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## Protein-Primed Replication of Bacteriophage $\Phi$ 29 DNA

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### 1. Introduction

Unlike circular genomes in which the conventional replication fork machinery can accomplish the copy of the complete molecule, the requirement of a DNA/RNA molecule to prime DNA synthesis imposes replication strategies to avoid the loss of genetic information contained at the very end of the lagging strand in linear chromosomes since DNA polymerases are unable to start *de novo* DNA synthesis. Thus, once the most terminal primer is removed, a short region of unreplicated single-stranded DNA (ssDNA) will remain at the end of the chromosome that would eventually lead to a continuous shortening of the daughter DNA molecule after successive rounds of DNA replication (the end-replication problem). Therefore, it is essential to guarantee replication of the chromosome ends, that otherwise would cause cell death. Organisms containing linear genomes have developed novel replication strategies to overcome such a problem by either yielding head-tail concatemers, most of them making use of terminal redundancies as phages T4, T7 and SPP1, or by the circularisation and further rolling circle replication of their chromosomes, as it occurs in phage  $\lambda$  [reviewed in (Salas & de Vega, 2008)]. In higher eukaryotes telomerase extends directly the 3' end, producing an overhanged ssDNA end (Kornberg & Baker, 1992) that finally can invade homologous double-stranded telomeric tracts, enlarging and protecting chromosome ends (Verdun & Karlseder, 2007).

Other organisms, as bacteriophages, animal viruses as adenovirus and human hepatitis B virus, mitochondrial plasmids, linear chromosomes and plasmids of *Streptomyces* (Salas, 1999), as well as several virus infecting Archaea, as halovirus (Bamford et al., 2005; Bath et al., 2006), possess replication origins, constituted by inverted terminal repetitions (ITR) together with a terminal protein (TP), placed at both ends of their linear chromosomes (Salas, 1991). In these cases, the location of the two replication origins allows both strands to be replicated continuously, without requiring asymmetric complexes of the replicative DNA polymerase with other accessory proteins to control the different mechanics of continuous and discontinuous synthesis (Blanco et al., 1989). The TP provides the OH- group of a specific serine, threonine or tyrosine to prime initiation of DNA replication from the ends of the linear chromosome, circumventing the end replication problem, the TP remaining covalently linked to such 5'-termini of the genome (TP-DNA) (Salas, 1991, 1999; Salas et al., 1996).

## 2. The protein-primed replication mechanism

The development of a soluble *in vitro* replication system with highly purified proteins and TP-DNA from bacteriophage  $\phi 29$  of *Bacillus subtilis* has allowed us to lay the foundations of the so-called protein-primed mechanism of DNA replication (Salas et al., 1995; Salas et al., 1996).

Figure 1 shows a summary of the protein-priming mechanism of  $\phi 29$  DNA replication (Salas, 1991). Initiation of replication starts by the formation of a TP/DNA polymerase heterodimer that recognises the TP-containing DNA ends which are the origins of replication. On the other hand, the formation of a nucleoprotein complex of the  $\phi 29$ -encoded double-stranded (ds)DNA binding protein p6 (DBP) at the DNA ends has been proposed to facilitate opening of the latter (see below) and, in the presence of the initiating nucleotide, dATP, stimulates the formation of the covalent linkage between dAMP and the OH group of a specific serine residue in the TP, catalysed by the  $\phi 29$  DNA polymerase (hereafter  $\phi 29$  DNAPol). Afterwards, the same polymerase molecule starts the elongation step (DNA-primed) of replication. This results in the formation of the type I replication intermediates that consist in full-length dsDNA molecules with one or more ssDNA tails of different

