The functional role of the \( \phi29 \)-encoded integral membrane protein p16.7 in phage DNA replication was studied using a soluble variant, p16.7A, lacking the N-terminal membrane-spanning domain. Because of the protein-primed mechanism of DNA replication, the bacteriophage \( \phi29 \) replication intermediates contain long stretches of single-stranded DNA (ssDNA). Protein p16.7A was found to be an ssDNA-binding protein. In addition, by direct and functional analysis we show that protein p16.7A binds to the stretches of ssDNA of the \( \phi29 \) DNA replication intermediates. Properties of protein p16.7A were compared with those of the \( \phi29 \)-encoded single-stranded DNA-binding protein p5. The results obtained show that both proteins have different, non-overlapping functions. The likely role of p16.7 in attaching \( \phi29 \) DNA replication intermediates to the membrane of the infected cell is discussed. Homologues of gene 16.7 are present in \( \phi29 \)-related phages, suggesting that the proposed role of p16.7 is conserved in this family of phages.

Studies on DNA replication and related processes have provided detailed insights into the function of many proteins involved in these processes (for review, see Ref. 1). Despite this, little is known about the in vivo organization of DNA replication. To gain a better insight into this fundamental process, we studied the in vivo DNA replication of the Bacillus subtilis bacteriophage \( \phi29 \) (2). The detailed knowledge of its in vitro mechanism of DNA replication (for reviews, see Refs. 3 and 4) made \( \phi29 \) an attractive system for this study.

The genome of \( \phi29 \) is a linear double-stranded DNA (dsDNA) of 19,285 base pairs that contains a terminal protein (TP) covalently linked at each 5’ end. Fig. 1A shows a schematic representation of the genetic and transcriptional organization of the \( \phi29 \) genome. Regulation of \( \phi29 \) DNA transcription, which can be divided into an early and a late stage, has been studied extensively in vivo as well as in vitro (for reviews, see Refs. 3 and 5). The late expressed genes, all transcribed from a single operon present in the central part of the genome, encode the structural proteins of the phage, proteins involved in morphogenesis, and those required for lysis of the infected cell. The early expressed genes are present in two operons that flank the late operon. The early operon located at the left side of the \( \phi29 \) genome encodes the transcriptional regulator protein p4 and various proteins that are directly involved in phage DNA replication, such as the DNA polymerase, TP, single-stranded DNA-binding protein (SSB), double-stranded DNA-binding protein, and protein p1. The operon located at the right side of the \( \phi29 \) genome encodes, in addition to proteins p17 and p16.7, four putative proteins of unknown function.

A schematic overview of the in vitro \( \phi29 \) DNA replication mechanism is shown in Fig. 1B. Initiation of \( \phi29 \) DNA replication occurs via a so-called protein-primed mechanism (reviewed in Refs. 3, 4, and 6). The TP-containing DNA ends constitute the origins of replication. Initiation of DNA replication starts by recognition of the origin by a heterodimer formed by the \( \phi29 \) DNA polymerase and the primer TP. The DNA polymerase then catalyzes the addition of the first dAMP to the primer TP. Next, after a transition step, these two proteins dissociate, and the DNA polymerase continues processive elongation until replication of the nascent DNA strand is completed. Replication, which starts at both DNA ends, is coupled to strand displacement. This results in the generation of so-called type I replication intermediates consisting of full-length double-stranded \( \phi29 \) DNA molecules with one or more single-stranded DNA (ssDNA) branches of varying lengths. When the two converging DNA polymerases merge, a type I replication intermediate becomes physically separated into two type II replication intermediates. Each of these consists of a full-length \( \phi29 \) DNA molecule in which a portion of the DNA, starting from one end, is double-stranded, and the portion spanning to the other end is single-stranded.

Over the last decades convincing evidence has been presented that replication of bacterial genomes, including that of resident plasmids and infecting phages, occurs at the bacterial cell membrane (for review, see Ref. 7). Also, replication of \( \phi29 \) DNA takes place at the membrane of the infected cell (2, 8, 9). Gene 16.7, present in the early expressed operon located at the right side of the \( \phi29 \) genome (see Fig. 1A), encodes an integral membrane protein of 130 amino acids. The efficiency of in vivo \( \phi29 \) DNA replication is affected in the absence of protein p16.7 (10). In this work we analyzed the functional role of p16.7 in \( \phi29 \) DNA replication using purified p16.7A, a soluble variant of p16.7 lacking the N-terminal transmembrane-spanning domain. We found that protein p16.7A can functionally substitute the \( \phi29 \) SSB p5 in in vitro \( \phi29 \) DNA amplification assays,
suggested that it is a ssDNA-binding protein. This inference was further supported by direct assays such as electron microscopy and gel retardation studies. Thus, in addition to a classical SSB p5, $\phi 29$ encodes a membrane-localized ssDNA-binding protein. Contrary to the SSB p5, p16.7A has no helix-stabilizing activity, and p16.7 is not synthesized in stoichiometric amounts in infected cells. These and other results show that p16.7 and SSB p5 have non-overlapping functions. Based on the properties determined in this work together with the features described before, it is most likely that p16.7 attaches $\phi 29$ DNA to the membrane of the infected cells by binding to the stretches of ssDNA present in the replication intermediates.

