Homologous-pairing Activity of the *Bacillus subtilis* Bacteriophage SPP1 Replication Protein G35P*

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Genetic evidence suggests that the SPP1-encoded gene 35 product (G35P) is essential for phage DNA replication. Purified G35P binds single-strand DNA (ssDNA) and double-strand (dsDNA) and specifically interacts with SPP1-encoded replicative DNA helicase G40P and SSB protein G36P. G35P promotes joint molecule formation between a circular ssDNA and a homologous linear dsDNA with an ssDNA tail. Joint molecule formation requires a metal ion but is independent of a nucleotide cofactor. Joint molecules formed during these reactions contain a displaced linear ssDNA strand.

Electron microscopic analysis shows that G35P forms a multimeric ring structure in ssDNA tails of dsDNA molecules and left-handed filaments on ssDNA. G35P promotes strand annealing at the AT-rich region of SPP1 oriL on a supercoiled template. These results altogether are consistent with the hypothesis that the homologous pairing catalyzed by G35P is an integral part of SPP1 DNA replication. The loading of G40P at a φ-loop (ori DNA or at any stalled replication fork) by G35P could lead to replication fork reactivation.

* Bacillus subtilis* bacteriophage SPP1 encapsidates its dsDNA into an empty procapsid by a processive headful packaging mechanism, using a linear head-to-tail concatamer as a substrate (1). SPP1 replication begins at a “unique” origin and proceeds unidirectionally (2–4). However, two SPP1 replication intermediates have not been observed (3–5). The generation of concatemeric SPP1 DNA is at least dependent on phage-encoded (G38P, G39P, G40P, G41.1P, G35P, and G36P, see Fig. 1 below) and host-encoded (DNA PolIII, DnaG, and DNA topoisomerases) replication proteins but is independent of host-encoded components of the primosome (e.g. DnaB, DnaD, and DnaI) and recombination proteins (RecA, AddAB, and RecF) (5–10). Furthermore, we could show that the SPP1 plating efficiency is indistinguishable on wt, priA1, and priA1 dnaB75 strains.

Analysis of SPP1 conditional-lethal mutants for their capacity to synthesize phage DNA has lead to the identification of two different complementation groups. Whereas mutants in genes 38, 39, and 40 show a block in DNA replication, mutants in genes 34.1 and 35 show a normal initiation but a replication arrest phenotype (5, 6, 10). Genes 38, 39, and 40 and genes 34.1, 35, 36, and 36.1 are early transcribed genes that form part of two different operons (Fig. 1A). G36P, which is a helix-stabilizing single-stranded binding protein and shares 46% identity with *B. subtilis* SSB protein, does not seem to be essential under laboratory growth conditions (10). The activity of G36.1P, which shares a significant degree of identity with group I endonuclease proteins, remains to be characterized.

The initiation of theta-type replication at SPP1 oriL has been defined. In *vitro* studies reveal that multiple copies of G38P bound to its cognate site (AB and ab boxes, Fig. 1B) induce local unwinding of the adjacent AT-rich sequence (DUE region) present within oriL or oriR (7, see Fig. 1B). The helicase loader, G39P, specifically interacts with the replisome organizer, G38P, and with G40P-ATP (6, 11). G40P-ATP is a *bona fide* hexameric DNA helicase with a 5' to 3' unwinding polarity (12–14). G39P directs the assembly of G40P-ATP to G38P-bound oriL and allows the loading and subsequent activation of the G40P-ATP helicase (11). G40P-ATP bound to the ssDNA region at the unwound AT-rich region interacts with DnaG and the σ subunit of DNA PolIII and then theta type replication initiates (7, 12). However, very little is known about the mechanisms involved in the recovery of collapsed replication forks and in the generation of linear concatemeric SPP1 DNA.

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† The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; nt, nucleotide; AGE, aguroo gel electrophoresis; AS, ammonium sulfate; dME, μ-mercaptoethanol; BSA, bovine serum albumin; BIR, break-induced replication; DSβ, double-strand break; DUE, DNA unwinding element; EMSA, electrophoretic mobility shift assay; G38P, gene X product; ndPAGE, non-denaturing PAGE; PEI, polyethyleneimine; PolIII, DNA polymerase III; SSA, single-strand annealing; wt, wild type; CHAPS, 3-[3-cholamidopropyl(dimethy lamino)-1-propanesulfonic acid; EM, electron microscopy; HSV, herpes simplex virus.

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‡ S. Ayora and J. C. Alonso, this work.

§ M. Martinez, P. Mesa, and J. C. Alonso, unpublished results.

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The picture of how replication proceeds has changed over the last decade (reviewed in Refs. 15–17). It is now assumed that the one or replication forks formed at the replication origin become inactivated at high frequency, for example as a result of roadblocks in or on the template strands. In bacteria, the repair of this stalled replication fork requires the RecA, RecBCD, RecF, RecG, and/or RuvABC recombination proteins that may create a D-loop that is recognized by a component of the primosome (see Refs. 15–17). It has been shown that the PriA protein bound to the branched DNA molecule (recombination intermediate) directs the assembly of a new replication fork at the site through the loading of a primosome for theta type DNA replication (17). Furthermore, recent studies demonstrate that recombination intermediates formed between a linear duplex and supercoiled DNA are substrates for a DNA synthesis reaction where products longer than unit length are generated (18).