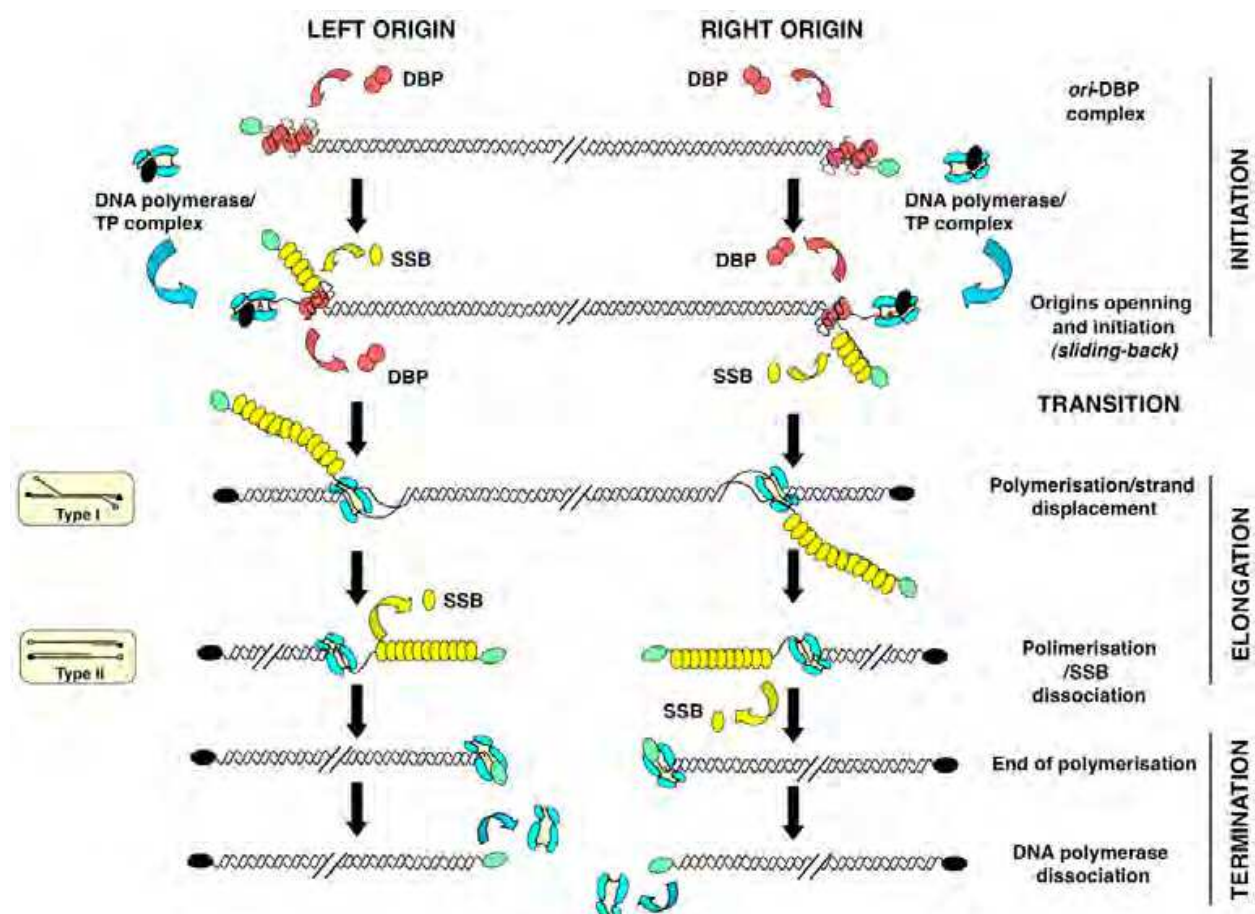


Fig. 1. Schematic representation of bacteriophage  $\phi 29$  TP-DNA replication. Primer and parental TP are shown in black and green, respectively.  $\phi 29$  DNAPol, DBP and SSB are coloured in blue, red and yellow, respectively. An scheme of the type-I and type-II replication intermediates is shown at the left. See text for details.

lengths that are stretched by the binding of the phage-encoded single-stranded DNA binding protein (SSB). When the two replication forks, that have been initiated at each DNA end, are encountered, type I molecules give rise to two type II molecules that consist of a full-length  $\phi$ 29 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. Type II molecules are elongated by the  $\phi$ 29 DNApol with concomitant dissociation of the SSB protein to yield two fully replicated  $\phi$ 29 TP-DNA molecules. The final dissociation of the DNA polymerase from each DNA molecule should allow the formation of new heterodimers with free TP molecules to initiate a new round of replication.

These steps and the proteins involved are dissected in the following sections.