MATERIALS AND METHODS

**Bacterial Strains, Bacteriophages, and Growth Conditions**—B. subtilis 110NA (trpC2, spoOA3, su’ (11)) was used as the non-suppressor strain for $\phi 29$ infections. Cells, grown at 37 °C in LB medium supplemented with 5 mM MgSO$_4$, were infected with phage $\phi 29$ mutant sus4/1242 (12) at a multiplicity of infection of 5. Phage $\phi 29$ sus4/1242 contains a suppressor-sensitive mutation in gene 14 that encodes the holin gene. As a consequence, cell lysis is delayed, which allowed determination of the amounts of p16.7 and SSB at late stages in the infection cycle. The mutation has no effect on phage DNA replication or phage morphogenesis and, therefore, is considered as wild-type phage in these studies. Escherichia coli strain JM109 (F $\alpha$ traD56 lacIq $\Delta$(lacZAM15 proA $^-$ B-lac $\mu$ ProcA $^-$ $\Delta$(lac-proAB) thi gyrA96 (Nalr) endA1 hsdR17 (r- $\mu$ K-) relA1 supE44 recA1) harboring plasmid pUSH16.7A (10) was used for overexpression of protein p16.7A.

**DNA Techniques**—All DNA manipulations were carried out according to Sambrook et al. (13). [γ-$^{32}$P]ATP and [γ-$^{32}$P]ATP (3000 Ci/mmol) were obtained from Amersham Biosciences, Inc. DNA fragments were isolated from agarose gels using the Qiaex gel extraction kit (Qiagen, Inc., Chatsworth, CA).

**PCR Techniques**—PCR reactions were carried out with proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, MA) using conditions as described before (10). Oligonucleotides were purchased from Isogen Bioscience BV (Maarsen, The Netherlands).

**Overexpression and Purification of p16.7A**—Protein p16.7A was overexpressed and purified using a Ni$^{2+}$-nitrilotriacetic acid resin column as described before (10). $\phi 29$ Protein-primed Initiation, TP-DNA Replication, and Amplification Assays—These assays were performed as described before (14). The reaction mixtures of the $\phi 29$ TP-DNA replication assays contained the indicated amount of protein p16.7A or SSB p5. The amplification assays were stopped after an incubation period of 20 min.

**Psoralen Cross-linking and Spreading of DNA Molecules for Electron Microscopy**—Replication reactions were carried out in the absence or in the presence of p16.7A or SSB p5 as described below. After 30 min at 30 °C the samples were stopped by adding 0.05 volumes of 4.5 M trimethylpsoralen (200 μg/ml in 100% ethanol) on ice. The samples were then irradiated with 366-nm UV light on ice for 1 h with 2 psoralen additions, as described by Sogo and Thoma (15). These cross-linking conditions were sufficient to produce essentially complete cross-linking of the DNA molecules in the absence of protein. After psoralen cross-linking, the samples were digested with proteinase K (500 μg/ml) for 2 h at 56 °C and extracted with phenol, and the DNA was precipitated with ethanol. Denaturation and spreading of the psoralen cross-linked DNA for electron microscopy were carried out according the BAC technique, as described by Sogo et al. (16). Electron micrographs were taken with a Philips 420 electron microscope at 80 kV, routinely at a magnification of 20,000-fold.

**Gel Mobility Shift Assays**—Unless stated otherwise, the incubation mixtures contained, in a final volume of 20 μl, 25 mM Heps, pH 7.5, 4% Ficoll 400, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 10 mM dithiothreitol, the indicated labeled DNA fragment, and the indicated amount of protein p16.7A or $\phi 29$ SSB p5. After incubation for 10 min at 4 °C, the samples were subjected to electrophoresis in 4% non-denaturing polyacrylamide (80:1) gels containing 12 mM Tris acetate, pH 7.5, and 1 mM EDTA and run at 4 °C using a running buffer containing 12 mM Tris acetate, pH 7.5, and 1 mM EDTA at 70 V for 6 h. Next, the gels were dried and autoradiographed.

**Glycerol Gradients**—Twenty μg of protein p16.7A was subjected to a 15–30% linear glycerol gradient containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 7 mM β-mercaptoethanol and run as described before (14). After fractionation of the gradient, aliquots of each fraction were analyzed by SDS-PAGE and gel retardation assays.