Although the presence of redundant pathways that could account for the recovery of an arrested replication fork and for the accumulation of concatemeric SPP1 DNA cannot be ruled out, we have previously shown that SPP1 replication does not require host-encoded homologous recombination proteins and components of the D-loop primosome assembly (Refs. 19 and 20, and this work). Furthermore, there are several lines of evidence that support the idea that SPP1 encodes in its genome recombination functions that may drive the restart of stalled replication forks and the switch from theta to sigma type of replication. First, plasmid transduction is markedly increased when homology between the plasmid and SPP1 is provided (9). Second, SPP1 infection triggers the appearance of long concatemeric plasmid linear molecules whose synthesis is dependent on homology, and phage-encoded functions, but independent of host-encoded recombination or primosome assembly functions (e.g. RecA, DnaB, etc.), whereas in non-infected cells synthesis of concatemeric plasmid molecules is dependent of both RecA and DnaB (21, 22). It is likely, therefore, that SPP1 may encode for a system that coordinates the processing of inactivated replication forks and the subsequent fork reactivation and the synthesis of concatemeric SPP1 DNA.

G35P and G34.1P share an overall identity of 40 and 18% with the Escherichia coli recombination proteins RecT and RecE, respectively. RecT is a SSA protein and RecE is an ATP-independent 5’–3’ exonuclease, both encoded by the defective lambdaib Rac prophage (reviewed in Ref. 15). Unlike the homologous recombination systems of lambdoid phages (e.g. λ-red, P22-erf, etc.) that are required for growth in recA mutants (reviewed in Refs. 23 and 24), G35P and G34.1P are essential SPP1 replication proteins in both wt and recA host strains (3, 5, 10).

Sedimentation studies of DNA synthesized by SPP1tsI20F (impaired in G35P, see Fig. 1A) or SPP1tsI17 (impaired in
gene 35) in wt-infected cells, at the restrictive temperature, revealed that only a small percentage of the phage DNA can be recovered in a fast sedimenting form (concatemeric DNA). In both cases SPP1 particles of less than unit length (30–35 kb in size) accumulated (5). Consistent with this DNA arrest phenotype, and considering the unidirectional movement of the SPP1 replication fork, we can assume, that any event initiated at oriL stops 35–35 kb away, in G35P and G34.1P mutants at non-permissive temperature, suggesting that G35P and G34.1P are involved in fork reactivation and the generation of concatemeric DNA. Furthermore, this distance is consistent with the hypothesis that G35P bound at oriR might work as a roadblock that would collapse any replication fork started at oriL, being the generated replication intermediates of ~32 kb (22).

To understand how concatemeric DNA initiates, we began the characterization of G35P. We found that G35P binds ssDNA and forms filaments. G35P preferentially catalyzes, in an ATP-independent manner, SSA between a circular ssDNA and the homologous AT-rich region of oriL on a supercoiled molecule and specifically interacts with the G40P DNA helicase and G36P SSB proteins. The results presented provide the first evidence that the SPP1 replication/recombination protein, G35P, might direct the assembly of the hexameric replicative helicase G40P at a β-structure by a new primosome assembly mechanism that does not require the primosome assembly proteins of *B. subtilis*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Phages—**E. coli strain JM103 (25) and BL21(DE3) (26) were used. *B. subtilis* subvar. pei (7) and its isogenic priA1::Em and priA1::Em dnaB75 derivatives have been previously described (7, 8). Plasmids pBT318, pBT320, and pBT430 (6); pBT233 (11); pCB163 (7); pLYS8 (26); and pUC18 and phage M13mp18 (25) have been previously described. The plating efficiency of an SPP1 stock was measured in four independent experiments for wt, priA1, and priA1 dnaB75 strains.

**Enzymes and Reagents—**Isozyme-1-thio-β-D-galactosanase and rifampin were from Calbiochem. DNA restriction and modification enzymes were purchased from MBI or Roche Molecular Biochemicals. [α-32P]dATP, [γ-32P]ATP, Sephadex G-100, DEAE, Superose 12, and Q-Sepharose were from Amersham Pharmacia Biotech. Phosphocellulose was from Whatman. PEI was from Sigma.

**DNA Manipulations and Substrates—** The 6065-bp HindIII:MseI-cleaved M13mp18 duplex DNA fragment was gel-purified. Linear 6065-bp M13mp18 DNA molecules having an ssDNA 3’ terminus (5’-tailed dsDNA) or an ssDNA 5’ terminus (5’-tailed dsDNA) were prepared by incubation of DNA with T7 gene 6 exonuclease or with *E. coli* EcoIII, respectively. About 50% of both substrates had an ssDNA tail with a length of ~140 nt, measured as the percent of substrate resistive to Peul or RflJ digestion, which are located 140 or 170 bp, respectively, from the restriction site dsDNA end.

**Enzymological Assays—**Bacterial strains, plasmids, and phages—E. coli strain JM103 (25) and BL21(DE3) (26) were used. *B. subtilis* subvar. pei (7) and its isogenic priA1::Em and priA1::Em dnaB75 derivatives have been previously described (7, 8). Plasmids pBT318, pBT320, and pBT430 (6); pBT233 (11); pCB163 (7); pLYS8 (26); and pUC18 and phage M13mp18 (25) have been previously described. The plating efficiency of an SPP1 stock was measured in four independent experiments for wt, priA1, and priA1 dnaB75 strains.

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previously described (28), using a reference-free algorithm to generate averages (29).

