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Compartmentalization of phage ϕ29 DNA replication: interaction between the primer terminal protein and the membrane-associated protein p1

Alicia Bravo, Belén Illana[1] and Margarita Salas[2]

Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

[1]Present address: EnWare SA (Grupo ABS), Nuñez de Balboa 51, 28001 Madrid, Spain

[2]Corresponding author
e-mail: msalas@cbm.uam.es

The bacteriophage ϕ29 replication protein p1 (85 amino acids) is membrane associated in *Bacillus subtilis*-infected cells. The C-terminal 52 amino acid residues of p1 are sufficient for assembly into protofilament sheet structures. Using chemical cross-linking experiments, we demonstrate here that p1ΔC43, a C-terminally truncated p1 protein that neither associates with membranes *in vivo* nor self-interacts *in vitro*, can interact with the primer terminal protein (TP) *in vitro*. Like protein p1, plasmid-encoded protein p1ΔC43 reduces the rate of ϕ29 DNA replication *in vivo* in a dosage-dependent manner. We also show that truncated p1 proteins that retain the N-terminal 42 amino acids, when present in excess, interfere with the *in vitro* formation of the TP·dAMP initiation complex in a reaction that depends on the efficient formation of a primer TP–ϕ29 DNA polymerase heterodimer. This interference is suppressed by increasing the concentration of either primer TP or ϕ29 DNA polymerase. We propose a model for initiation of *in vivo* ϕ29 DNA replication in which the viral replisome attaches to a membrane-associated p1-based structure.

Keywords: compartmentalization/protein–protein interactions/viral DNA replication

Introduction

Studies with eukaryotic cells have shown that DNA replication occurs at numerous fixed locations within the nucleus. Each site constitutes a replication factory containing many polymerizing machines working on different templates (Newport and Yan, 1996). Replication of adenovirus, a linear DNA genome with a terminal protein (TP) bound to the ends, also takes place at distinct subnuclear sites (Pombo *et al*., 1994). In this case, the nuclear matrix is thought to function as a scaffold to which the different replication proteins are recruited (for review see de Jong and van der Vliet, 1999). All eukaryotic positive-stranded RNA viruses appear to utilize intracellular membranes as a site of replication. Membranes appear to function not just as a way of compartmentalizing virus RNA replication, but also appear to have a central role in the organization and function of the replication complex (Buck, 1996; Ahola *et al*., 1999).

In the bacterium *Bacillus subtilis*, the machinery for replicating DNA, as well as the transcriptional and translational machinery, have specific subcellular localizations (Lemon and Grossman, 1998; Lewis *et al*., 2000). Direct visualization of the replicative DNA polymerase in living *B.subtilis* cells has revealed that it is localized at discrete intracellular positions, predominantly at or near midcell, rather than being randomly distributed along the nucleoid mass (Lemon and Grossman, 1998). This finding supports the idea that in bacteria, as in eukaryotes, the replisome remains static by becoming attached to larger structures, and DNA is pulled through (Cook, 1999).

The genome of the *B.subtilis* bacteriophage ϕ29 consists of a linear double-stranded DNA molecule with a TP covalently linked to each 5′ end (TP-DNA). Recently, it has been reported that phage ϕ29 DNA replication occurs at sites different from the host cell replication factories (Meijer *et al*., 2000). At the onset of replication, ϕ29 DNA nearly always localizes as a single focus towards one end of the host cell nucleoid. Later on, phage replication is redistributed to multiple sites around the nucleoid periphery. The early viral protein p16.7 is thought to be involved in the spatial redistribution of phage DNA replication sites (Meijer *et al*., 2000). Furthermore, cell fractionation studies have shown that parental viral DNA, newly synthesized ϕ29 DNA molecules and some viral replication proteins are membrane associated in infected cells, suggesting an essential role of the bacterial membrane for ϕ29 DNA replication (Ivarie and Pène, 1973; Bravo and Salas, 1997; W.J.J.Meijer, A.Serna-Rico and M.Salas, submitted). Despite these observations, little is known of the molecular basis for the compartmentalization of ϕ29 DNA replication. Experiments performed in the 1970s (Ivarie and Pène, 1973) indicated that association of parental ϕ29 DNA with the bacterial membrane is mediated by early viral-encoded proteins. Subsequently, we found that the viral protein p1, which is expressed early after infection, behaves as a membrane-associated protein during ϕ29 DNA replication. Membrane association of protein p1 also occurs in the absence of other viral components, suggesting that protein p1 contacts the bacterial membrane directly (Bravo and Salas, 1997). This small protein (85 amino acids) affects the rate of ϕ29 DNA replication *in vivo* in a temperature-dependent manner (Bravo and Salas, 1998). These features, together with its capacity to self-interact *in vitro*, generating highly ordered structures, led us to propose that protein p1 could assemble *in vivo* to form a viral structure in close association with the bacterial membrane (Bravo and Salas, 1998). This membrane-associated p1-based structure might provide a site for recruitment of viral replication proteins before the initiation of ϕ29 DNA synthesis by a protein-priming mechanism (for review see Salas *et al*., 1996). In fact, when initiation of *in vivo* viral DNA