## 2.1 Replication origins of TP containing genomes

$\phi$ 29 is a lytic phage that infects the gram-positive bacterium *B. subtilis* (Anderson et al., 1966). It has a linear dsDNA genome 19.3 kbp long, with a 6 bp ITR (3'-TTTCAT) (Escarmis & Salas, 1982; Yoshikawa & Ito, 1982) and a 31 kDa TP covalently linked to each 5' end by a phosphoester bond between Ser<sup>232</sup> and 5'-dAMP (the initiator nucleotide) (Salas et al., 1978), forming the minimal replication origin.

Other *B. subtilis* phages related to  $\phi$ 29 that also contain linear dsDNA and TP of similar size are classified in three groups: 1)  $\phi$ 15, PZA and PZE that belong to the  $\phi$ 29 group; 2) Nf, M2 and B103; and 3) GA-1. The DNA of all these phages has a short ITR six nucleotides long (3'-TTTCAT) for  $\phi$ 29,  $\phi$ 15, PZA and B103, eight nucleotides long (3'-TTTCATTC) for Nf and M2, and seven nucleotides long (3'-TTTATCT) for GA-1 (Salas, 1991).

Phage Cp-1, that infects the Gram-positive bacterium *Streptococcus pneumoniae*, contains a 19.3 kbp linear dsDNA (Martín et al., 1996b) with a TP of 28 kDa covalently linked to the 5' DNA ends by a phosphoester bond between a still undetermined Thr and 5'-dAMP (García et al., 1983; García et al., 1986). Cp-1 DNA has an ITR of 236 bp with the reiteration 3'-TTT (Martín et al., 1996a).

TP-DNA genomes have also been observed in phages infecting Gram-negative bacteria as it is the case of phage PRD1, a member of a family of lipid-containing phages that infect *Escherichia coli*. The 5' termini of the 15 kbp long linear dsDNA of PRD1 (Bamford et al., 1991) are linked to a 28 kDa TP by a phosphoester bond between Tyr<sup>190</sup> and 5'-dGMP (Bamford & Mindich, 1984; Shiue et al., 1991). The TP-DNA of PRD1 and related phages has 110 bp long ITR and the reiteration 3'-CCCC at the ends.

Adenoviruses also contain a linear dsDNA genome 36 kbp long with two replication origins located at the 100 bp long ITRs. The Adenovirus type-5 origin sequence starts with the reiteration 3'-GTAGTA. The 5' ends are covalently linked to the 55 kDa TP by a phosphoester bond between Ser<sup>580</sup> and 5'-dCMP [reviewed in (Coenjaerts & van der Vliet, 1995; Van der Vliet, 1995)].

The product of  $\phi$ 29 early gene 6 is a DBP that binds preferentially to the  $\phi$ 29 DNA ends every 24 nucleotides and with a defined phase, being essential for the activation of the initiation of DNA replication (Serrano et al., 1990). This protein has been described as a histone-like protein that self-associates into elongated oligomers doughnut-shaped that grow into right-handed double-helical filaments (Abril et al., 1999). These filaments form a scaffold tightly wrapped by a DNA right-handed superhelix (Serrano et al., 1990) restraining its positive supercoiling (Prieto et al., 1988; Serrano et al., 1993b) and helping to open the DNA ends, activating initiation of TP-DNA replication most probably by allowing



replication origin recognition by the DNA polymerase/primer TP heterodimer (Serrano et al., 1993a). In support of this hypothesis,  $\phi$ 29 DBP is absolutely required for the *in vitro* formation of the TP-dAMP complex at low temperature (Serrano et al., 1994). Our results were pioneers in proposing the structural basis for the activation of replication origins, a biological event shown later to be universally conserved in both prokaryotic and eukaryotic initiators (Clarey et al., 2006; Erzberger et al., 2006; O'Donnell & Jeruzalmi, 2006).