Isolation of G40P-Loaded on Joint Molecules—Joint molecules were prepared with supercoiled plasmid pCB163, oligonucleotides 1–4, and G35P, in the presence of 1 mM ATP, as described before. After incubation during 15 min at 30 °C, G40P (80 nm) was added, and the reaction was continued for another 10 min at 30 °C. Aliquots of the reaction were taken and deproteinized to analyze and quantify the extent of the D-loops formed, and isolation of G40P bound to the joint molecules from unbound G40P was performed by gel filtration chromatography on a Sepharose CL-6B as previously described (11). G40P was detected in the different fractions by Western blot.

**RESULTS**

**Characterization of G35P**—Soluble G35P, which was purified to 99% homogeneity as assayed by SDS-PAGE and quantitative analysis of the amino-terminal amino-acid sequence, was assayed for its ability to act as dsDNA or ssDNA nuclease (exo- or endonuclease), to hydrolyze ATP in the presence or absence of ssDNA or dsDNA, to unwind DNA, to bind ssDNA or dsDNA, and to interact with SPP1-encoded replication proteins. From these activities tested we observed that purified G35P protein is able to bind DNA and to interact with at least G40P and G36P.

G35P consists of 287 amino acid residues corresponding to a molecular mass of 32,000 Da (10). The native molecular mass of purified G35P was estimated by size fractionation through a Superose 12 fast protein liquid chromatography gel filtration column in buffer A containing 100 mM NaCl. In the presence or in the absence of 5 mM MgCl2, at low G35P concentrations (7 μM), G35P elutes in two peaks of similar area, one narrow peak corresponding to M, 65,000 and one broad peak corresponding to M, 250,000–350,000. At high G35P concentrations (30 μM), G35P elutes mainly in a broad peak, with M, 250,000–350,000, in the presence or in the absence of MgCl2. If we assume that G35P is spherical in shape, it is likely that, under the G35P concentrations assayed, G35P is in an equilibrium between a dimer and a higher order oligomer in solution. We cannot rule out, however, that G35P is an elongated monomer with a large Stokes radius.

**G35P Interacts Preferentially with ssDNA**—The affinity of G35P for linear 7,250-nt M13mp18 ssDNA (1 μM) was determined by filter binding assays by following the rate of complex formation as a function of G35P concentration (Fig. 2A). G35P-ssDNA complex formation was not linear at low protein concentrations and appeared to be sigmoidal at high G35P concentrations. The K app of the G35P-ssDNA complex, which in this case is equal to protein concentration midpoint, was estimated to be ~14 nM and ~26 nM at pH 7.5, 30 °C in buffer B in the absence and the presence of 5 mM MgCl2, respectively. It is likely, therefore, that, both in the absence or presence of 5 mM MgCl2, G35P interacts with ssDNA with a weak cooperativity. To analyze the type of complexes formed by G35P with ssDNA, EMSA was used. G35P does not bind to segments as short as the 26-nt ssDNA fragment of different sequence, indicating that there is a minimum length required for stable binding, but the formation of G35P-194-nt ssDNA complexes was observed by EMSA (Fig. 2B). At molar ratio of 1 G35P per 80-nt or 40-nt (1:80 or 1:40), G35P forms multiple and diffuse complexes with the 194-nt 32P-ssDNA, whereas at higher molar ratios (1:20 and 1:10) one discrete complex was observed (Fig. 2B, lanes 2, 3, and 10). The K app value of the G35P-ssDNA complex was estimated by EMSA to be ~18 and ~36 nM at pH 7.5, 30 °C in buffer B in the absence and the presence of 5 mM MgCl2, respectively.

The affinity of G35P for linear 7250-nt M13mp18 dsDNA (1 μM) was determined by filter binding assays also in the absence or the presence of Mg2+ (5 mM MgCl2) (Fig. 2A). The G35P-dsDNA complex retained by the filter was less than 65%.

**FIG. 2.** Effect of magnesium in binding of G35P to DNA. A, M13 mp18 γ-32P-ssDNA (1 μM in nt) (open and filled squares) or M13 mp18 α-32P-dsDNA (1 μM in bp) (open and filled circles) was brought to room temperature in buffer B, containing 5 mM MgCl2 (filled symbols) or in the absence of the metal ion (open symbols); increasing amounts of G35P dimers (0.75 to 1560 nM) were added and the incubation was continued for 10 min at 30 °C. Binding of G35P was analyzed by calculating the DNA retained on the filter as described under “Experimental Procedures.” B, G35P-ssDNA; C, G35P-dsDNA complex formation analyzed by EMSA. The 194-nt γ-32P-ssDNA (1 μM in nt, B) or 194-bp α-32P-dsDNA (1 μM in bp, C) was incubated with increasing concentrations of G35P in buffer B, in the presence or absence of 5 mM MgCl2 for 10 min at 30 °C and then loaded onto a 6% nPAGE. In lanes 1 and 9, no protein was added. In B, increasing amounts of G35P were added in lanes 8 to 2, and 16 to 10 (1.5 to 100 μM). In C, the amount of G35P added doubles from 4.5 to 300 nM in lanes 8 to 2, and 16 to 10. CI and CI-IV, denote the protein-DNA complexes, and FD denotes free DNA.
In the plateau region, where all the blunted linear dsDNA is presumably saturated with G35P, only 55–62% retention are observed, even at a G35P concentration greater than 1.5 μM. Similar results were observed at 25 and 50 mM NaCl; hence, G35P binds with linear dsDNA complexes show a poor stability. Under these experimental conditions, we observed that, in the absence of Mg2+, at pH 7.5 and 30 °C, G35P binds to linear 32P-M13mp18 dsDNA with a K