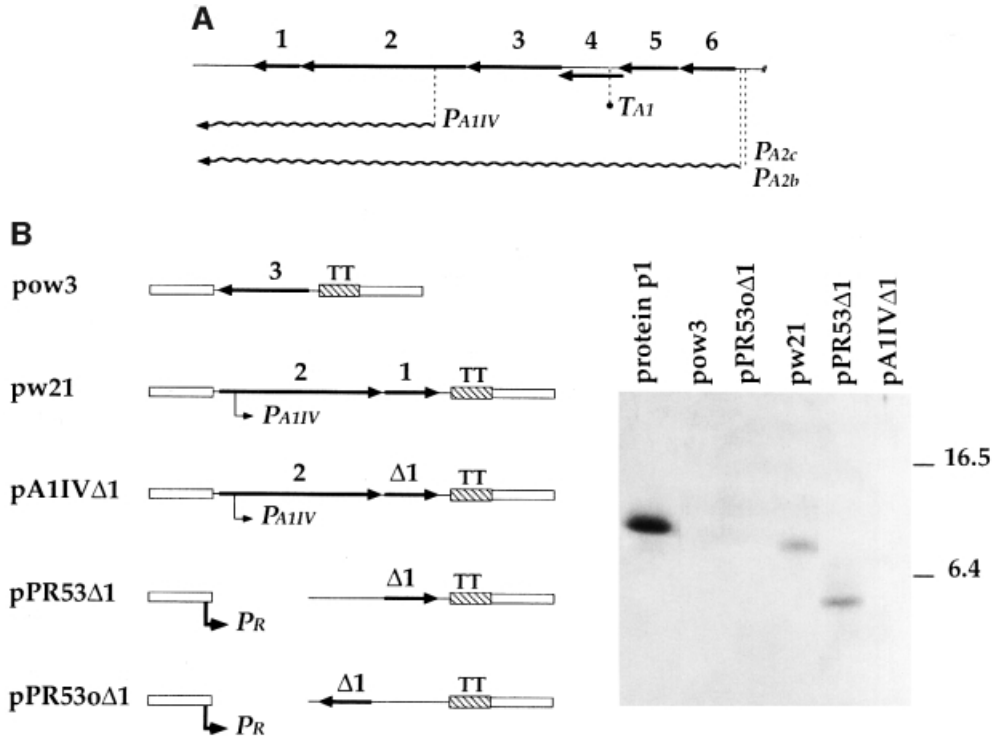


Fig. 1. (**A**) Genetic and transcriptional map of the left end of the phage ϕ29 genome. Only relevant features are shown. The position and approximate length of each gene are indicated. Genes 6 (encoding protein p6), 5 (single-stranded DNA-binding protein), 3 (terminal protein), 2 (DNA polymerase) and 1 (protein p1) are involved in viral DNA replication. Gene 4 encodes protein p4, which activates late transcription. Transcripts from the different promoters are shown. A significant number of transcripts starting from the main early promoters *PA2b* and *PA2c* terminate at the transcription terminator *TA1* (reviewed by Salas and Rojo, 1993). (**B**) Left: partial genetic map of plasmids carrying different ϕ29 DNA sequences. Maps are not drawn to scale. ϕ29 genes 3 (encoding TP), 2 (DNA polymerase) and 1 (protein p1) are indicated. Gene Δ1 has a deletion of one nucleotide, which leads to a truncated p1 protein of 45 amino acids, conserving the 42 N-terminal residues (protein p1ΔC43) (Bravo and Salas, 1997). The position of the promoters *PA1IV* and *PR* is shown. Open boxes indicate pUB110 DNA (McKenzie *et al*., 1986). The hatched box represents a DNA fragment containing the transcriptional termination sites (TT) of the *E.coli rrn*B rRNA operon (Brosius *et al*., 1981). Right: immunoblot analysis of whole-cell extracts from plasmid-containing *B.subtilis* 110NA cells using anti-p1 serum. Equivalent amounts of the cell extracts were loaded on to the gel. Purified protein p1 was run in the same gel. The molecular weight of pre-stained proteins used as markers is indicated in kilodaltons.

replication is blocked due to the lack of the viral protein p6 (histone-like protein), large amounts of ϕ29 DNA polymerase are recovered in membrane fractions together with protein p1 and free TP, which acts as a primer (Bravo and Salas, 1997). Nevertheless, full understanding of the function of protein p1 requires a detailed knowledge of the molecular interactions between protein p1 and other viral replication components.

In this report, we demonstrate that the N-terminal 42 amino acid residues of protein p1 are sufficient for binding to the primer TP *in vitro*. In addition, we show that an excess of protein p1 at the beginning of the infective process reduces the rate of ϕ29 DNA synthesis. The function of protein p1 involved in this *in vivo* interference has also been located within the N-terminal 42 amino acid region. Truncated p1 proteins that retain the N-terminal 42 amino acids, when present in excess, interfere with the *in vitro* formation of the TP·dAMP initiation complex in a reaction that depends on the efficient formation of a primer TP–ϕ29 DNA polymerase heterodimer. This *in vitro* interference can be explained as competition between the truncated p1 protein and the ϕ29 DNA polymerase for binding to the primer TP. A model for the initiation of *in vivo* ϕ29 DNA replication in which the viral replisome attaches to a membrane-associated p1-based structure is discussed.

Results

Plasmid-encoded protein p1 specifically decreases the rate of ϕ29 DNA synthesis

The gene encoding protein p1 (gene 1) is located at the left end of the ϕ29 genome, adjacent to gene 2, which encodes the viral DNA polymerase (Figure 1A). Gene 1 is mainly transcribed from two strong early promoters, *PA2b* and *PA2c*, in a polycistronic RNA, which contains other replication genes (for review see Salas and Rojo, 1993). In addition, *in vivo* S1 mapping experiments revealed the existence of a weak and early transcription initiation site within the DNA polymerase coding region (Barthelemy *et al*., 1986). This transcription initiation site is close to the reported *Escherichia coli* RNA polymerase binding site, A1IV (Sogo *et al*., 1984). These findings suggest that gene 1 could also be transcribed from the weak promoter *PA1IV* (Figure 1A). To construct a protein p1-producing *B.subtilis* strain, plasmid pw21, which carries gene 1 and the *PA1IV* promoter, was introduced into *B.subtilis* 110NA cells (Figure 1B). To find out whether such a strain synthesizes protein p1, whole-cell extracts were prepared and total proteins were analyzed by immunoblot techniques using polyclonal antibodies against protein p1. As a control, extracts from cells containing plasmid pow3, which lacks gene 1, were analyzed. As shown in Figure 1B,

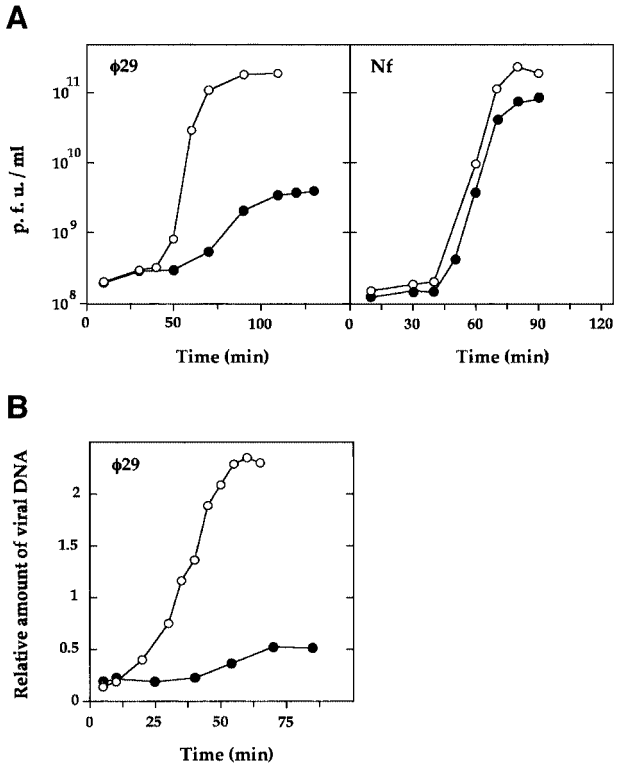


Fig. 2. *In vivo* interference mediated by protein p1. (**A**) Development of ϕ29 and Nf phages in *B.subtilis* 110NA cells carrying plasmid pow3 (open circles) or pw21 (filled circles). The production of infective particles (measured as p.f.u./ml) was determined by plating on 110NA cells without plasmid. (**B**) Time course of the accumulation of ϕ29 DNA in the indicated strains under one-step phage growth conditions.