**Oligonucleotide Degradation and Helix Destabilization Assays**—Oligonucleotide degradation assays and the helix destabilization assays,
the latter using 125 ng of primed M13mp18 DNA, were carried out as described by Gascón et al. (17). Oligonucleotide 40 Universal and the same with a 5′ (5 thymidines) or a 3′ (5 adenines) extension were end-labeled with T4-polynucleotide kinase using [γ-32P]ATP for 1 h at 37°C.

Preparation of Crude Cell Extracts, Western Blot, and Quantification of p16.7A and SSB p5—To determine the intracellular levels of the viral proteins p16.7 and SSB throughout the course of the φ29 infection cycle, B. subtilis cells were infected with phage φ29 mutant sus14(1242) as described above. At different times after infection, 1.5-ml samples were withdrawn and processed as described before (10). Known amounts (ng) of the corresponding purified proteins (p16.7A or SSB p5) were run in the same gel to determine the standard curve. Polyclonal antisera from rabbits against φ29 p16.7 or against φ29 SSB p5 were diluted 2,500 and 1,000 times, respectively.

RESULTS

Protein p16.7A Has No Effect on In Vitro φ29 DNA Initiation of Replication—Previous results show that the absence of protein p16.7 affects the efficiency of in vivo φ29 DNA replication, especially at early infection times (10). Protein p16.7 might stimulate φ29 DNA replication by enhancing the rate-limiting step of initiation of DNA replication. To study this possibility in vitro, φ29 DNA replication initiation assays were performed in the absence or presence of increasing amounts of purified p16.7A. Although efficient initiation requires the presence of φ29 template TP-DNA, the DNA polymerase is also able to carry out the TP-deoxynucleotidylation reaction in the absence of TP-DNA (18). Therefore, in vitro φ29 DNA initiation experiments were performed in reaction mixtures either lacking or containing template TP-DNA and in the absence or presence of increasing amounts of protein p16.7A. No significant effect of p16.7A on the initiation reactions was obtained (not shown), indicating that p16.7A plays no role in the in vitro initiation of φ29 DNA replication.

Protein p16.7A Can Functionally Substitute the SSB p5 in φ29 DNA Amplification Assays—The possibility that p16.7A has a role in φ29 DNA replication after the initiation step was analyzed by studying the possible effects of protein p16.7A in an in vitro φ29 DNA amplification system. This system, which allows the amplification of very low amounts of φ29 template DNA, requires the following four φ29-encoded proteins: DNA polymerase, TP, double-stranded DNA-binding protein p6, and the SSB p5 (19). It has been described that omission of SSB p5 from the reaction mixtures results in the generation (19) and amplification (20) of short φ29 DNA products. Esteban et al. (20) demonstrated that the short φ29 DNA products are of palindromic nature and that they are caused by a DNA polymerase template-switching event during replication. Binding of SSB p5 to the displaced stretches of ssDNA present in type I DNA replication intermediates avoids the DNA polymerase to switch template, thus preventing the generation of short DNA products. Interestingly, we found that the addition of increasing amounts of protein p16.7A to reaction mixtures lacking SSB p5 resulted in the synthesis of increasing amounts of full-sized φ29 DNA and a concomitant decrease in the amounts of short DNA products (Fig. 2). In fact, the generation of the short DNA products was fully prevented when the reaction mixtures contained 14 or 28 μM protein p16.7A. These results show that, under the conditions tested, the presence of protein p16.7A prevents the DNA polymerase from switching template and, therefore, that it can functionally substitute the SSB p5. The most likely explanation for these results is that the p16.7A protein binds to the stretches of displaced ssDNA present in the type I replication intermediates. The amount of full-sized φ29 DNA synthesized in the presence of 14 or 28 μM protein p16.7A is slightly lower than that compared in the presence of 7 μM. Probably, this decrease is due to binding of protein p16.7A to double-stranded φ29 template DNA at these elevated p16.7A concentrations (see below), causing a small effect on the efficiency of the φ29 DNA amplification.