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of ~90 nM and 300 nM at pH 7.5, 30 °C in buffer B in the absence and the presence of 5 mM MgCl2, respectively (Fig. 2A). Both, in the absence or the presence of 5 mM MgCl2, at low G35P molar ratios (1:27) one discrete G35P-194-bp 32P-dsDNA complex was formed (CI complex), and at larger ratios (1:13 to 1:3.3) slow moving protein-DNA complexes were observed (CII to CIV) (Fig. 2C, compare lanes 5 with 2 and 13 with 10). Under these conditions, the K

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value of G35P-dsDNA complex formation was ~100 and ~300 nM in the absence or the presence of 6 mM MgCl2, respectively.

Hence, in the absence of Mg2+, G35P binds with 5-fold higher affinity to ssDNA over dsDNA, and this preference is even larger in the presence of 10 mM MgCl2. In the latter case, the protein binds with 8-fold higher affinity to ssDNA than to dsDNA.

G35P Promotes the Formation of Joint Molecules—G35P shares a 40% overall identity with the RecT protein (data not shown). RecT has been shown to promote the formation of joint molecules between a circular ssDNA and a homologous linear dsDNA having an ssDNA tail (30). To address whether G35P also catalyzes joint molecule formation, linear blunt-ended 6065-bp M13mp18 dsDNA was incubated with circular M13mp18 ssDNA and G35P in buffer D containing either 25 or 100 mM NaCl during 10 min at 30 °C. The samples were deproteinized, and the DNA forms separated by AGE. As shown in Fig. 3, lanes 2 and 8, G35P fails to form any new product that migrates slower than the linear dsDNA, when the dsDNA is blunt-ended. However, when linear 6065-bp M13mp18 dsDNA, with ~50% of the molecules containing an ssDNA tail with an average of ~140 nt either at the 5’ or the 3’, was incubated with circular M13mp18 ssDNA and G35P, the accumulation of a discrete new band that migrates more slowly than linear dsDNA was observed (Fig. 3). End products of the strand exchange reaction (relaxed dsDNA and linear ssDNA) as detected in the presence of B. subtilis RecA protein did not accumulate (data not shown).

In the presence of low NaCl (25 mM) annealing of the circular ssDNA and the linear dsDNA containing a 3’-tail accounted to ~37% of the total dsDNA, whereas ~17% of annealed molecules were observed when the 5’-tail dsDNA template was used with a G35P concentration of 780 nM (1 dimer per 35 nt of ssDNA) (Fig. 3, lanes 6 and 4). No further increase in the yield of the products was obtained at higher protein concentrations (data not shown). Because the length of the ssDNA tail is not uniform in both substrates, due to exonuclease treatment, we cannot rule out that the different yield observed with the 3’-tail over 5’-tails substrates is a consequence of any difference in length in both substrates. To address this question we have compared joint molecule formation with the same substrates, at low or high NaCl concentrations. At 100 mM NaCl concentration, the circular ssDNA annealed to the 3’-tailed dsDNA by ~21% and to the 5’-tailed dsDNA by ~7% (Fig. 3, lanes 12 and 10). Hence, at low NaCl, G35P shows a preference for substrates having 3’-tails over 5’-tails of ~2-fold, and this difference is enlarged in the presence of 100 mM NaCl. It is likely, therefore, that the protein might bind better to linear DNA having a 3’-tail than to linear DNA having a 5’-tail and that G35P shows some polarity in the reaction. Identical results are obtained when G35P is first incubated with linear dsDNA and the pre-complexed is then incubated with circular ssDNA or when the first incubation is with ssDNA. The formation of this new product, which was dependent on incubation with G35P, was not observed when proteinase K was added at the same time that G35P or MgCl2 was omitted or EDTA was added. Titration with MgCl2 shows an optimum for joint molecule formation at ~5 mM (data not shown). The formation of this new product, which could be reversed by heating at 100 °C, does not require the addition of a nucleotide cofactor, and its presence does not alter the yield of the reaction. It is likely, therefore, that G35P is free of contaminating enzymes capable of generating an ssDNA tail on the linear dsDNA substrate (e.g. exonucleases). Because the formation of SSA between tailed-dsDNA and homologous ssDNA is independent of the presence of a nucleotide cofactor, we can rule out that a DNA helicase could separate both DNA strands: the reaction lacks a nucleotide cofactor that is essential for all DNA helicases.

Visualization of the Joint Molecules Formed by G35P—To analyze whether the annealed products observed by AGE are the product of the annealing reaction of the complementary ssDNA tails present in the linear substrate with the circular ssDNA or if some exchange has taken place, G35P, at a concentration of 1 G35P molecule per 35 nt of ssDNA, was incubated with circular M13mp18 ssDNA and linear 6065-bp M13mp18 dsDNA having a 3’-ssDNA end, the reaction was deproteinized, and the products were examined by EM. Under these conditions, about 20–25% of total linear 3’-tailed M13mp18 dsDNA molecules were joined to homologous circular M13mp18 ssDNA (Fig. 3). At short times of incubation,
dsDNA, and G-containing 50 mM NaCl were incubated at 30 °C for 10 min, and the deproteinized products were analyzed by EMSA. A gallery of partially double-stranded circles with dsDNA and ssDNA branches attached to the circle. The branch of dsDNA and ssDNA is indicated by an arrowhead. ss denotes the presence of an adjacent single circular M13mp18 ssDNA molecule. B, schematic representation of the joint molecules presented in A.