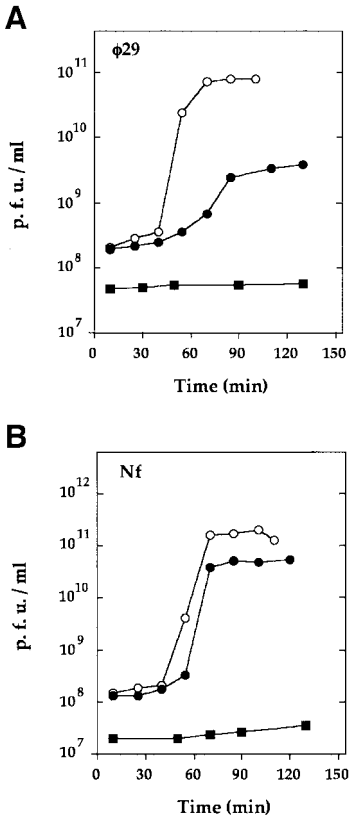


Fig. 3. *In vivo* interference mediated by protein p1ΔC43. (**A**) Phage ϕ29 development under one-step conditions in *B.subtilis* 110NA cells carrying plasmid pPR53oΔ1 (open circles), pA1IVΔ1 (filled circles) or pPR53Δ1 (filled squares). (**B**) Phage Nf development under one-step conditions in the same strains.

protein p1 was only detected in plasmid pw21-containing cells.

Preliminary experiments showed that the plating efficiency of phage ϕ29 on *B.subtilis* cells containing plasmid pw21 (protein p1-producing cells) was much less than that obtained on plasmid pow3-carrying cells. Phage ϕ29 formed very tiny plaques on protein p1-producing cells (not shown). These observations suggest that plasmid-encoded protein p1 interfered with phage ϕ29 growth. To get a more accurate measurement of this interference, phage development experiments under one-step growth conditions were performed (Figure 2A). To this end, cells producing or not producing protein p1 were infected with phage ϕ29 or the related phage Nf. When protein p1-producing cells (plasmid pw21) were infected with phage ϕ29, a viral yield of ~40 phages/cell was obtained. This production was 45-fold lower than that obtained in cells not producing protein p1 (plasmid pow3). Interestingly, no reduction in viral production was observed when protein p1-producing cells (plasmid pw21) were infected with phage Nf, indicating that the amount of protein p1 provided by plasmid pw21 specifically interfered with phage ϕ29 growth.

The interference phenotype was further analyzed by measuring synthesis of viral DNA under one-step growth conditions (Figure 2B). In this experiment, cells producing or not producing protein p1 were infected with phage ϕ29 and, at various times after infection, total intracellular DNA was isolated and analyzed by agarose gel electrophoresis. In protein p1-producing cells (plasmid pw21), phage DNA replication was markedly delayed and the amount of viral DNA accumulated in 55 min was much lower than that accumulated in cells not producing protein p1 (plasmid pow3).

Collectively, the above results suggest that intracellular accumulation of protein p1 before ϕ29 infection specifically decreases the rate of viral DNA synthesis and, as a consequence, viral production is reduced.

The N-terminal 42 amino acid residues of protein p1 are sufficient for in vivo interference

In contrast to protein p1, a truncated p1 protein that lacks the C-terminal 43 amino acids (p1ΔC43) does not behave like a membrane-associated protein *in vivo* (Bravo and Salas, 1997). It cannot self-interact in solution, as determined by sedimentation through glycerol gradients (see below). To investigate whether protein p1ΔC43 was still able to interfere with phage ϕ29 growth, we constructed plasmids pA1IVΔ1 and pPR53Δ1 (Figure 1B). In these plasmids, the gene encoding p1ΔC43 (gene Δ1) would be transcribed from the weak promoter *PA1IV* or from the strong promoter *PR*, respectively. As a control, plasmid pPR53oΔ1, which carries gene Δ1 inserted in the opposite orientation to the *PR* promoter, was used. Extracts from *B.subtilis* cells carrying the different plasmids were prepared, and total proteins were analyzed by immunoblot techniques using anti-p1 polyclonal antibodies. In cells

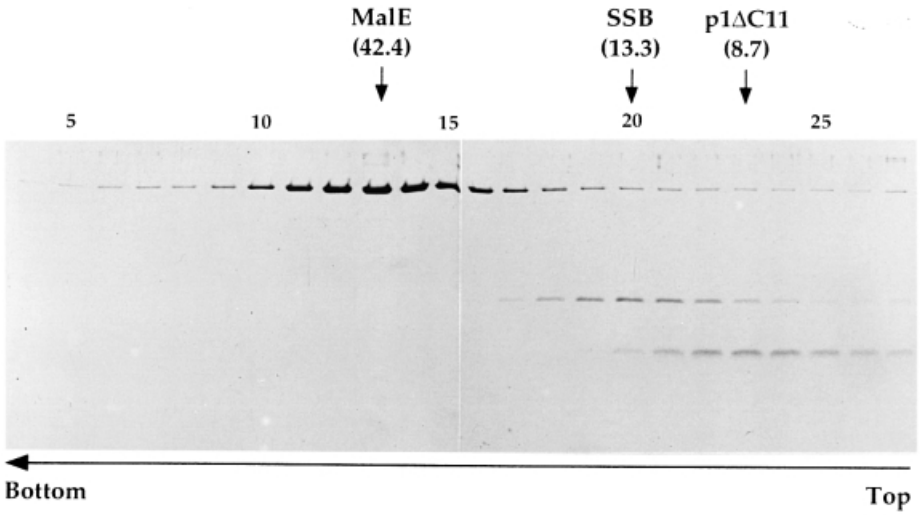


Fig. 4. Oligomeric state of protein p1ΔC11 in solution. The oligomeric state of p1ΔC11 was studied by sedimentation through glycerol gradients. A MalE-p1ΔC11 protein preparation (21 μM) was completely digested with protease factor Xa at a w/w ratio of 2% the amount of fusion protein. The reaction mixture was incubated at 4°C. This protease cleaves after the sequence IEGR, which separates the MalE (42.4 kDa) and p1ΔC11 (8.7 kDa) domains. Two hundred microliters of the totally digested preparation were loaded on to a glycerol gradient (15–30% in 20 mM Tris–HCl pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol) and subjected to centrifugation at 62 000 r.p.m. and 4°C in a Beckman SW65 rotor for 22 h. As a marker, ϕ29 single-stranded DNA-binding protein (SSB; 13.3 kDa), which behaves as a monomer in solution (Soengas *et al*., 1994), was loaded in the same gradient. After fractionation of the gradient, aliquots from each fraction were analyzed by SDS–Tricine–PAGE (Schagger and Jagow, 1987). The gel was stained with Coomassie Blue. The fraction at which the maximal amount of each protein appears is indicated. The molecular weight of the proteins is indicated in kilodaltons.