Protein p16.7A Protects the Displaced ssDNA from Psoralen Cross-linking—Psoralen can cross-link portions of a dsDNA molecule that are free of protein (15) as well as folded-back regions in ssDNA (21). The presence of the φ29 SSB p5 was shown to prevent the displaced ssDNA of the φ29 DNA replication intermediates from psoralen cross-linking, demonstrating that it binds to ssDNA (22–24). To study whether protein p16.7A indeed binds to the ssDNA portions of φ29 DNA replication intermediates, as indicated by the results of the φ29 DNA amplification assays described above, we used the psoralen cross-linking technique. Thus, φ29 DNA replication reactions, carried out in the absence or presence of either protein p16.7A or SSB p5, were treated with psoralen as described under “Materials and Methods.” Next, after the reaction products were treated with proteinase K, extracted with phenol, and purified, they were spread under denaturing conditions and analyzed by electron microscopy. As expected, in all three samples full-length dsDNA molecules with one or more ssDNA tails (type I) and full-length DNA molecules formed by a dsDNA portion of variable length from one DNA end plus an ssDNA portion spanning to the other DNA end (type II) were observed. A representative type I replication intermediate of each sample is shown in Fig. 3. As described before (22–24), the displaced ssDNA regions of replication intermediates produced in the absence (Fig. 3A) or the presence of SSB p5 (Fig. 3C) appeared as collapsed and well unfolded structures, respectively. Fig. 3B shows that the ssDNA portions in replication intermediates produced in the presence of protein p16.7A also had a well unfolded structure. These results, therefore, show that protein p16.7A, like the SSB p5, prevents the ssDNA from psoralen cross-linking, demonstrating that it binds to the ssDNA portions of the replication intermediates generated during φ29 DNA replication.

Protein p16.7A Has Higher Affinity for ssDNA Than the SSB p5—Binding of p16.7A to ssDNA was further analyzed by gel mobility shift assays. For this purpose, the 175-base pair right-
end fragment of the ϕ29 genome was end-labeled with $^{32}$P (see “Materials and Methods”), heat-denatured, and used in gel retardation assays in the absence or presence of increasing amounts of purified protein p16.7A. Although retardation of the ssDNA fragment was observed in the presence of 45 nM p16.7A, full retardation of all the ssDNA molecules required a p16.7A concentration of 360 nM (Fig. 4A). Similar results were obtained with various other DNA fragments (results not shown). To gain an insight in the global ssDNA binding of p16.7A compared with that of the well studied ϕ29 protein p5, the experiment shown in Fig. 4A was carried out in parallel using purified SSB p5. Whereas in agreement with previously published results (25–27), some retardation of part of the ssDNA molecules occurred in the presence of 3.3 μM SSB p5, full retardation of all the ssDNA molecules required a concentration of 13.3 μM (Fig. 4B). Together, these results show that the ssDNA binding activity of p16.7A is about 50 times higher than that of the ϕ29 SSB. To study possible binding of protein p16.7A to dsDNA, the same fragment of the ϕ29 genome used in Fig. 4, A and B, but in its double-stranded form, was used in gel retardation assays. The results, presented in Fig. 4C, show that p16.7A binds to dsDNA, although the amount of p16.7A required to obtain full retardation of all the dsDNA molecules was about 20-fold higher than that required to bind the same DNA molecules in their single-stranded form. The observation that a smear of retarded DNA species is observed at low p16.7A concentrations (45–180 and 180–720 nM for sa- and dsDNA, respectively) indicates that the nucleoprotein complex formed at these concentrations is rather unstable. In addition, the increasing mobility shift caused by the various concentrations of p16.7A protein analyzed suggests the binding of more than one protein molecule per DNA molecule. This inference is further supported by the findings that a considerable amount of protein p16.7A is required (i) to prevent the generation of short ssDNA products in amplification assays lacking the SSB p5 (see above) and (ii) to cover the complete circular M13 ssDNA in order to prevent binding of the ϕ29 DNA polymerase (see below).

To confirm that the observed retardation was due to protein p16.7A and not to a possible minor contaminant, the purified p16.7A protein was subjected to a glycerol gradient, after which aliquots of the gradient were analyzed for ssDNA binding by gel retardation. The results presented in Fig. 4D show that the ssDNA binding activity was restricted to those fractions that contained protein p16.7A.

Protein p16.7A Binds to Circular ssDNA—The following approach was used to study possible binding of p16.7A to circular ssDNA. The ϕ29 DNA polymerase has strong affinity for naked ssDNA (28) but does not bind ssDNA when it is covered with the SSB p5 (17, 23). In addition, free ϕ29 DNA polymerase, but not when bound to M13 ssDNA, can degrade a single-stranded oligonucleotide due to its 3′-5′ exonucleolytic activity. M13 DNA that is complexed with an ssDNA-binding protein, therefore, is unable to trap the ϕ29 DNA polymerase, which can be measured by the 3′-5′ exonucleolytic activity. Thus, ϕ29 DNA polymerase was added to either naked M13 ssDNA or the M13 ssDNA that was preincubated with increasing amounts of protein p16.7A. Then, 1 min after a 5′ labeled oligonucleotide was added to the mixtures, samples were analyzed for degradation of the oligonucleotide. The assays were carried out in parallel using SSB p5. Fig. 5 shows, as expected, that the oligonucleotide was degraded by the ϕ29 DNA polymerase when the M13 ssDNA trap was omitted (lane 2), but it was not degraded when the reaction mixtures contained naked M13 ssDNA (lane 3). In agreement with previously published results (17), preincubation of the M13 ssDNA with increasing amounts of SSB p5 resulted in increasing levels of degradation of the oligonucleotide (lanes 4–7). Similar results were obtained when the M13 ssDNA had been preincubated with protein p16.7A (lanes 9–12). The oligonucleotide was not degraded when it was only incubated with the highest concentration of SSB p5 or p16.7A (lanes 8 and 13, respectively). This excludes the possibility that the observed degradation of the oligonucleotide would be the consequence of a contaminant exonuclease in the purified protein preparations. These results, therefore, indicate that protein p16.7A binds to circular M13 ssDNA and that this prevents it from ϕ29 DNA polymerase binding.