Fig. 4. Electron microscopic analysis of the products of the strand exchange reaction. Reactions (10 μl) containing circular M13mp18 ssDNA (30 μM), 3′-tailed (30 μM) M13mp18 linear dsDNA, and G35P (780 nm) in buffer D containing 50 mM NaCl were incubated at 30 °C for 10 min, and the deproteinized products were analyzed by EM. In A, a gallery of partially double-stranded circles with dsDNA and ssDNA branches attached to the circle. The branch of dsDNA and ssDNA is indicated by an arrowhead. ss denotes the presence of an adjacent single circular M13mp18 ssDNA molecule. B, schematic representation of the joint molecules presented in A.

the complex formed by the annealing of the single-stranded region of the linear duplex and the homologous circular ssDNA molecule, leading to a sigma-shaped structure, was observed (data not shown). After 10 min of incubation, one of the DNA strands from the linear duplex DNA was displaced by the circular ssDNA (alpha-shaped structures). As revealed in Fig. 4, the short displaced strand is of ssDNA nature and a stretch of dsDNA of roughly comparable length has been formed on the circular ssDNA molecule. Neither sigma- nor alpha-shaped structures were observed when G35P was omitted (data not shown). The base pairing of the linear 3′-tailed DNA with the circular ssDNA did not exceed 2000 nt (Fig. 4B, data not shown), and the end products of the strand exchange reaction (relaxed dsDNA and linear ssDNA) were not observed.

G35P Forms Ring Structures on Short ssDNA Tails and Nucleoprotein Filaments with ssDNA—To visualize the type of complexes formed by G35P with the substrates used for the strand exchange reaction, the protein, at a concentration of 1 G35P molecule per 35 nt of ssDNA, was incubated with circular M13mp18 ssDNA and linear 6065-bp M13mp18 dsDNA having a 3′ ssDNA end, and the products were directly examined by EM without deproteinization. G35P bound to ssDNA changed the compact bush-like structures typical of protein-free ssDNA to relatively open, circular structures resembling pearls of a necklace (see Fig. 5A). G35P-circular M13mp18 ssDNA complexed with 3′-tailed M13mp18 dsDNA (joint molecules) was also observed (Fig. 5B). In accordance with its preferential binding to ssDNA, G35P-dsDNA complexes were not observed at the protein concentration used, but the ssDNA ends of the 3′-tailed dsDNA were covered by doughnut-shaped oligomers of G35P (Fig. 5B, denoted by arrowheads). From the volume occupied by the G35P ring structure, it could be predicted that it is an oligomer formed by seven to eight protomers. This is consistent with the observation that G35P has a mass of 250,000–350,000 Da in solution (see above).

Inspection of the electron micrographs of negatively stained G35P-ssDNA complexes revealed a striated pattern, which is suggestive of helical filaments, and these helices were determined to be left-handed by rotary shadowing (data not shown). To analyze these structures, 8867 short segments were selected from these filament images. Analysis of these segments showed that the helix-start helix with a variable pitch, a range greater than 85–105, and a mean of ~95 (Fig. 5C). The filaments have a clear polarity, but the periodicities arising from subunits along this continuous helix must be extremely weak, because they were not observed.

The observation that the filaments were formed only in the presence of ssDNA suggests that these filaments contain ssDNA. Unfortunately, no information is available about the location of the ssDNA within this protein filament.

G35P Promotes SSA at the Unwound AT-rich Region on a Supercoiled DNA Substrate and Loads G40P at This Region—Previously, it has been reported that (i) the DNA helix at the tandemly repeated AT-rich region present at replication origins is thermodynamically unstable and (ii) supercoiling of DNA, in the absence of the initiator proteins, induces localized unwinding (DUE) at the same sequence opened by the initiator protein (31). The ability of G35P to catalyze the assimilation of ssDNA, into the homologous supercoiled plasmid-borne SPP1 replication origin oriL (SPP1-DUE) or into a region with lower prediction of unwinding by supercoiling, was assayed. Four different oligonucleotides (1 to 4) have been synthesized (Fig. 6A). The 32P-labeled 230-nt-long oligonucleotides 1 and 4 have a region of homology of 40 nt, at either the 5′- or 3′-ends, respectively, with the AT-rich region of oriL; whereas the 32P-labeled 215-nt-long oligonucleotides 2 and 3 show only a short region of homology (10 nt) with the AT-rich region of oriL at the 5′- or 3′-ends, respectively (Fig. 6A). G35P has a similar affinity for the four ssDNA segments as measured by EMSA (data not shown).