containing plasmid pPR53Δ1, a polypeptide migrating more quickly than protein p1 (plasmid pw21) and with the mobility expected for protein p1ΔC43 (5.1 kDa) was detected (Figure 1B). This product was not detected in cells carrying pA1IVΔ1 or pPR53oΔ1, even when more extract or different blotting conditions were used. However, phage development experiments performed with the different strains (see below) indicated that cells carrying plasmid pA1IVΔ1 synthesize protein p1ΔC43, although less of it than plasmid pPR53Δ1-containing cells. Since gene Δ1 in plasmid pA1IVΔ1 is transcribed from the *PA1IV* promoter, as is the case for gene 1 in plasmid pw21 (see Figure 1B), we assume that these two genes are similarly transcribed. Therefore, the lack of detection of protein p1ΔC43 in cells carrying plasmid pA1IVΔ1, but not of protein p1 in pw21-containing cells, suggests that the stability of the truncated p1 protein could be lower than that of the intact protein and/or that the anti-p1 antibodies could react less efficiently with the truncated p1 protein.

To study the effect of plasmid-encoded protein p1ΔC43 on ϕ29 development, cells carrying plasmid pA1IVΔ1 or plasmid pPR53Δ1 were infected with phage ϕ29 under one-step growth conditions (Figure 3A). In cells containing pA1IVΔ1, the latent and rise periods were slightly extended, and phage production was ~25-fold lower than that in cells carrying the control plasmid (pPR53oΔ1). Therefore, the amount of protein p1ΔC43 accumulated in cells carrying plasmid pA1IVΔ1, although not detected by immunoblot analysis (Figure 1B), was sufficient to decrease viral production. The effect of protein p1ΔC43 on ϕ29 development was much more severe in cells carrying plasmid pPR53Δ1 (which have higher levels of p1ΔC43). In this case, phage growth was totally blocked (Figure 3A). These results demonstrate that plasmid-encoded protein p1ΔC43 interferes with phage ϕ29 development in a dosage-dependent manner.

We also analyzed the growth of the ϕ29-related phage Nf in cells producing different levels of protein p1ΔC43 (Figure 3B). In contrast to phage ϕ29, low levels of protein p1ΔC43 (encoded by plasmid pA1IVΔ1) did not significantly affect Nf viral production. However, like phage ϕ29, Nf development was totally blocked in cells producing high levels of p1ΔC43 (encoded by plasmid pPR53Δ1).

All these observations led us to conclude that the N-terminal region of protein p1 is involved in its interference function. Therefore, such interference cannot be due to the capacity of protein p1 to associate with the bacterial membrane nor to its ability to self-associate; rather, it is probably due to its capacity to interact with other viral replication protein(s). In addition, it appears that protein p1 from phage ϕ29 is able to interact with its corresponding Nf target, although with less affinity.

p1ΔC11 and p1ΔC43 form a complex with primer TP in vitro

In contrast to protein p1, which self-associates into highly ordered structures (Bravo and Salas, 1998), protein p1ΔC11 (8.7 kDa), which lacks the C-terminal 11 amino acids, cannot self-interact in solution, as determined by sedimentation assays through glycerol gradients. In such experiments, only one population of p1ΔC11 molecules, sedimenting at a position corresponding to monomers, was observed (Figure 4). A similar result was obtained with the smaller protein p1ΔC43 (not shown).

To search for viral replication proteins that interact directly with protein p1, *in vitro* chemical cross-linking experiments, using dithiobis(sulfosuccinimidylpropio-

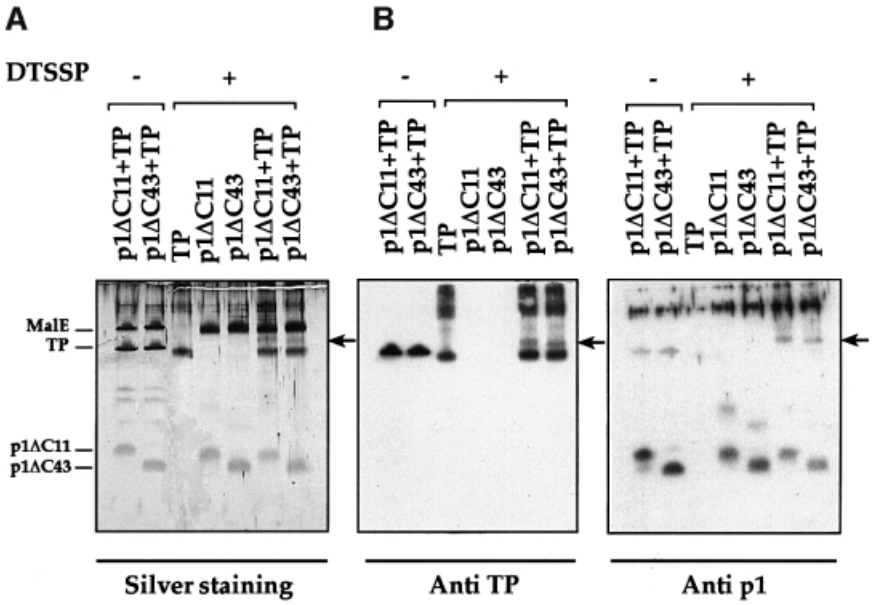


Fig. 5. Cross-linking of C-terminal truncated p1 proteins and primer TP. Protein samples were treated with DTSSP and separated by SDS–Tricine–PAGE (Schagger and Jagow, 1987). The gel was either (**A**) stained with silver (Morrissey, 1981) or (**B**) processed for western blotting. The position of MalE (42.4 kDa), TP (31 kDa), p1ΔC11 (8.7 kDa) and p1ΔC43 (5.1 kDa) is indicated. The arrow shows the position of the p1ΔC11/TP and p1ΔC43/TP cross-linked products.

nate) (DTSSP) as a cross-linking agent, were performed. To this end, we used both C-terminal truncated p1 proteins, p1ΔC11 and p1ΔC43. The latter was used taking into account that the N-terminal 42 amino acids of protein p1 are sufficient for the interference phenotype (see above). Various viral replication proteins were tested, but we only obtained positive results with primer TP (Figure 5). The truncated p1 protein preparations used in these experiments contained maltose-binding protein (MalE; 42.4 kDa) due to the purification procedure (see Materials and methods). A specific cross-linked product with the mobility expected for a complex of p1ΔC11 (8.7 kDa) and primer TP (31 kDa) was detected when both proteins were together but not when only one of them was present in the reaction (Figure 5A). A specific cross-linked product was also observed when p1ΔC43 (5.1 kDa) and primer TP were together. In both cases, the cross-linked product was recognized by anti-TP and anti-protein p1 polyclonal antibodies (Figure 5B). We conclude, therefore, that both p1ΔC11 and p1ΔC43 can interact *in vitro* with the primer TP.