By site-directed and deletion mutagenesis we showed that the N-terminal region of p6 is involved in DNA binding. Specifically, mutation in residues Lys<sup>2</sup> or Arg<sup>6</sup> resulted in p6 proteins impaired in DNA binding (Freire et al., 1994; Otero et al., 1990). On the other hand, residues critical for the dimerization of protein p6, identified by random mutagenesis, are Ile<sup>8</sup> and Ala<sup>44</sup>. In addition to impaired dimer formation ability, mutations at these two residues showed reduced DNA binding affinity and they were affected in the initiation of  $\phi$ 29 DNA replication. Thus, dimers seem to be the active form of  $\phi$ 29 DBP for DNA binding (Abril et al., 2000).

## 2.2 Initiation of $\phi$ 29 TP-DNA replication

The first step in  $\phi$ 29 TP-DNA replication is the recognition of the replication origins by a heterodimer formed by two viral encoded proteins: the replicative DNA polymerase and a free molecule of TP.

### 2.2.1 $\phi$ 29 DNAPol

$\phi$ 29 DNAPol is a monomeric enzyme of only 66 kDa, fully responsible for viral DNA replication (Blanco & Salas, 1985a). Based on amino acid sequence similarities and its sensitivity to specific inhibitors, we included  $\phi$ 29 DNAPol in the family B (eukaryotic-type) of DNA-dependent DNA polymerases (Bernad et al., 1987). As any of them, it accomplishes sequential template-directed addition of dNMP units onto the 3'-OH group of a growing DNA chain, with insertion discrimination values ranging from  $10^4$  to  $10^6$  and with an efficiency of mismatch elongation  $10^5$  to  $10^6$ -fold lower than that of a properly paired primer terminus (Esteban et al., 1993). In addition,  $\phi$ 29 DNAPol catalyses 3'-5' exonucleolysis, i.e., the release of dNMP units from the 3' end of a DNA strand (Blanco & Salas, 1985b), showing a preferential degradation of a mismatched primer-terminus, in agreement with a role in proofreading of DNA insertion errors that enhances replication fidelity  $10^2$ -fold (Esteban et al., 1994; Garmendia et al., 1992), as it occurs in most DNA replicases.

An extensive mutational analysis of individual residues contained in regions of high amino acid similarity among family B DNA polymerases, as well as the construction of deletion mutants (Blanco & Salas, 1996) allowed us to identify the  $\phi$ 29 DNAPol catalytic residues required for these activities and those responsible for the stabilisation of the primer-terminus at both active sites. As these residues are evolutionarily conserved the results obtained with  $\phi$ 29 DNAPol could be extrapolated to most DNA polymerases [reviewed in (Blanco & Salas, 1995, 1996)]. Thus, sequence alignments and site-directed mutagenesis served to identify the catalytic and ssDNA ligand residues responsible for the 3'-5' exonuclease activity, located at the N-terminal one-third of the enzyme (exonuclease domain), and to propose the hypothesis, widely demonstrated later, of an evolutionary conserved 3'-5' exonuclease active site among distantly related DNA-dependent DNA polymerases (Bernad et al., 1989). Such an active site is formed by three N-terminal amino acid motifs, named ExoI, ExoII, and ExoIII, invariantly containing four carboxylate groups

that bind two metal ions and one tyrosine residue that orients the attacking water molecule (Bernad et al., 1989), as it had been shown to occur in *E. coli* Pol I (Derbyshire et al., 1991). In addition, these analyses led us to identify a new motif (K<sub>x</sub>h) whose lysine residue plays an auxiliary role in catalysis, specifically in family B DNA polymerases (de Vega et al., 1997). Similarly, our site-directed mutagenesis studies of  $\phi$ 29 DNApol pioneered the functional analyses of specific amino acids at motifs Y<sub>x</sub>GG, D<sub>x</sub>SLYP, K<sub>x</sub><sub>3</sub>NS<sub>x</sub>YG, T<sub>x</sub><sub>2</sub>GR, Y<sub>x</sub>DTDS, and K<sub>x</sub>Y, highly conserved at the C-terminal two-thirds of eukaryotic DNA polymerases from family B. These investigations demonstrated the overlapping between polymerisation and protein-primed initiation domains, and served to identify the amino acids involved in metal binding and catalysis, as well as DNA, TP and dNTP ligands (Blanco & Salas, 1995, 1996; Pérez-Arnaiz et al., 2010).