Protein p16.7A Has No Helix-destabilizing Activity and Has No Stimulatory Effect On in Vitro ϕ29 TP-DNA Replication—Possible helix destabilization activity of protein p16.7A was studied by its ability to displace a 5′-labeled 17-mer oligonucleotide hybridized to its complementary sequence in circular
M13 ssDNA molecules. This substrate was incubated without or with increasing amounts of protein p16.7A, after which the samples were analyzed by polyacrylamide gel electrophoresis. The experiments were carried out in parallel using \( \phi 29 \) SSB p5. As shown in Fig. 6, incubation in the absence of protein did not result in release of the labeled oligonucleotide, indicating that the hybrid substrate was stable throughout the experiment. In agreement with previously published results (17, 24), the \( \phi 29 \) SSB p5 was able to displace the labeled oligonucleotide from M13 DNA. However, no displacement of the oligonucleotide was detected when the hybrid substrate was incubated with protein p16.7A up to a concentration of 112 \( \mu \text{M} \). To facilitate the oligonucleotide displacement, these assays were also performed using an oligonucleotide that contained, in addition to the 17 complementary nucleotides, either a non-complementary 5'-(5 thymidines) or a 3'-(5 adenines) tail. Contrary to the SSB p5, protein p16.7A was also unable to displace these oligonucleotides (results not shown). Because protein p16.7A is able to bind M13 ssDNA (see above), these results strongly suggest that protein p16.7A has no helix-stabilizing activity.

The \( \phi 29 \)-encoded TP and the DNA polymerase are the only two proteins required in a minimal in vitro \( \phi 29 \) DNA replication system (29). Contrary to the \( \phi 29 \) DNA amplification system, the minimal replication system requires high concentrations of template TP-DNA and is limited to one or two rounds of \( \phi 29 \) DNA replication. To study a possible effect of protein...
p16.7A in this system, in vitro φ29 DNA replication assays were performed in the absence or presence of protein p16.7A using the SSB p5 as a control. The amount of incorporated [α-32P]dTTP was determined (Fig. 7A), after which the samples were subjected to alkaline-agarose gel electrophoresis to determine the size of the synthesized DNA (Fig. 7B). As reported previously (23, 25), the SSB p5 stimulated the in vitro φ29 DNA replication, especially at long incubation times. However, no stimulatory effect on replication was observed in the presence of protein p16.7A in this assay.

Relative Amounts of Protein p16.7 and SSB p5 Synthesized during the Infection Cycle—The intracellular amount of protein p16.7 accumulated in B. subtilis culture was determined before by quantitative immunoblotting (10). Although it has been described that the SSB p5 is one of the most abundantly expressed φ29-encoded proteins in infected cells (25), a kinetic analysis of the accumulation of the SSB p5 during the infection cycle had not been performed. We were especially interested in the ratio between p16.7 and SSB p5 molecules per infected cell at the various times analyzed (Fig. 8B). Whereas an infected cell contained a maximum of about 180,000 molecules of p16.7, the SSB p5 reached levels up to 2 × 10^5. Fig. 8C shows that at 6 min after infection, the ratio between the SSB p5 and protein p16.7 is about 2, but this value increased rapidly during the next 10 min of infection to a value of about 12.

**DISCUSSION**

p16.7 Is a Membrane-localized, Dimeric, ssDNA-binding Protein—Here we demonstrated by functional and direct analysis that protein p16.7A has high ssDNA binding activity and that it binds ssDNA without an apparent sequence preference. Thus, protein p16.7A is an ssDNA-binding protein, and hence, the genome of φ29 encodes for two ssDNA-binding proteins, protein p16.7 and SSB p5. Previously, we demonstrated that protein p16.7A forms dimers in solution, and in vivo cross-linking experiments suggested that native p16.7 also exists as a dimer in infected cells (10). In addition, we demonstrated that the native protein p16.7 is an integral membrane protein and...
that the N-terminal region of the protein, constituting a trans-membrane-spanning domain, is responsible for its membrane localization (2, 10). Together with the results obtained in this work, we conclude that the native protein p16.7 is a membrane-localized, dimeric, ssDNA-binding protein. To the best of our knowledge this is the first prokaryotic protein described with such characteristics.