G35P, at a concentration of 1 G35P per 135 bp of supercoiled DNA, forms a 3′-tailed complex with 32P-labeled 230-nt-long oligonucleotides 1 and 4 having a region of homology of 40 nt, at either the 5′- or 3′-ends, respectively, with the AT-rich region of oriL; whereas the 32P-labeled 215-nt-long oligonucleotides 2 and 3 show only a short region of homology (10 nt) with the AT-rich region of oriL at the 5′- or 3′-ends, respectively (Fig. 6A). G35P has a similar affinity for the four ssDNA segments as measured by EMSA (data not shown).
dsDNA, was incubated with the labeled oligonucleotides and then with the homologous supercoiled plasmid-borne SPP1-DUE. The samples were deproteinized and analyzed by AGE. G\textsubscript{35}P preferentially promoted the formation of a stable complex between oligonucleotide 4 and the plasmid DNA that co-migrated with supercoiled plasmid DNA on an agarose gel (Fig. 6B). The amount of SSA in three independent experiments was quantified and found to be 13%, 3%, 6%, and 24% for oligonucleotides 1 through 4, respectively. The oligonucleotides 4 and 3, which have a region homologous to the AT-rich region of oriL at their 3’-end, base-paired to one strand of the supercoiled plasmid molecule with 2-fold higher efficiency than that of their respective counterparts. The reaction required an Mg\textsuperscript{2+} cation but did not require a nucleotide cofactor. Similarly, to join the molecule formation, the optimum Mg\textsuperscript{2+} concentration was 5 mM (data not shown) and the protein showed some preference for pairing the oligonucleotide that enters the SPP1-DUE region in a 3’ orientation (Fig. 6B, lanes 4 and 16). The percentage of D-loops formed was independent of the order of incubation of the DNA substrates with G35P. The migration distance of the reaction product on the agarose gel was identical to that of the D-loop product formed by B. subtilis RecA. SSA was not observed when the plasmid molecule was relaxed with DNase I prior incubation with G35P (data not shown).

When an ssDNA segment complementary to another region of the supercoiled plasmid DNA was used, 3–5% joint molecule formations were observed, similar to the yield with ssDNA segments 2 and 3, which only have 10-nt annealing to the AT-rich region (data not shown). It is therefore likely that G35P promotes SSA between a pre-existing unwound region (e.g. AT-rich region of oriL, DUE region) and that the region of homology needs to have a minimum length (see Ref. 20 and this work).

We have previously shown that (i) the loading of G40P at the oriL region is dependent on the replisome organizer, G38P, and the helicase loader G39P (11) and (ii) G40P preferentially binds

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**Fig. 6. D-loop formation at SPP1 oriL.** A, the SPP1 oriL region. The double line represents the DNA, the shaded regions within are the G38P cognate sites (boxes ab and AB), and the bubble region denotes the adjacent AT-rich segment. The relevant restriction sites are indicated. Schematic shows the oligonucleotides used in the experiment. Oligonucleotide 1 and 4, and 2 and 3, are complementary, respectively. B, γ\textsuperscript{32}P-labeled oligonucleotides (120 nM) were incubated with supercoiled pCB163 DNA containing oriL (8 μg) and 62 nM G35P in buffer D containing 100 mM NaCl at 30 °C for 30 min. The reaction products were deproteinized and separated by 0.8% AGE. Gels were dried and analyzed by autoradiography. The symbols + and – denote the presence or absence of the indicated product.
to ssDNA regions with no potential secondary structures and to ssDNA as short as 10–12 nt in length (13). In both E. coli and B. subtilis, the loading of the replicative helicase onto collapsed replication forks is a PriA-dependent step (8, 17). Because SPP1 replication is independent of PriA, DnaB, DnaD, and DnaI primosomal proteins (Ref. 4 and this work), it can be assumed that D-loops catalyzed by G35P might have some role on the loading of G40P into ssDNA. To address whether G35P creates an ssDNA region of sufficient length to which the replicative helicase G40P can be loaded and, therefore, the replisome established at this region, joint molecules promoted by G35P between supercoiled plasmid pCB163 and oligonucleotides 1–4, in the presence of 1 mM ATP, were prepared. Then G40P (80 nM) was added, and the reaction was incubated for another 10 min at 30 °C. The free G40P was separated from G40P bound to the joint molecules by gel filtration chromatography, and the amount of G40P loaded on the different D-loops formed was quantitated. Accordingly to the yield of D-loops formed with the different ssDNA segments, different yields in the loading of G40P could be observed. When D-loops were formed with oligonucleotides 2 and 3 and supercoiled plasmid pCB163, no G40P could be detected in the DNA-containing fractions, whereas when oligonucleotides 1 and 4 were used about 6 and 15% of total input G40P, respectively, was present and associated with the joint molecules (data not shown). It is likely, therefore, that in both cases the region of ssDNA created by the annealing of the invading oligonucleotide to the complementary strand promoted by G35P has a length that is sufficient to load G40P at the D-loop region. Alternatively, the loading of G40P is stimulated by a direct protein-protein interaction, and G35P is sufficient for loading G40P at the D-loop, as suggested by the high efficiency in the loading of G40P at joint molecules (compare the percentage of input G40P present in the D-loops with the percentage of D-loops formed with oligonucleotides 1 and 4).

G35P Interacts with G40P and G36P—G35P preferentially catalyzes strand invasion on a pre-existing unwound region; i.e. as the AT-rich region at the origins of replication of SPP1 (see above). To address whether G35P could recruit any replication protein at this site, we analyzed whether G35P physically interacts with four (G36P, G38P, G39P, and G40P) of the six SPP1-encoded replication products (except for the G34.1P and G36.1P endonucleases). We immobilized the proteins G35P or BSA (6 μM), as a control, on an Affi-Gel 10 matrix. G36P, G38P, G39P, and G40P were then loaded (1 μM) separately onto the matrix. As shown in Table I, the DNA helicase protein, G40P, and the SSB, G36P, were retained by G35P bound to the column, whereas the replisome organizer, G38P, the helicase loader, G39P, or BSA were not retained by the same column. None of the proteins bind to the BSA column. It is likely, therefore, that G35P physically interacts with both G36P and G40P.