We have shown that  $\phi$ 29 DNApol has three distinctive functional features compared to most replicases. First, it initiates DNA replication at the origins located at both ends of the linear genome by catalysing the addition of the initial dAMP onto the hydroxyl group of Ser<sup>232</sup> of the phage TP, which acts as primer (see below) [reviewed in (Salas 1991, 1999; Salas & de Vega, 2006)]. Second, unlike most replicases that rely on accessory proteins to be stably bound to the DNA, as thioredoxin in the case of T7 DNA polymerase (Huber et al., 1987; Tabor et al., 1987), the  $\beta$ -subunit of *E. coli* Pol III holoenzyme (Kong et al., 1992), or the eukaryotic clamp protein, PCNA (Jonsson & Hübscher, 1997; Kelman, 1997),  $\phi$ 29 DNApol performs DNA synthesis without the assistance of processivity factors, displaying the highest processivity described for a DNA polymerase [ $>70$  kb; (Blanco et al., 1989)]. A third distinctive property of  $\phi$ 29 DNApol is the efficient coupling of processive DNA polymerisation to strand displacement. This capacity enables the enzyme to replicate the double-stranded genome without the need for a helicase (Blanco et al., 1989). These two features, high processivity and intrinsic strand displacement activity, are currently being exploited for the use of  $\phi$ 29 DNApol in isothermal multiple displacement amplification (MDA) (Dean et al., 2002; Dean et al., 2001). These amplification technologies based on  $\phi$ 29 DNApol have two main advantages respect to classical PCR DNA amplification: first, no previous sequence information is required, due to the use of random hexamer primers, any DNA being susceptible to be amplified, and second, amplicons performed by the  $\phi$ 29 DNApol are much larger than those obtained with PCR. On the other hand, the ability displayed by  $\phi$ 29 DNApol to use circular multiply primed ssDNA as template has led to the development of the multiply primed rolling circle amplification, one of the most robust technologies to amplify circular templates of variable size (Dean et al., 2001). This amplification technology is being widely used for genome sequencing, efficient amplification and detection of known and unknown circular viral genomes (Johne et al., 2009), genotyping of single nucleotide polymorphisms (Qi, et al., 2001), whole genome analysis of noncultivable viruses (Johne et al., 2009), detection and identification of circular plasmids in zoonotic pathogens (Xu et al., 2008), and for the description of new metagenomes (López-Bueno et al., 2009). Recently we have achieved improvements of isothermal MDA by fusing DNA binding domains to the C-terminus of  $\phi$ 29 DNApol (de Vega et al., 2010). The results showed that the addition of Helix-hairpin-Helix domains increases DNA binding of the hybrid DNA polymerases without hindering their replication rate. In addition, the chimerical DNA polymerases displayed an improved DNA amplification efficiency on both circular plasmids and genomic DNA and are unique  $\phi$ 29 DNApol variants with enhanced amplification performance. These chimerical DNA polymerases will contribute to make  $\phi$ 29 DNA amplification technology one of the most powerful tools for genomics, consolidating MDA technology as the alternative to PCR for many applications.