**φ29 Encodes for Two ssDNA-binding Proteins with Different Characteristics**—In most systems SSBs stimulate DNA replication (for reviews, see Refs. 1, 30, and 31). This stimulation is the result of direct and/or indirect effects that SSBs have on DNA replication. The direct effect, reported for several SSBs, involves specific interactions between the SSB and its cognate DNA polymerase. The indirect effect can be the consequence of one or more of the following mechanisms. First, binding of the SSB may help to load the helicase (for reviews, see Refs. 32 and 33). Second, upon binding, SSBs protect ssDNA from nuclease degradation (25, 26). Third, it prevents non-productive binding of the DNA polymerase to ssDNA (17, 23). Fourth, it prevents and removes the formation of DNA secondary structures (23, 24). And finally, it is required in in vitro DNA amplification assays when low amounts of template TP-DNA are used (19).

Here we demonstrated that protein p16.7A has high ssDNA binding activity. Contrary to SSB p5, however, protein p16.7A has no helix destabilization activity, and its presence in the minimal in vitro DNA replication assay did not stimulate DNA replication. These results indicate that, although both proteins bind ssDNA, they have non-overlapping functions. This view is further supported by the following results. First, the SSB p5 is essential for in vivo DNA replication (36), indicating that protein p16.7 cannot replace all of the functions of the SSB p5. Second, although both proteins are expressed early after infection, much lower amounts of protein p16.7 are synthesized than the SSB p5 during the middle and late stages of phage infection. The rapid amplification of the φ29 genome during the middle stage of the infection cycle generates large amounts of ssDNA. The huge increase of SSB p5 synthesis during this infection stage is in agreement with the view that
the SSB p5, as most other SSBs, is required in stoichiometric amounts.

Model of the Function of Protein p16.7 in in Vivo $\phi 29$ DNA Replication—As a consequence of its protein-primed mechanism of replication and the absence of lagging strand synthesis, the $\phi 29$ DNA replication intermediates contain very long stretches (up to $>10,000$ nucleotides in length) of displaced ssDNA (see Fig. 1B). Binding of protein p16.7A to these stretches of ssDNA was demonstrated directly by electron microscopy analysis. Also, the observation that protein p16.7A prevented the accumulation of short DNA products in the in vitro $\phi 29$ DNA amplification assays lacking SSB p5 strongly indicates that it binds to these stretches of ssDNA. Importantly, these latter results also show that the binding of p16.7A to the stretches of ssDNA hardly interfered with DNA replication in the dynamic $\phi 29$ DNA amplification system. Rather, under these conditions, protein p16.7A had a positive effect on the efficiency of full-length $\phi 29$ DNA synthesis. Based on these results, the characteristics of p16.7A described above, and taking into account that native protein p16.7 is membrane-localized (10), we propose, as schematically presented in Fig. 9, that the principal role of p16.7 in $\phi 29$ DNA replication involves the attachment of replicating $\phi 29$ DNA molecules to the membrane of infected cells. Although not shown in the model, it is possible that both protein p16.7 and SSB p5 will be bound simultaneously to the ssDNA portions of the $\phi 29$ DNA replication intermediates under natural conditions. The percentage of the $\phi 29$ ssDNA portions that is bound by either protein probably varies during the infection cycle because of the different expression patterns of these proteins. Nevertheless, based on the relative ssDNA binding activities, it is most likely that p16.7 binds more rapidly to a displaced ssDNA strand of a $\phi 29$ replication intermediate than the SSB p5, especially at early infection times when the amount of p16.7 in the infected cell, compared with the SSB p5, is relatively high. Interestingly, in vivo $\phi 29$ DNA replication is affected, especially at early infection times in the absence of protein p16.7 (10), indicating that it has an important role during early infection times. Attachment of $\phi 29$ DNA replication intermediates to the membrane by protein p16.7 will result in compartmentalization of the phage DNA within the infected cell. Besides a possible role of p16.7 in the organization of in vivo $\phi 29$ DNA replication, compartmentalization of replicating $\phi 29$ DNA probably stimulates in vivo $\phi 29$ DNA replication directly.