p1ΔC11 and p1ΔC43, when present in excess, interfere with the formation of the TP·dAMP complex in vitro

It has been demonstrated that a free molecule of TP (primer TP) becomes covalently bound to 5′-dAMP (TP·dAMP complex) in a reaction catalyzed by the ϕ29 DNA polymerase in the presence of 5′-dATP and ϕ29 TP–DNA as template (DNA-dependent initiation reaction) (Blanco and Salas, 1985). To understand p1-mediated *in vivo* interference, we studied the effect of both p1ΔC11 and p1ΔC43 on the *in vitro* formation of the TP·dAMP initiation complex (Figure 6A). We used increasing concentrations of the corresponding truncated p1 protein and low concentrations of primer TP and ϕ29 DNA polymerase (1.2 nM). The truncated p1 protein was added to the reaction before primer TP and ϕ29 DNA polymerase. As shown in Figure 6A, truncated p1 protein reduced the amount of TP·dAMP complexes in a dosage-dependent manner. A similar result was obtained when a 259 bp DNA fragment from the ϕ29 left end (*ori*L) lacking the parental TP was used as template (Figure 6B). These results indicate that both p1ΔC11 and p1ΔC43, when present in excess, interfere with formation of the TP·dAMP initiation complex *in vitro*. This interference is independent of the presence of the parental TP.

We next studied the formation of the TP·dAMP complex in the absence or presence of protein p1ΔC11 as a function of ϕ29 TP–DNA template concentration (Figure 7A). Again, this experiment was carried out using low concentrations of both primer TP and ϕ29 DNA polymerase (1.2 nM). In the absence of p1ΔC11, the initiation reaction increased linearly when the TP–DNA concentration was increased from 0.08 to 0.32 nM; in contrast, in the presence of an excess of p1ΔC11 (2 μM), the reaction did not increase significantly with the amount of TP–DNA. We conclude that p1ΔC11-mediated inhibition is not due to a reduction in the amount of functional template molecules (active origins).

p1ΔC11-mediated inhibition is suppressed by increasing the concentration of primer TP or ϕ29 DNA polymerase

Formation of the TP·dAMP initiation complex requires the efficient formation of a primer TP–ϕ29 DNA polymerase heterodimer (Blanco *et al*., 1987). Since protein p1ΔC11, like p1ΔC43, was able to bind primer TP in the *in vitro* cross-linking assay (Figure 5), it is possible that an excess of p1ΔC11 reduces the amount of primer TP molecules available to interact with ϕ29 DNA polymerase. This in turn would reduce the amount of primer TP–ϕ29 DNA

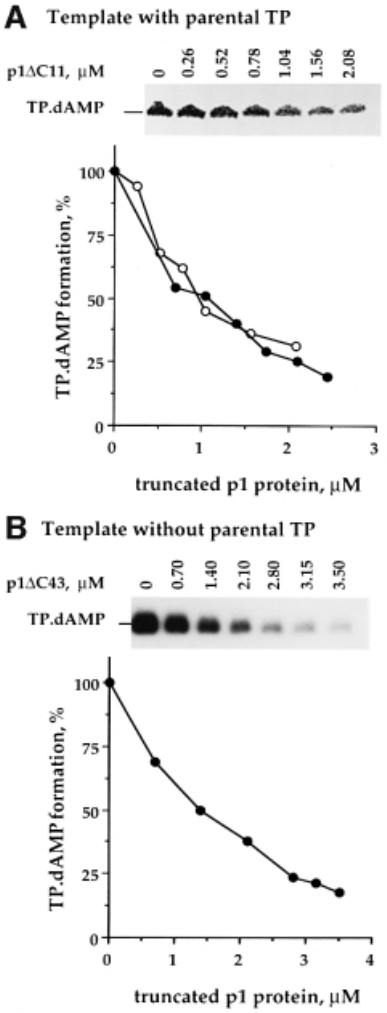


Fig. 6. Effect of C-terminal truncated p1 proteins on the *in vitro* formation of the TP·dAMP initiation complex. (**A**) The assay was carried out in the presence of 0.25 μM [α-^{32}P]dATP (1 μCi), ϕ29 TP–DNA (0.08 nM), DNA polymerase (1.2 nM), primer TP (1.2 nM) and the indicated amounts of p1ΔC11 (open circles) or p1ΔC43 (filled circles) as described in Materials and methods. $MgCl_2$ (10 mM) was used as a metal activator. (**B**) The assay was performed by incubation of 0.25 μM [α-^{32}P]dATP (1 μCi), DNA polymerase (3 nM), primer TP (3 nM), a 259 bp DNA fragment from the ϕ29 left end (0.6 nM) and the indicated amounts of p1ΔC43. $MnCl_2$ (1 mM) was used as a metal activator.

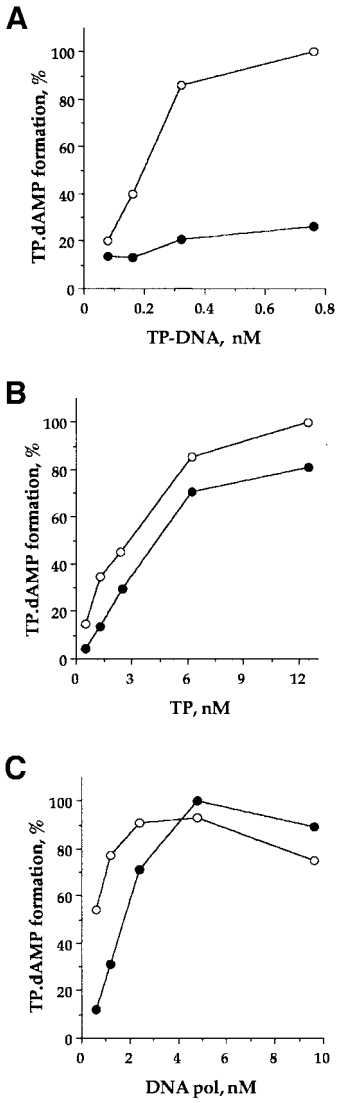


Fig. 7. Inhibition of the *in vitro* initiation reaction by p1ΔC11. The TP·dAMP complex was formed in the absence of p1ΔC11 (open circles) and in its presence (filled circles) using (**A**) DNA polymerase (1.2 nM), primer TP (1.2 nM) and different amounts of ϕ29 TP–DNA; (**B**) DNA polymerase (1.2 nM), ϕ29 TP–DNA (0.08 nM) and different amounts of primer TP; (**C**) primer TP (1.2 nM), ϕ29 TP–DNA (0.08 nM) and different amounts of DNA polymerase.

polymerase heterodimers, resulting in a decrease in the amount of TP·dAMP complexes. If this were the case, it would be possible to suppress p1ΔC11-mediated inhibition simply by increasing the concentration of primer TP or ϕ29 DNA polymerase. To test this hypothesis, TP–DNA-dependent initiation reactions were performed in the presence of 0 or 1 μM p1ΔC11 as a function of primer TP concentration (Figure 7B). In both cases, the initiation reaction increased linearly when the primer TP concentration was increased from 0.5 to 6.25 nM. Interestingly, as the primer TP concentration increased, the ratio between the amount of TP·dAMP complexes formed in the absence of p1ΔC11 and the amount that formed in its presence (inhibitory effect) decreased (Table I). Moreover, the maximum reaction obtained in the presence of p1ΔC11 was only slightly lower (1.2-fold) than that obtained in its absence.

