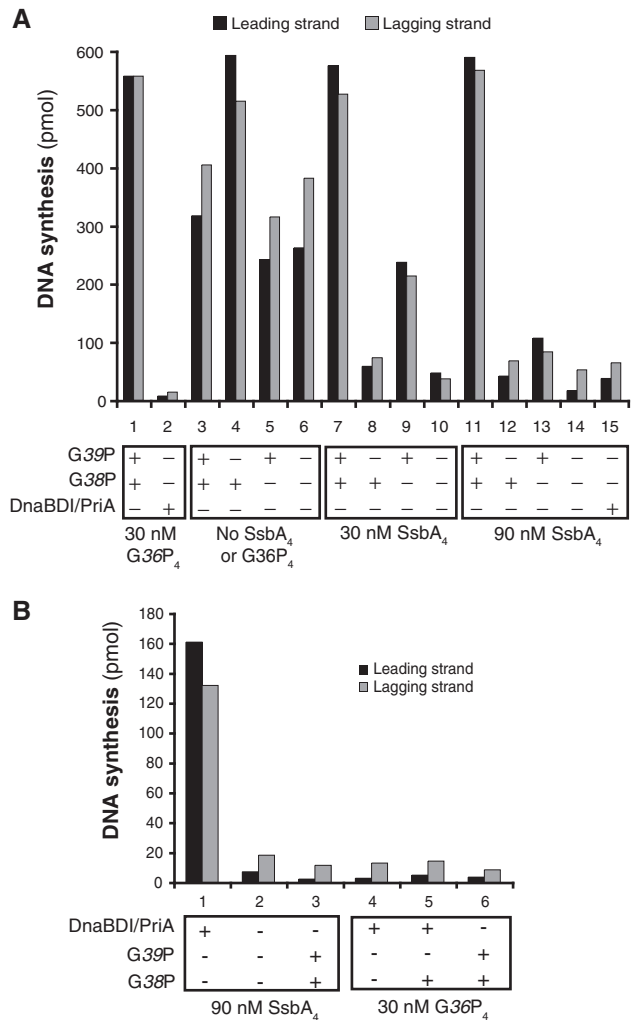


Figure 4. Interchangeable components of the replication machinery of *B. subtilis* and SPP1. (A) The SPP1 replication fork can use SsbA or G36P. *In vitro* replication of reconstituted SPP1 replication forks assembled in the presence of 30 nM G36P₄ (lanes 1–2), in the absence of an SSB (lanes 3–6) or in the presence of 30 nM or 90 nM SsbA₄ (lanes 7–10 and 11–15, respectively). The presence of G39P (helicase loader) and G38P (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 owns helicase loading system. *In vitro* replication of reconstituted *B. subtilis* replication forks assembled in the presence of 90 nM SsbA₄ or 30 nM G36P₄ and the indicated components. The data shown are the mean of at least three independent experiments.

15 min incubation, the cells were infected with SPP1, and the cultures were incubated for 2 h at 50°C. Titrations of three independent experiments yielded a mean of 3 × 10⁹ phage/ml and 1 × 10⁹ phage/ml for infections of *ssbA35* cells at permissive and non-permissive temperature, respectively. 3 × 10⁹ and 4 × 10⁹ 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 G36P

The preceding results show that G36P is not able to support replication in the full *B. subtilis* replication