The proposed role of p16.7 in in vivo $\phi 29$ DNA replication may explain several observations made by Ivarie and Pène (8), who demonstrated for the first time that $\phi 29$ DNA replication occurs at the membrane of infected cells. These authors showed (i) that it took at least 10 min before the parental infected $\phi 29$ DNA molecules became attached to the membrane, (ii) that
Membrane Protein p16.7 Is an ssDNA Binding Protein

Gene 16.7 Is Conserved in \(\phi 29\)-related Phages—The generation of replication intermediates containing large stretches of displaced ssDNA is a characteristic feature of the protein-primed mechanism of DNA replication. Thus, it is conceivable that gene 16.7 is conserved in the genome of other phages that replicate by the protein-primed mechanism. Phage \(\phi 29\) is the paradigm of a family of \textit{Bacillus} phages that all use the protein-primed mechanism of DNA replication (for review, see Ref. 6). These \(\phi 29\)-like phages can be divided into three groups. The first group includes, in addition to \(\phi 29\), phages PZA, \(\phi 15\), and BS32. The second group comprises B103, Nf, and M2Y (M2), and the third, most distantly related group, contains GA-1 as its sole member. Except for phages Nf and M2Y, the DNA sequences of the right part of these phage genomes are available. Analysis of these DNA sequences revealed that they all contain an open reading frame whose deduced protein sequence (ranging from 130 to 133 amino acids) shares significant similarity to that of the \(\phi 29\) protein p16.7. In addition, Western blot analysis using polyclonal antibodies against p16.7 of \(\phi 29\) provided evidence that phage Nf (group II) encodes a p16.7 homologue (10). Together, these data show that gene 16.7 is conserved in most and probably all \(\phi 29\)-related phages, sup-

early expressed viral protein(s) was required for membrane association, (iii) that replicating \(\phi 29\) DNA molecules were attached to the membrane and that the fully replicated double-stranded \(\phi 29\) DNA molecules were first released from the membrane before they were packaged into the phage particles, and (iv) that phage DNA of a \(\phi 29\) mutant containing a temperature-sensitive mutation in gene 2 (later shown to encode the DNA polymerase) did not become associated to the membrane when infected at the non-permissive temperature. Replication of \(\phi 29\) DNA does not start until about 10 min after infection (2, 8), implying that until this time no ssDNA-containing replication intermediates are present in the infected cell. This explains why the infecting \(\phi 29\) DNA molecules, present in its double-stranded form, are not associated to the membrane at the very early infection times even though p16.7 is already detected as soon as 6 min after infection (this work and Ref. 10). Also the observation that the replicating DNA molecules, but not the fully replicated phage DNA molecules, are attached to the membrane is in agreement with the proposed role of protein p16.7. By immunofluorescence microscopy we have in fact confirmed that the majority of the replicated double-stranded \(\phi 29\) DNA molecules present at late infection times were no longer localized at the membrane. Rather, these molecules were found within the bulk of the host nucleoid (2). Finally, although the possibility, proposed by Ivarie and Pène (8), that the \(\phi 29\) gene 2 product, the DNA polymerase, could be directly involved in attachment of replicating \(\phi 29\) DNA to the membrane still holds, another perhaps more likely explanation can be considered. Replication will not occur in the absence of a functional DNA polymerase. As a consequence, the infecting DNA, despite the synthesis of protein p16.7 and other viral proteins, will remain double-stranded, which explains why it did not become attached to the membrane.

By immunofluorescence microscopy it was shown that the first rounds of \(\phi 29\) DNA replication localized nearly always to the distal end of the bacterial nucleoid both in the presence or absence of protein p16.7. However, whereas a few minutes later, phage DNA replication was found to occur at multiple sites at the membrane in the wild-type situation, phage DNA replication remained restricted to the initial replication site for a prolonged time in the absence of p16.7. These results indicated that protein p16.7 is involved in the efficient distribution of replicating phage DNA from its initial to additional replication sites in the infected cell. During the middle stage of infection protein p16.7 localizes throughout the membrane, and this pattern is consistent with the localization of the viral DNA at these times (2). This may suggest that the \textit{in vivo} distribution of phage DNA is a passive process due to the dispersed p16.7 localization. However, active distribution of phage DNA within the infected cell cannot be ruled out.

**FIG. 9. Model of the p16.7-mediated attachment of the \(\phi 29\) DNA replication intermediates to the membrane of the infected cell.** The membrane bilayer, protein p16.7 dimer, and \(\phi 29\) DNA replication intermediate type I (A) and type II (B) are indicated (see "Discussion" for details).
porting the view that the proposed mechanism of p16.7-mediated attachment of replicating phage DNA to the membrane of the infected cell is conserved in all these phages.

In summary, we found that the integral membrane protein p16.7 of φ29 is an ssDNA-binding protein. Based on the results reported here and those reported previously (2, 10), it is most probable that the principal role of p16.7 involves the attachment of replicating φ29 DNA to the membrane of the infected cell, resulting in the efficient distribution of replicating φ29 DNA from its initial to additional replication sites. Probably, this mechanism is conserved in all members of the φ29 family of phages. As such, these results are an important contribution to the better understanding of protein-primed φ29 DNA replication.