Table I. Effect of protein p1ΔC11 (1 μM) on the DNA-dependent initiation reaction

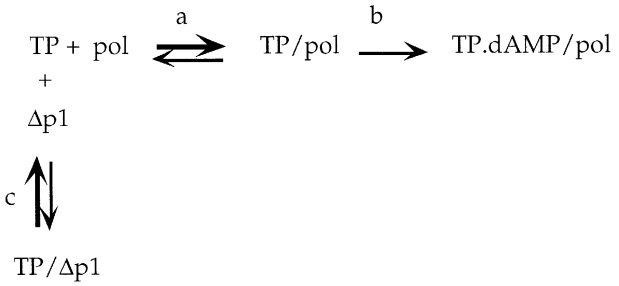
DNA polymerase (1.2 nM)		TP (1.2 nM)	
TP (nM)	Inhibitory effect[a]	DNA polymerase (nM)	Inhibitory effect[a]
0.50	3.5	0.60	4.5
1.28	2.5	1.20	2.5
2.50	1.5	2.40	1.3
6.25	1.2	4.80	0.9
12.50	1.2	9.60	0.8

[a]The inhibitory effect refers to the ratio between the amount of initiation complexes formed in the absence of p1ΔC11 and that formed in its presence. It was calculated from data presented in Figure 7.

We next carried out TP–DNA-dependent initiation reactions in the absence or presence of p1ΔC11 as a

function of ϕ29 DNA polymerase concentration (Figure 7C). In this assay, in which the concentration of primer TP was 1.2 nM, the p1ΔC11-mediated inhibitory effect decreased as the DNA polymerase concentration increased (Table I). In the presence of p1ΔC11, a higher DNA polymerase concentration was required to reach the saturation of the initiation reaction, and the maximum reaction obtained was similar to that obtained in the absence of p1ΔC11.

We conclude that the inhibition of the initiation reaction mediated by C-terminal truncated p1 proteins can be explained by competition between these proteins and the ϕ29 DNA polymerase for binding to the primer TP. Thus, the initiation reaction in the presence of p1ΔC11 or p1ΔC43 can be schematized as follows:



where formation of the primer TP/ϕ29 DNA polymerase (pol) heterodimer (reaction a) is greatly favored under the experimental conditions used (Blanco *et al*., 1987); in the presence of 5′-dATP and ϕ29 TP–DNA, DNA polymerase catalyzes the linkage of dAMP to the primer TP (Blanco and Salas, 1985) (reaction b); and the C-terminal truncated p1 protein (Δp1) binds to the primer TP with very low affinity, leading to the formation of a non-functional complex (reaction c).

Discussion

Protein p1 is membrane associated in *B.subtilis*-infected cells, both during the synthesis of viral DNA and after blocking initiation of viral DNA replication (Bravo and Salas, 1997). Moreover, the C-terminal 52 amino acid residues of p1 are sufficient for assembly into protofilament sheets (Bravo and Salas, 1998). The results of this study, using chemical cross-linking techniques, show that truncated p1 proteins that retain the N-terminal 42 amino acids (p1ΔC11 and p1ΔC43) bind to primer TP *in vitro*. Although the formation of p1ΔC11–primer TP and p1ΔC43–primer TP complexes was not greatly favored under the experimental conditions used, the existence of this interaction is supported by *in vivo* and *in vitro* functional studies. First, an excess of protein p1 at the beginning of the infective process reduced the rate of viral DNA replication. A similar dose-dependent interference was observed in cells producing p1ΔC43, a truncated p1 protein that neither binds to membranes *in vivo* (Bravo and Salas, 1997) nor self-associates *in vitro*. Hence, the *in vivo* interference mediated by protein p1 is probably related to its ability to interact with primer TP. Secondly, both p1ΔC11 and p1ΔC43, when present in excess, interfered with the *in vitro* formation of the TP·dAMP initiation complex in a reaction that requires the efficient formation of a primer TP–ϕ29 DNA polymerase heterodimer. This *in vitro* interference was suppressed by increasing the concentration of primer TP or ϕ29 DNA polymerase. Thus, the inhibition of the initiation reaction imposed by a high concentration of the C-terminal truncated p1 protein can be explained by competition between such a protein and the ϕ29 DNA polymerase for binding to the primer TP.

Why does an excess of plasmid-encoded p1 inhibit ϕ29 DNA replication? Initiation of viral DNA replication, like any biological process, needs an ordered assembly of the different components involved in the process. This can be efficiently achieved if the intracellular concentration of each component is maintained precisely. In fact, the molar ratio of protein p1 to primer TP and to ϕ29 DNA polymerase remains constant throughout the course of infection (Bravo and Salas, 1997). Therefore, we explain the p1-mediated *in vivo* interference as a consequence of the increase in the molar ratio of protein p1 with respect to primer TP and ϕ29 DNA polymerase. Although we do not know in which order these three proteins interact to initiate phage DNA replication, the interference caused by p1 when it is accumulated before ϕ29 infection suggests that the first assembly step could be the formation of the primer TP–ϕ29 DNA polymerase complex. These heterodimers, which can be isolated from ϕ29-infected cells (Matsumoto *et al*., 1984), could further interact with protein p1. According to this view, it is conceivable that a high intracellular concentration of p1 (or p1ΔC43) at the beginning of the infective process sequesters the phage-encoded primer TP, leading to the formation of non-functional complexes. As a result, assembly of primer TP–ϕ29 DNA polymerase heterodimers would be impaired and the efficiency of the viral DNA replication process would decrease. This interpretation is supported by the *in vitro* results, which showed that a high concentration of protein p1ΔC43 reduces the amount of TP·dAMP initiation complexes, probably due to a reduction in the amount of primer TP–ϕ29 DNA polymerase heterodimers.