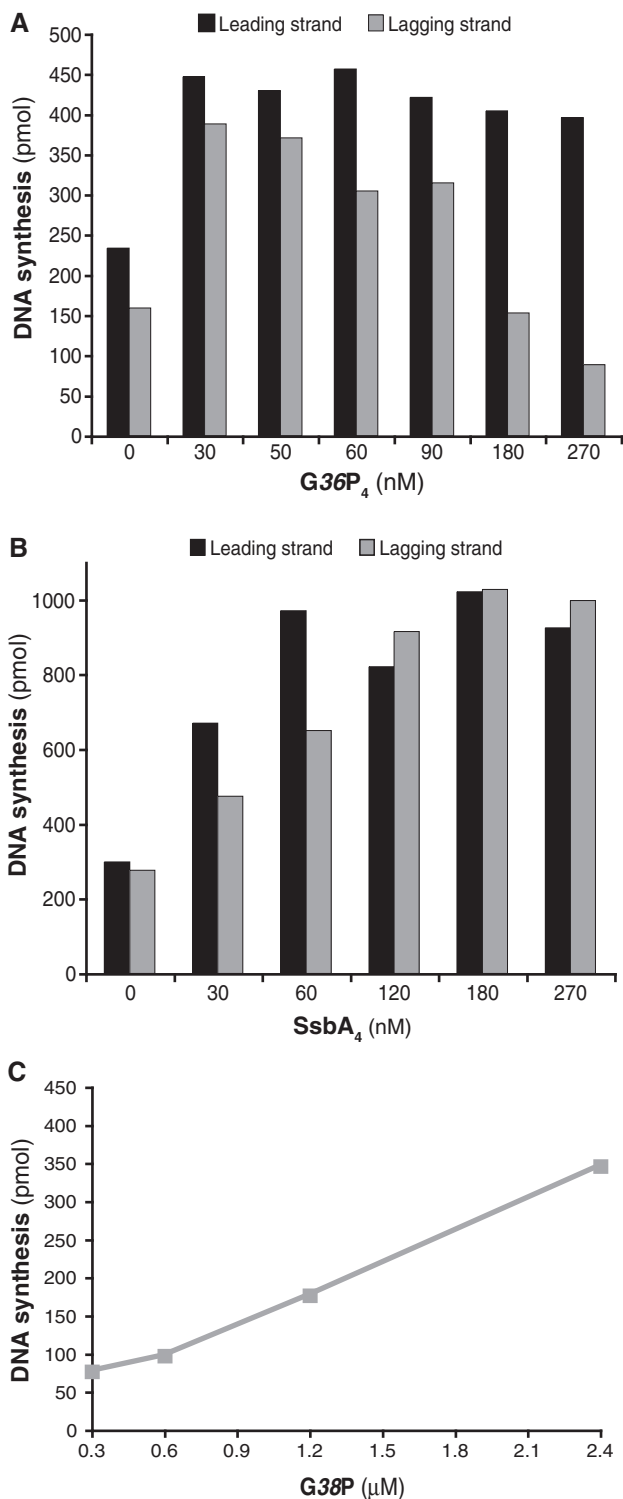


Figure 5. Increasing concentrations of G36P protein inhibit lagging strand DNA synthesis; synthesis can be restored by increasing G38P. (A) G36P₄ titration in SPP1 replication fork reconstitution. Standard reactions to measure DNA synthesis at leading and lagging strands were performed at the indicated G36P₄ concentrations. (B) High SsbA₄ concentrations do not inhibit lagging strand synthesis. (C) Effect on lagging strand synthesis of increasing G38P at high G36P₄ (180 nM). The values represented are the mean of three independent experiments.

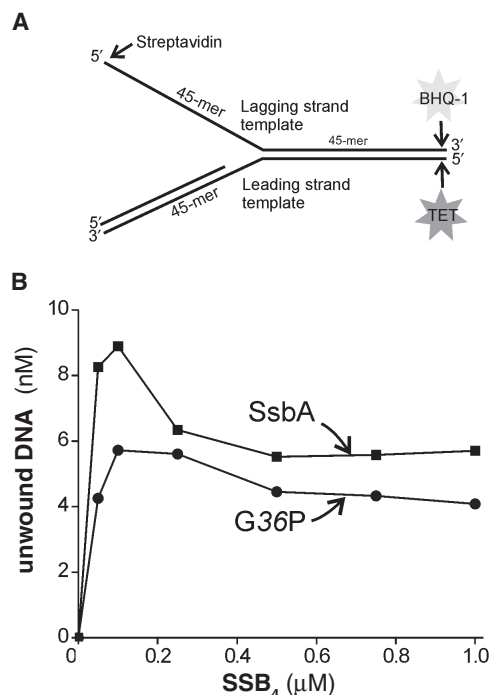


Figure 6. Either SsbA or G36P will support *B. subtilis* helicase loading and unwinding. (A) DNA substrate used in unwinding reactions. The fluorescence of TET on the 5' terminus increases on being separated from a quencher (BHQ-1) on the opposing strand. The 5'-end of the lagging strand template is 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 G36P. DNA substrate was combined with varying amounts of either SsbA or G36P, and 150 nM PriA, 75 nM DnaD, 75 nM DnaB, 12 nM DnaC₆, and 50 nM DnaI.