**DISCUSSION**

**G35P Is a Bona Fide ATP-independent SSA Protein**—The characterization of G35P revealed a significant biochemical similarity with evolutionarily distinct families of ATP-independent SSA proteins that form rings, filaments, or both. Many of these families appear to be primarily of bacteriophage origin, namely the RecT/Redβ, Erf, and Rad52 families (see Ref. 32). The RecT/Redβ family comprises the Rec-deficient prophage RecT, phage λ β protein (λβ), and SPPI G35P proteins, the Erf family comprises the phage P22 Erf protein (P22-Erf), and the Rad52 family comprises the Rad52 eukaryotic protein and putative proteins of phage origin (see Ref. 32). A biochemical similarity with ATP-independent SSA complex proteins of eukaryotic origin, namely the hXRC2c-hRad51c/hRad51L2 and hXRC2c/hRad51D/hRad51L3 (33–35), was also observed. One common feature in these families of proteins is their ability to form rings composed by a divergent number of subunits with different requirements. Although ring formation seems to be independent of Mg2+ for the eukaryotic proteins hRad52 and hXRC2c/hRad51D (33, 35, and 36) and G35P (this work), Mg2+ seems to be essential for ring formation for RecT and λβ (37, 38). In the case of G35P, ring formation seems to be concentration-dependent, whereas this possibility has not been analyzed for the eukaryotic ATP-independent strand-annealing proteins. Furthermore, the broad peak obtained with G35P suggests a heterogeneity in the number of subunits that compose the ring.

The presence of the polymorphism observed with the λβ protein when examined by electron microscopy (38) and suggests a dynamic behavior in all this family of proteins.

λβ protein seems to be the only protein of this family that does not form filamentous structures on ssDNA (38). The filamentous structures with ssDNA observed under the electron microscope for hRad52, hXRC2c/hRad51D complex, and the hXRC2c/hRad51C complex do not seem to have a helical structure and can be composed of stacked rings packed in an edge to edge manner (33–36). However, the filaments observed for G35P with ssDNA are helical and have a clear polarity (Fig. 5C). Furthermore, λβ protein seems to be the only protein of this family to filament on dsDNA, and filament formation required the dsDNA substrate to have an ssDNA tail to start filamentation, where the protein first assembles as a ring structure (38). Similarly to λβ, G35P, binds to dsDNA with an ssDNA tail forming a ring structure with the tails (Fig. 5B), but accordingly to its preferential binding to ssDNA, no protein was bound to the dsDNA region of the DNA substrate. The ssDNA tails on the linear dsDNA used in Fig. 5B were obtained by exonuclease treatment and are no longer then ~140 nt; therefore, it is likely that, depending on the length of the ssDNA, G35P forms rings or filaments with a helical structure with ssDNA.

G35P and RecT catalyze joint molecule formation between a linear dsDNA having an ssDNA tail and its homologous circular ssDNA, where some strand displacement is observed. RecT preferentially catalyzes this reaction at a low concentration of Mg2+ (~0.35 mM MgCl2) (30, 39), with similar efficiency with substrates having a 3′- or a 5′-tail (39); G35P, however, performs limited strand exchange at high Mg2+ concentrations (5 mM) with 2- to 3-fold higher efficiency with substrates having a 5′-tail.

G35P (Fig. 6), RecT (39), hRad52 (33), hXRC2c/hRad51D complex (34), and the hXRC2c/hRad51C complex (35) have been shown to catalyze DNA strand invasion between an oligonucleotide and a homologous supercoiled plasmid DNA, but the requirements for this reaction seem to be different. Although RecT does not require Mg2+ for D-loop formation, it is required for D-loops formed with G35P and the hXRC2c-hRad51D and hXRC2c-hRad51C complexes, but the optimum Mg2+ concentrations are in each case different (34, 35). The differences in the Mg2+ requirements correlate with their preferential binding for dsDNA or ssDNA and suggest that,
and binds to them in an ATP-independent fashion, opening the adjacent AT-rich region, where G
polymerize on it. Although the products of the reaction are the same, mechanistically this group of proteins may act in a different way. Many proteins of these family such as RecT (39) and hRad52 (33) bind preferentially to dsDNA at no or low Mg$^{2+}$ concentrations, and this is the optimum Mg$^{2+}$ concentration required for θ-loop formation. However, incubation of the protein first with dsDNA inhibits the joint molecule reaction and suggests that these proteins need to contact dsDNA but in a prefixed order. G35P, however, performs θ-loop formation and limited strand exchange at high Mg$^{2+}$ concentrations, where the protein does not bind to dsDNA (Fig. 2).

Although all these proteins have been considered so far as recombination proteins, the functional similarities between the SPP1 replication protein, G35P, and phage-encoded λ-β, P22-erf, and RecT suggest that they could work in the processing of inactivated replication forks and the generation of concatameric linear substrates that are encapsidated into empty procapsids. Furthermore, the functional similarity between the phage-encoded homologous-pairing proteins and hRad52 and the hXrec2-hRad51D and hXrec2-hRad51C complexes, despite lacking obvious sequence homology, suggest that the human proteins may also act in the assembly of replication forks after stalling. In fact, yeast Rad52 has been shown to play a major role in BIR, a process that has been shown to be rad51-independent (40). In the case of the phage recombination systems, a limited exonuclease activity associated with the SSA protein may play an active role in the annealing process (41), although the presence of an exonuclease in not obvious in the human recombination proteins.