Some lines of evidence, both in prokaryotes and eukaryotes, suggest a model for chromosomal DNA replication in which DNA polymerases are immobilized by attachment to larger structures (stationary replisome model) (for review see Cook, 1999). This model necessarily implies direct interactions between the DNA replication machinery and the cellular substructure. The results presented here are of particular relevance because they support a comprehensive model for the initiation of *in vivo* ϕ29 DNA replication (Figure 8). The model postulates that the viral replisome, constituted at least by the primer TP–ϕ29 DNA polymerase heterodimer, assembles on a membrane-associated p1-based viral structure. Proper assembly of this multiprotein complex would demand a very precise balance in the intracellular concentration of each component. Attachment of the ϕ29 replisome to the membrane-associated p1 structure would be achieved by protein–protein interactions between primer TP and p1. In addition, direct contacts between primer TP and the bacterial membrane could take place because, in the absence of other viral components, primer TP has shown affinity for membranes (Bravo and

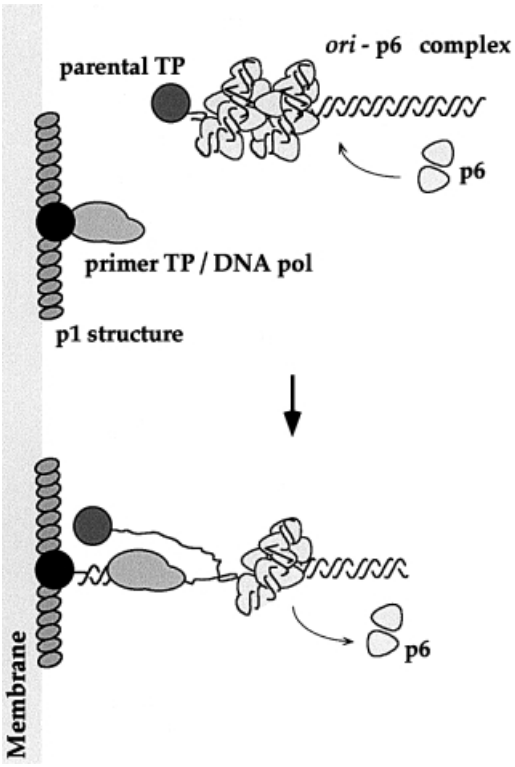


Fig. 8. Model for initiation of *in vivo* ϕ29 DNA replication. For simplicity, only one ϕ29 DNA end (replication origin) is represented. Viral DNA replication would start with the assembly of a protein p1-based structure on the bacterial membrane. This structure would provide an anchoring site for the viral replisome, constituted at least by the primer TP–ϕ29 DNA polymerase heterodimer. The membrane-associated replisome would further interact with the replication origins, located at both ends of the parental ϕ29 TP–DNA molecule. This stage is activated by the viral protein p6, which forms a multimeric nucleoprotein complex at the replication origins. This activation requires specific recognition of the protein p6 nucleoprotein complex by the primer TP–ϕ29 DNA polymerase heterodimer (Freire *et al*., 1996). Protein p6 is absolutely required for ϕ29 DNA replication *in vivo* (Carrascosa *et al*., 1976; Mellado *et al*., 1980). See Discussion for more details.

Salas, 1997). Once the replisome is anchored in place, it would specifically interact with the replication origins, located at both ends of the ϕ29 genome. Then, initiation of viral DNA replication would occur by a protein-priming mechanism in which the viral DNA polymerase catalyzes the linkage of dAMP to the primer TP (for review see Salas *et al*., 1996). Since the primer TP molecule remains covalently bound to the newly synthesized DNA, the model accounts for the specific attachment of the parental ϕ29 DNA molecule to the membrane-associated p1 structure when it is replicated. The early viral protein, p16.7, an integral membrane protein that has non-specific DNA-binding affinity, could also contribute to the membrane localization of phage DNA replication (Meijer *et al*., 2000; W.J.J.Meijer, A.Serna-Rico and M.Salas, submitted). Our model also explains previous results showing that (i) parental ϕ29 DNA–membrane complexes are formed near the onset of viral DNA synthesis, and an apparent maximum is reached by the middle of the DNA synthesis period; (ii) formation of these complexes requires the synthesis of early viral-encoded proteins (Ivarie and Pène, 1973); (iii) membrane association of viral DNA encoding a thermosensitive DNA polymerase does not occur at the restrictive temperature unless cells are co-infected with the wild-type virus (Ivarie and Pène, 1973; Mellado *et al*., 1976); and (iv) large amounts of ϕ29 DNA polymerase are sequestered in membranes, in addition to protein p1 and primer TP, when initiation of *in vivo* ϕ29 DNA replication is blocked due to the lack of the viral protein p6 (Bravo and Salas, 1997).

In conclusion, protein p1, in addition to its affinity for membranes *in vivo* and its ability to polymerize *in vitro*, has the capacity to interact with primer TP. This previously unknown function is located within the N-terminal 42 amino acid residues of the protein. These findings support a model for initiation of *in vivo* ϕ29 DNA replication in which the ϕ29 replisome is attached to a membrane-associated p1-based viral structure. Therefore, the viral replication components would be located at specific sites within the cell.