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 G36P. We exploited the fluorescence-based assay (Figure 6A). The results showed that SPP1 G36P and SsbA are interchangeable in the helicase assays (Figure 6B). Thus, the defect in the *B. subtilis* assay in the presence of G36P occurs after helicase loading and DNA unwinding.

G36P 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 G36P, we tested for its ability to stimulate reactions catalysed by the DnaE and PolC holoenzymes. We observed that SsbA and G36P stimulate the DnaE holoenzyme with similar efficiency (Figure 7A). However, G36P 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 G36P-supported *B. subtilis* reaction likely resides in its inability to stimulate the PolC holoenzyme.

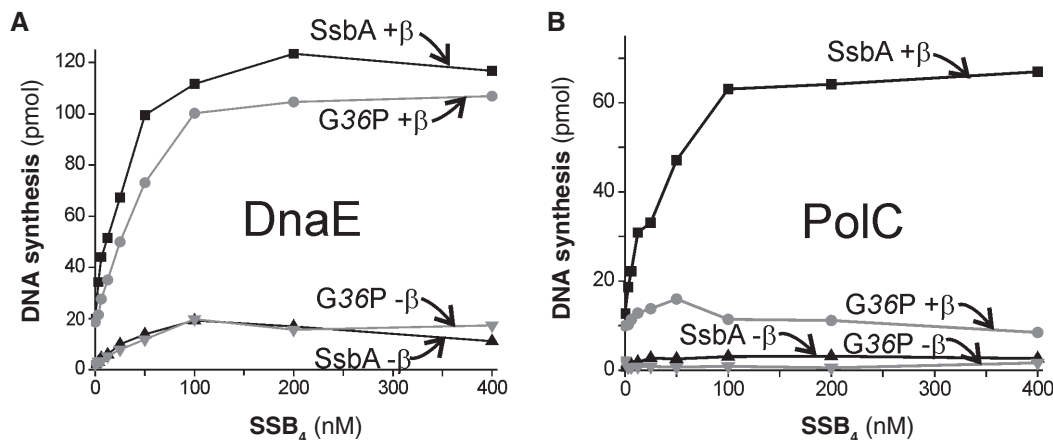


Figure 7. SPP1 G36P stimulates DnaE holoenzyme, but not PolC holoenzyme. (A) Extension of a DNA primer by the DnaE holoenzyme using either SsbA or G36P. SsbA or G36P were titrated in a DNA-primed M13_{Gori} reaction containing 3 nM DnaE in the presence (+) or absence (-) of β₂ as indicated. (B) Extension of a DNA primer by the PolC holoenzyme (2 nM) using either SsbA or G36P. Total DNA synthesis was analysed by quantification of incorporated [³H]dTTP.

G36P blocks host DNA replication

The observation that G36P does not support *B. subtilis* replication led us to investigate whether 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 SsbA₄ and increasing concentrations of G36P₄. Both leading and lagging strand synthesis were significantly inhibited even at the lowest G36P 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 (G40P), its loading protein (G39P) and the host DnaG primase and PolC DNA polymerase. We also observed a requirement for the G36P protein. Neither the *B. subtilis* helicase loading proteins nor the helicase are required, but all of the polymerase elongation components are necessary, including β₂, 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 used.