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REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) DNA Replication, W. H. Freeman and Co., San Francisco
2. Meijer, W. J. J., Lewis, P. J., Errington, J., and Salas, M. (2000) EMBO J. 19, 4182–4190
3. Salas, M., and Rojo, F. (1993) in Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sørensen, A. L., Hoch, J. A., and Losick, R., eds) pp. 843–858, American Society for Microbiology, Washington, D. C.
4. Salas, M., Miller, J. T., Leis, J., and DePamphilis, M. L. (1996) in DNA Replication in Eukaryotic Cells (DePamphilis, M. L., ed) pp. 131–176, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
5. Rojo, F., Menéndez, M., Monsalve, M., and Salas, M. (1998) Prog. Nucleic Acid Res. Mol. Biol. 60, 29–46
6. Meijer, W. J. J., Horcajadas, J. A., and Salas, M. (2001) Microbiol. Mol. Biol. Rev. 65, 261–287
7. Firestein, W. (1989) Annu. Rev. Microbiol. 43, 89–120
8. Ivarie, R. D., and Pène, J. J. (1973) Virology 52, 351–362
9. Bravo, A., and Salas, M. (1997) J. Mol. Biol. 269, 102–112
10. Meijer, W. J. J., Serna-Rico, A., and Salas, M. (2001) Mol. Microbiol. 39, 731–746
11. Moreno, F., Camacho, A., Viñuela, E., and Salas, M. (1974) Virology 62, 1–16
12. Jiménez, F., Camacho, A., de la Torre, J., Viñuela, E., and Salas, M. (1977) Eur. J. Biochem. 73, 57–72
13. Sogo, J. M., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Serna-Rico, A., Illana, B., Salas, M., and Meijer, W. J. J. (2000) J. Biol. Chem. 275, 40529–40538
15. Sogo, J. M., and Thomas, F. (1989) Methods Enzymol. 170, 142–165
16. Sogo, J. M., Stasiak, A., DeBernadin, W., Losa, R., and Keller, T. (1987) in Electron Microscopy in Molecular Biology. A Practical Approach (Sommerville, J., and Scheer, U., eds) pp. 61–79, IRL Press at Oxford University Press, Oxford
17. Gascón, I., Lázaro, J. M., and Salas, M. (2000) Nucleic Acids Res. 28, 2034–2042
18. Blanco, L., Bernad, A., Esteban, J. A., and Salas, M. (1992) J. Biol. Chem. 267, 1225–1230
19. Blanco, L., Lázaro, J. M., de Vega, M., Bonnin, A., and Salas, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12196–12202
20. Esteban, J. A., Blanco, L., Villar, L., and Salas, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2921–2926
21. Wollenzen, P. L. (1988) in Psoralen-DNA Photobiology (Gasparro, F. P., ed) pp. 51–85, CRC Press, Inc., Boca Raton, FL
22. Gutiérrez, C., Sogo, J. M., and Salas, M. (1991) J. Mol. Biol. 222, 983–994
23. Gutiérrez, C., Martin, G., Sogo, J. M., and Salas, M. (1991) J. Biol. Chem. 266, 2104–2111
24. Soengas, M. S., Gutiérrez, C., and Salas, M. (1995) J. Mol. Biol. 253, 517–529
25. Martin, G., Lázaro, J. M., Méndez, E., and Salas, M. (1989) Nucleic Acids Res. 17, 3663–3672
26. Soengas, M. S., Esteban, J. A., Salas, M., and Gutiérrez, C. (1994) J. Mol. Biol. 239, 213–226
27. Gascón, I., Gutiérrez, C., and Salas, M. (2000) J. Mol. Biol. 296, 989–999
28. Blanco, L., Bernad, A., Lázaro, J. M., Martin, G., Garimendia, C., and Salas, M. (1989) J. Biol. Chem. 264, 8935–8940
29. Blanco, L., and Salas, M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6404–6408
30. Meyer, R. R., and Laine, P. S. (1990) Microbiol. Rev. 54, 342–380
31. Chase, J. W., and Williams, K. R. (1988) Annu. Rev. Biochem. 51, 133–136
32. Kelmam, Z., and O’Donnell, M. (1994)Curr. Opin. Genet. Dev. 4, 185–189
33. Stillman, B. (1994) Cell 78, 725–728
34. Salas, M. (1991) Annu. Rev. Biochem. 60, 39–71
35. Salas, M., Freire, R., Soengas, M. S., Esteban, J. A., Méndez, J., Bravo, A., Serrano, M., Blasco, M. A., Lázaro, J. M., Blanco, L., Gutiérrez, C., and Hermoso, J. M. (1995) FEMS Microbiol. Rev. 17, 73–82
36. Mellado, R. P., Peñalva, M. A., Inciarte, M. R., and Salas, M. (1980) Virology 104, 64–96
37. Mellado, R. P., Moreno, F., Viñuela, E., Salas, M., Reilly, B. E., and Anderson, D. L. (1976) J. Virol. 19, 495–500