G35P Might Direct the Assembly of a New Replication Fork: A Model—Genetic evidence suggests that SPP1 mutants impaired in the putative 5′ to 3′ exonuclease G34.1P or homologous-pairing G35P accumulate less than unit-length DNA molecules (30–35 kb in length) under non-permissive conditions (5). Why would the failure to use the recombination proteins G34.1P or G35P alone lead to a DNA arrest phenotype? Previously, it was shown that such a defect cannot be overcome by any of the host recombination nor by the DnaA- and PriA-DnaB-DnaD-Dna1-dependent assembly pathways (see the introduction). Here we show that G35P catalyzes SSA and promotes θ-loop formation, which are features associated with bona fide homologous-pairing proteins. Because SPP1 replication is independent of host-encoded primosomal components, and G35P physically interacts with G40P and G30P, we assume that G35P may be involved in recombination-dependent replication and it might help in the re-establishment of a stalled replication fork at oriR or at any other stalled region.

We hypothesize that, after the initial phase of initiation of theta type SPP1 replication at oriL (6, 7), the progression of the replication fork might be stalled when the replication fork encounters G38P bound at oriR (roadblock) in the absence of an overt DNA damage (see Fig. 7) or at any region in the presence of a DNA damage. The stalled replication fork breaks, and the broken fork is rescued by a process dependent on phage-encoded G35P and G34.1P functions. After Skalka (23, 24), Formosa and Alberts (42), Viret et al. (18), and Kuzminov (15), we propose that G34.1P exonuclease may degrade the 5′-end of the linear dsDNA of a collapsed replication fork at oriR or at any

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**Fig. 7.** Roadblock as a model for the shift from theta to sigma replication. A. G38P bound to oriL or oriR blocks replication fork progression. After stalling, a nick in the leading strand (bottom part) will be processed by the putative 5′–3′ exonuclease, G34.1P, to generate a 3′-ssDNA tail on which G35P will polymerize, whereas a nick in the lagging strand (top part), will not require further processing, and G35P will polymerize on it. B. Model for SPP1 initiation of sigma type DNA replication. G38P recognizes AB boxes of the SPP1 replication origin (oriL or oriR), and binds to them in an ATP-independent fashion, opening the adjacent AT-rich region, where G36P binds with high affinity. A G35P:ssDNA filament pairs with the leading strand of the unwound region. By direct G32P-G36P interactions, a remodeling of both proteins can take place, so that the G39P-G40P-ATP complex can be loaded in the unwound region by the ATP-dependent ssDNA binding capacity of G34P, as well as the interactions between G35P and G40P, and G39P and G38P, respectively. G38P-G39P, which forms a heterodimer, dissociates G39P from the G39P-G40P-ATP complex, and releases G38P from the origin. G40P helps the assembly of DnaG and perhaps DNA PolIII at the AT-rich region. The 3′-OH end of the paired strand could be used to prime the leading strand and DnaG could provide the primer for lagging strand synthesis. The arrow indicates the direction of helicase movement.
other stalled region generating 3'- overhangs to which G35P binds (Fig. 7A). G35P-mediated joint molecule formation could provide a 3'-end to anneal at oriR on a second supercoiled SSP1 molecule (Fig. 7B). Recently it has been shown that the RecE/RecT and λ-α/λ-β pairs physically interact, and homologous pairing was favored with respect to the exonuclease activity (41). Because RecT, λ-β, and G35P belong to the same family of SSA proteins (32), it is likely that G35P physically interacts with G34.1P.

We envisage two pathways, one that is G35P-dependent and one G35P-independent, for the assembly of the hexameric replicative helicase G40P at a replication fork or at a re-established fork. In the case of the G35P-dependent pathway, G35P, by protein-protein interaction, might cause a local remodeling of G36P, bound to the G36P-promoted locally melted AT-rich region on oriR or oriL and catalyze SSA (Fig. 7B). G35P alone or in combination with G35P, again by protein-protein interaction, stimulates the loading of the G39P-G40P-ATP complex at the ssDNA region. The interaction of G38P with G35P remodels the G39P-G40P-ATP inactive complex and releases G40P-ATP of the G39P-G40P-ATP complex. Then, the interaction of G35P with G40P-ATP stabilizes the later at the open complex (Fig. 7B). In the case of the G35P-independent pathway, G35P would catalyze SSA at any pre-existing unwound homologous ssDNA region to which G36P is bound and cause a local remodeling of G36P. G35P would stimulate the loading of the G39P-G40P-ATP complex or free G40P-ATP at the unwound region by G35P-G40P-ATP interaction. However, a mechanism for activation/remodeling of the G40P hexameric DNA helicase has to be envisaged if G35P stimulates loading of the inactive G39P-G40P-ATP complex. Because G35P synthesis is halted and its relative amount drops after min 18 of post-infection injection, whereas G40P remains constant (43), we assume that G35P might load free G40P-ATP at the unwound region. Then, in both replication assembly pathways, G40P-ATP bound to ssDNA directs the assembly of DnaG and DNA PolIII in an ATP-dependent and or in combination with G35P remod-

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Homologous-pairing Activity of the Bacillus subtilis Bacteriophage SPP1 Replication Protein G 35P
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