In the well-characterized *E. coli* system, DnaG primase binds to the DnaB₆ 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

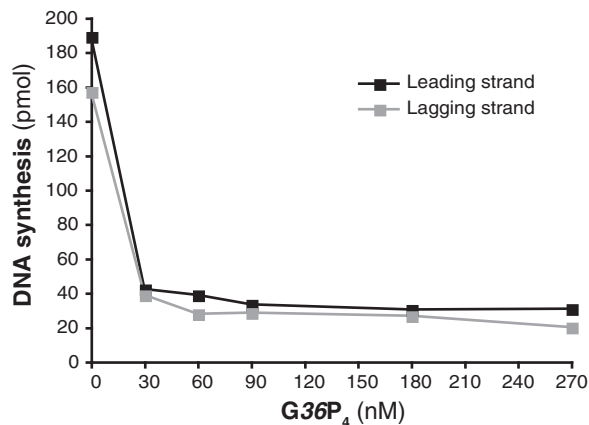


Figure 8. G36P inhibits *B. subtilis* rolling circle DNA replication even when SsbA is present at saturating concentrations. *In vitro* replication of reconstituted *B. subtilis* replication forks assembled in the presence of increasing concentrations of G36P₄ (30, 60, 90, 180 and 270 nM tetramer) and 90 nM SsbA₄. Both SSBs were added simultaneously to the substrate mix. The data shown are the mean of at least three independent experiments.

owing 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 G38P 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 G38P plays a dual role as an origin-specific and forked structure-specific initiation protein. Future studies directed at common features of PriA- and G38P-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 (G38P and G39P).

We found that both SPP1 G36P and *B. subtilis* SsbA supported the SPP1 replicative reaction, but with interesting differences. Under standard reaction conditions, ~180 nM SsbA₄ was required to achieve an optimal rate of leading and lagging strand synthesis. However, with G36P₄, an optimum rate is achieved at a 6-fold lower concentration. Further increases of G36P concentration result in marked inhibition of lagging strand synthesis. The most straightforward explanation for this observation is that G36P makes specific protein-protein interactions that sequester some viral component in inactive binary complexes. As elevated levels of G38P reverse the lagging strand inhibition induced by elevated G36P, 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 G38P is involved in both loading the replicative helicase and reversing the inhibition by high G36P 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, G36P, will not support a full host replisome-dependent replicative reaction. Dissection of the individual reaction steps shows that G36P is fully competent to support PriA/DnaD/DnaB/DnaI-dependent helicase loading and ensuing advancement to separate DNA strands. G36P is also able to stimulate a DnaE holoenzyme activity as good as the host SSB. The only defect detected was a failure of G36P 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 G36P of the *B. subtilis* replication fork and the efficient use in the SPP1 replication fork of the two SSBs (the viral G36P 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 G36P. 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 80 α , which transfers pathogenicity islands between staphylococci. This phage also encodes a G38P-like protein (47).

G36P 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). The C-terminus of G36P 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 G36P and SsbA on PolC holoenzyme stimulation and the specific inhibition of SPP1 replication at high G36P 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 coliphages, the only viral protein that is required for replication is a nicking/religation activity that functions after duplex formation to shift SS \rightarrow 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 G38P, 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 Φ 29 (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 of 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 G38P 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 Figures 1–7, Supplementary Materials and Methods and Supplementary Reference [56].

ACKNOWLEDGEMENTS

The authors thank Gloria E. García-Baca, and Begoña Carrasco for providing us with purified G36P and SsbA, María López for excellent technical assistance and Patrice Polard and Juan C. Alonso for providing us with strains. A.L.P. received a contract from the Community of Madrid. C.S.M. sabbatical leave was supported, in part, by the Spanish Ministry of Education (SAB20111-0026) and by the Burroughs Wellcome Fund 2012 Collaborative Research Travel Grant. J.Z. received partial stipend support from NIH Molecular Biophysics Training Grant T32 GM65013.

FUNDING

Spanish Ministry of Economy and Competitiveness [BFU2009-09520 to S.A.]; National Science Foundation [MCB-0919961 to C.S.M.]. Funding for open access charge: Spanish Ministry of Economy and Competitiveness.

Conflict of interest statement. None declared.

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