Materials and methods

Bacterial strains, bacteriophages, plasmids and growth conditions

Bacillus subtilis 110NA (Moreno *et al*., 1974) was used to grow the ϕ29 and Nf phages, and as a host for pUB110-based plasmids. *Escherichia coli* XL1-Blue (Stratagene) was used as a host for plasmids encoding MalE fusion proteins. Phage stocks were prepared essentially as reported previously (Talavera *et al*., 1971), except that the phage particles were concentrated as described (Bravo *et al*., 1990). To construct plasmids pw21 and pow3, the 114 bp *Sac*I–*Sph*I restriction fragment of the pUB110-based replicon, pPR53 (Bravo and Salas, 1997), was replaced by different ϕ29 DNA sequences: pw21 carries a *Hin*dIII–*Sca*I DNA fragment (coordinates 2899–706; Yoshikawa and Ito, 1982) and pow3 contains a *Dsa*I–*Hin*dIII DNA fragment (coordinates 3773–2899), which contains most of gene 3 except for the last 15 nucleotides. Plasmid pA1IVΔ1 was constructed in the same way as pw21, but DNA sequencing revealed a spontaneous mutation in gene 1. This mutant gene (gene Δ1) has a deletion of one nucleotide that leads to a truncated p1 protein of 45 amino acids, conserving the 42 N-terminal residues (Bravo and Salas, 1997). A *Hpa*II–*Sca*I DNA fragment (coordinates 1186–706), which contains gene Δ1 but not promoter *PA1IV*, was inserted into the expression vector pPR53 (Bravo and Salas, 1997) under control of the bacteriophage λ *PR* promoter (plasmid pPR53Δ1) and in the opposite orientation (plasmid pPR53oΔ1). To purify the C-terminally truncated p1 proteins p1ΔC11 and p1ΔC43, plasmids pMalE-p1ΔC11 and pMalE-p1ΔC43 were constructed. To construct pMalE-p1ΔC11, ϕ29 DNA sequences (coordinates 1130–873) were amplified by polymerase chain reaction (PCR) using the oligonucleotides *Sma*I (5′-GGAGAT-GTTTGTACCCGGGAAAATCTTCG-3′) and *Xba*IΔC11 (5′-GGAGCA-CCTCTAGATAACTACTTTATAACC-3′) as primers and plasmid pw21 as template. To construct plasmid pMalE-p1ΔC43, ϕ29 DNA sequences (coordinates 1130–809) were amplified by PCR using the oligonucleotides *Sma*I (see above) and *Xba*I (5′-CCGTCATCA-TCTAGAAGCAAC-3′) as primers and plasmid pPR53Δ1 as template. In both constructions, PCR fragments were digested with *Sma*I and *Xba*I, and ligated into the *Xmn*I and *Xba*I sites of the *E.coli* plasmid pMAL-c2 (New England Biolabs). The nucleotide sequence of the inserts was confirmed by DNA sequencing using the dideoxynucleotide chain-termination method (Sanger *et al*., 1977). Bacteria were grown in LB broth (Sambrook *et al*., 1989) supplemented with 5 mM $MgSO_4$ in phage infection experiments. Plasmid-containing *B.subtilis* cells were grown in LB medium supplemented with kanamycin (5 μg/ml) or phleomycin (0.8 μg/ml). Plasmid-containing *E.coli* cells were grown in LB medium supplemented with ampicillin (100 μg/ml).

Phage growth under one-step conditions

Bacillus subtilis 110NA cells carrying plasmid were exponentially grown at 30°C to ~10^8 viable cells/ml, and then infected with the indicated phage at a multiplicity of infection of 5. After 10 min of incubation with gentle shaking, unadsorbed phages were eliminated by centrifugation of the infected culture. Cells were resuspended in the same volume of pre-warmed medium and incubated with vigorous shaking at the same

temperature. These conditions ensure that the whole cell population is simultaneously infected and, consequently, that the infection process takes place synchronously and kinetic analyses can be carried out. At different times, aliquots were taken and the production of infective particles [measured as plaque-forming units (p.f.u.)/ml] was determined by plating on 110NA cells without plasmid. The time of phage addition was denoted time zero.

Synthesis of viral DNA under one-step conditions

The method has been described previously (Bravo *et al*., 1994). Basically, total intracellular DNA was isolated at different times after infection and analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed under UV light illumination. Bands corresponding to unit-length ϕ29 DNA were quantified by densitometry of the negatives in a Molecular Dynamics 300A densitometer. The relative amount of viral DNA refers to the intensity of the intracellular ϕ29 DNA band with respect to that of proteinase K-treated ϕ29 DNA used as internal marker.

Western blots

Plasmid-containing cells were exponentially grown at 30°C to ~10^8 viable cells/ml. Before cell disruption by sonication, bacteria were concentrated 10- to 20-fold in loading buffer (60 mM Tris–HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 30% glycerol). Aliquots of the cell extracts were loaded on to an SDS–polyacrylamide gradient gel (10–20%). After electrophoresis, proteins were transferred electrophoretically using a Trans Blot apparatus (Bio-Rad) at 100 mA and 4°C for 35 min as described (Bravo and Salas, 1997). Immobilon-P membranes (Millipore) were probed with the indicated serum. Antigen–antibody complexes were detected using anti-rabbit horseradish peroxidase-conjugated antibodies and ECL western blotting detection reagents (Amersham). In *in vitro* cross-linking experiments, proteins were transferred electrophoretically using a Mini Trans Blot (Bio-Rad) at 100 mA and 4°C for 80 min.

Protein purification

ϕ29 DNA polymerase was purified essentially as described by Lázaro *et al*. (1995). ϕ29 TP was purified as reported (Zaballos *et al*., 1989). To purify p1ΔC11 and p1ΔC43, we used a protein fusion system based on MalE (Kellerman and Ferenci, 1982). For this, plasmids pMalE-p1ΔC11 and pMalE-p1ΔC43 were constructed (see above). MalE fusion proteins were purified as described (Bravo and Salas, 1998) and concentrated to ⩾0.3 mg/ml using a Microcon microconcentrator 10 (Amicon). To separate the MalE and p1 domains, 1 μg of protease factor Xa (New England Biolabs) was added to 25–100 μg of fusion protein. The reaction was incubated at 4°C overnight. After total digestion, the reaction mixture was loaded on to a 5 ml glycerol gradient (15–30% in dialysis buffer: 20 mM Tris–HCl pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol) prepared in a Beckman polyallomer centrifuge tube (13 × 51 mm). Centrifugation was performed at 62 000 r.p.m. and 4°C in a Beckman SW65 rotor for 20 h. After centrifugation, 170 μl fractions were collected by puncturing the bottom of the tubes. Aliquots from each fraction were analyzed by SDS–Tricine–PAGE (Schagger and Jagow, 1987). Fractions containing the truncated p1 protein were pooled and dialyzed against dialysis buffer (see above). Protein preparations were concentrated using a Microcon microconcentrator.

In vitro formation of the TP·dAMP complex (initiation reaction)

The reaction mixtures (25 μl) contained 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM ammonium sulfate, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.25 μM [α-^{32}P]dATP (1 μCi), 25 ng (2 fmol) of ϕ29 TP–DNA as template and the indicated amounts of primer TP, viral DNA polymerase and truncated p1 protein. When indicated, 2.5 ng (15 fmol) of a 259 bp DNA fragment from the ϕ29 left end (*ori*L) were used as template. In this case, 1 mM $MnCl_2$ was used as metal activator. After incubation for 3 min at 30°C, the reactions were stopped by adding EDTA to 10 mM and SDS to 0.1%. Samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume was analyzed by SDS–PAGE. The relative amounts of incorporated [α-^{32}P]dAMP were calculated by PhosphorImager densitometric scans of an exposed Fuji BAS-IIIs imaging plate.

Cross-linking of proteins

Protein preparations were dialyzed against 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5. The cross-linker DTSSP (Pierce) was dissolved in the same buffer just before use. The reaction mixture (25 μl) contained 5 mM DTSSP, 880 ng of ϕ29 primer TP and 640 ng of p1ΔC11 or p1ΔC43. After incubation at room temperature for 30 min, Tris–HCl pH 7.5 was added at a final concentration of 30 mM to quench the reaction. The reaction mixture was incubated for an additional 15 min. Samples were analyzed by SDS–Tricine–PAGE in the absence of β-mercaptoethanol.

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