Despite the exhaustive mutational analyses carried out throughout  $\phi 29$  DNApol, they did not provide a structural rationale for both the intrinsic processivity and strand displacement capacity of the enzyme. Instead, resolution of the  $\phi 29$  DNApol structure gave the insights into these two unique properties of the enzyme. These structural studies, carried out in collaboration with Tom Seitz's lab (Yale University), showed  $\phi 29$  DNApol formed by an N-terminal exonuclease domain, containing the 3'-5' exonuclease active site, and a C-terminal polymerisation domain that, like in other DNA polymerases, is subdivided into the universally conserved palm (containing the catalytic and DNA ligand residues), fingers (containing the dNTP ligands) and thumb (which confers stability to the primer) subdomains (Kamtekar et al., 2004) (see Figure 2A). 3D-structural comparisons indicated that the main difference between other family B DNA polymerases and  $\phi 29$  DNApol was the presence in the latter of two additional subdomains, both corresponding to sequence insertions that we had previously identified as specifically conserved in the protein-primed subgroup of DNA polymerases, called TPR1 and TPR2 (Blasco et al., 1990; Dufour et al., 2000). TPR1 lies at the edge of the palm, while TPR2 contains a  $\beta$ -hairpin structure just facing the apex of the thumb subdomain. Palm, thumb, TPR1, and TPR2 subdomains form a doughnut-shaped structure that encircles the upstream duplex DNA at the polymerisation active site (Berman et al., 2007), constituting an internal clamp that provides the enzyme with the maximal DNA-binding stability required for its intrinsic processivity, mimicking and making unnecessary the sliding clamp used in other replisomes. On the other hand, TPR2, palm and fingers subdomains, together with the exonuclease domain, surround the downstream template strand (Berman et al., 2007), forming another tunnel whose narrow dimensions ( $\sim 10$  Å) do not allow dsDNA binding. Thus, downstream dsDNA has to be unwound to enable threading of the template strand through this tunnel to reach the polymerisation site, using the same topological mechanism as hexameric helicases to open dsDNA regions, and providing a structural basis for the strand displacement capacity of  $\phi 29$  DNApol (Kamtekar et al., 2004; Rodríguez et al., 2005).

3D resolution of  $\phi 29$  DNApol structure also gave us the clues about how primer-terminus switches between polymerisation and exonuclease active sites during proofreading of polymerisation errors. Comparison of the structures of many apo DNA polymerases with their corresponding binary complexes showed that the major conformational changes occur mainly in their thumb subdomains, composed of two microdomains with a clear helicoidal character linked by a flexible region (Beese et al., 1993; Doublie et al., 1998; Eom et al., 1996; Franklin et al., 2001; Li et al., 1998; Shamoo & Steitz, 1999). Conversely, the  $\phi 29$  DNApol thumb subdomain has an unusual structure since it is small and mainly constituted by a long  $\beta$ -hairpin without identifiable microdomains (Kamtekar et al., 2004). Moreover, comparison of the apo enzyme with the binary complexes showed that the thumb subdomain does not rotate upon DNA binding (Berman et al., 2007). We have shown that the prevention of a potential thumb movement by crosslinking the tips of the TPR2 and thumb subdomains did not affect the partitioning of the primer-terminus between the polymerisation and editing active sites (Rodríguez et al., 2009). The impeded motion of the TPR2 subdomain suggests that rotation of the DNA is not required to transfer the primer-terminus between the polymerisation and editing active sites in  $\phi 29$  DNApol, most likely as there is not any structural barrier in between. Then, how does the frayed terminus travel to the exonuclease active site? Considering the  $\phi 29$  DNApol thumb subdomain as a nearly static structure, the primer switching would be accomplished by a passive diffusion of the

frayed primer-terminus. The energetically unfavourable gradual melting of three-four base pairs should be progressively offset by new and specific interactions established with DNA ligands of the thumb subdomain, as suggested (Pérez-Arnaiz et al., 2006). Such interactions would also channel the primer-terminus in the appropriate orientation to contact with ssDNA ligands of the exonuclease domain responsible for the stabilisation of the primer-terminus at the editing active site (de Vega et al., 1996, 1998b; Kamtekar et al., 2004; Pérez-Arnaiz et al., 2006; Rodríguez et al., 2009).

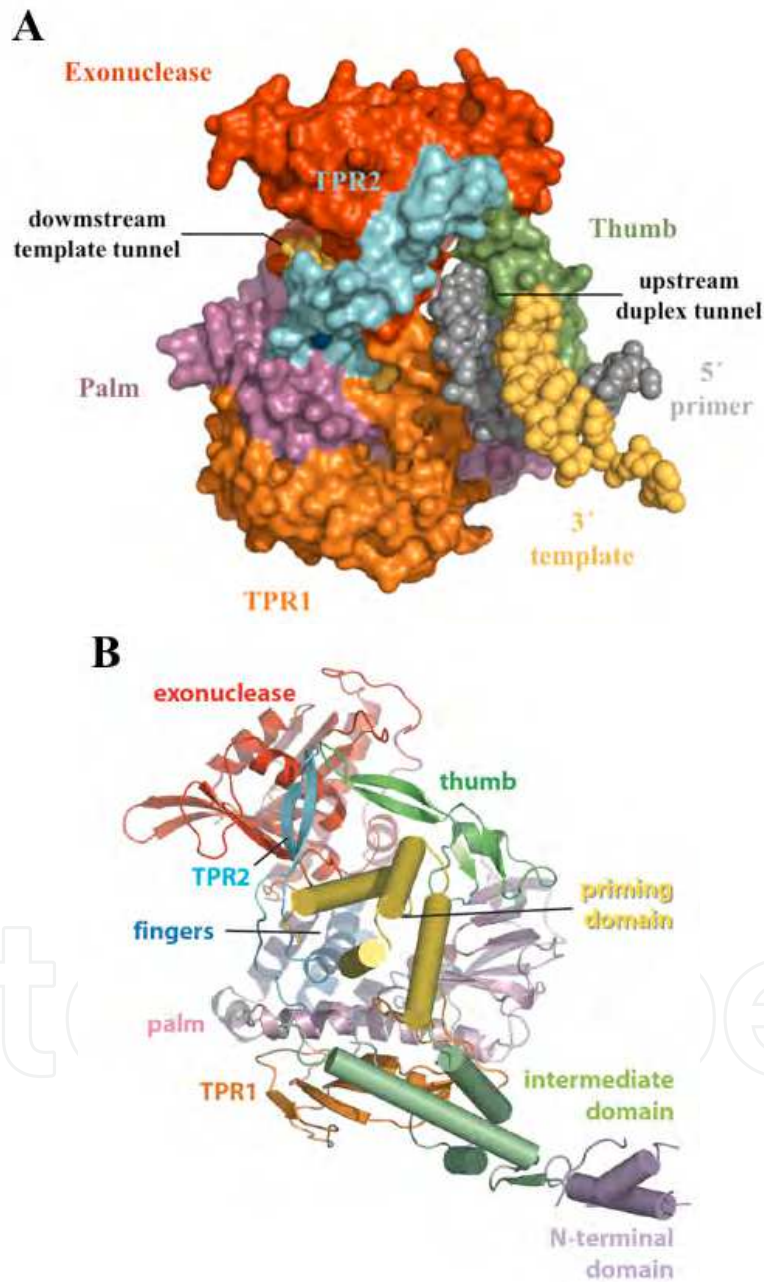


Fig. 2. (A)  $\phi$ 29 DNAPol-DNA complex. (B) Structure of the  $\phi$ 29 DNAPol-Terminal Protein complex. Reproduced with permission from Kamtekar, S., Berman, A.J., Wang, J., Lázaro, J.M., de Vega, M., Blanco, L., Salas, M. & Steitz, T.A. (2006). The phi29 DNA polymerase:protein-primer structure suggests a model for the initiation to elongation transition. *EMBO J.* Vol. 25, No. 6, pp. 1335-1343.



Additionally, recent resolution of  $\phi 29$  DNAPol tertiary complex structures has allowed us to dissect the subtle changes in the polymerisation active site that take place upon dNTP binding, providing the structural basis for the mechanism of translocation. Thus, once the catalysis of the phosphoester bond formation between the  $\alpha$ -phosphate of the incoming dNTP and the OH- group of the priming nucleotide takes place, the pyrophosphate produced leaves the DNA polymerase, breaking the electrostatic crosslink that kept the fingers subdomain in the closed state. Concomitantly to the fingers opening, residues Tyr<sup>254</sup> and Tyr<sup>390</sup> move back into the nucleotide insertion site, leading to one position backwards translocation of the nascent base pair out of the binding pocket, as now the nucleotide insertion site is sterically inaccessible (Berman et al., 2007). This translocation allows the 3' OH-group of the newly added nucleotide to be in a competent position to attack nucleophilically the  $\alpha$ -phosphate of the incoming nucleotide during the next nucleotide insertion event (Berman et al., 2007) (see Figure 3).

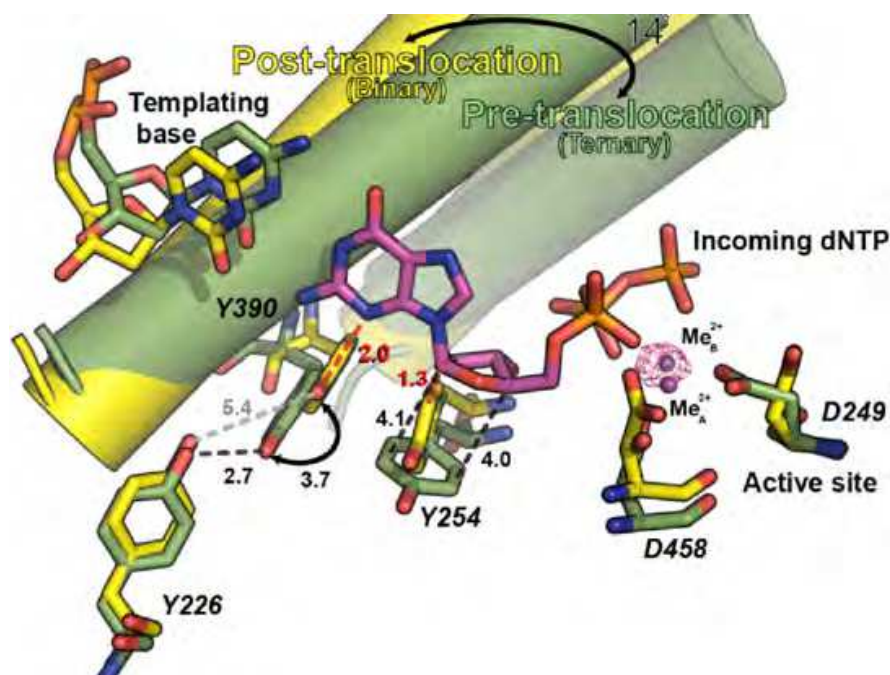


Fig. 3. Comparison of the binary (yellow) and ternary (green) complex structures of  $\phi 29$  DNAPol. The mechanistically significant amino acid movements are indicated. Reproduced with permission from Berman, A.J., Kamtekar, S., Goodman, J.L., Lázaro, J.M., de Vega, M., Blanco, L., Salas, M. & Steitz, T.A. (2007). Structures of phi29 DNA polymerase complexed with substrate: the mechanism of translocation in B-family polymerases. *EMBO J.* Vol. 26, No. 14, pp. 3494-3505.

### 2.2.2 $\phi 29$ Terminal protein

As already mentioned, the primer TP forms a heterodimer with the DNA polymerase for recognition and further initiation of TP-primed DNA replication. To discriminate between the two different functions, the TP molecule linked to the 5' DNA ends is called parental TP and the TP present in the complex with DNA polymerase is called primer TP. Crystallographic resolution of the structure of  $\phi 29$  DNAPol/primer TP heterodimer has shown that the TP has an elongated three-domain structure (Figure 2B) (Kamtekar et al.,

2006). The N-terminal domain (residues 1–73) is structurally disordered likely because it is not interacting with the polymerase (Kamtekar et al., 2006; Pérez-Arnaiz et al., 2007). The intermediate domain (residues 74–172) contains two long  $\alpha$ -helices and a short  $\beta$ -turn- $\beta$  structure and makes extensive contacts with the TPR1 subdomain of the polymerase. This interface has many charged residues and includes two salt bridges between arginine residues in the TP and glutamic acid residues in the TPR1 subdomain (R158:E291; R169:E322). It is connected through a hinge region to the C-terminal priming domain (residues 173–266), a region highly electronegative that has a four-helix bundle topology. Ser<sup>232</sup>, which provides the priming hydroxyl group for DNA synthesis, lies in a disordered loop (residues 227–233) at the end of the priming domain close to the active site of the DNA polymerase (see Figure 4, left panel). The priming domain structure shows interactions between many of their acidic residues and positively charged residues of the thumb subdomain of the polymerase (e.g., between E191:K575 and D198:K557), with residue R96 of the exonuclease domain and with TPR2 subdomain residues (Kamtekar et al., 2006; Rodríguez et al., 2004). Thus, the upstream duplex DNA “tunnel” of  $\phi$ 29 DNApol encircles the TP priming domain whose overall dimensions and its negative charge mimics DNA in

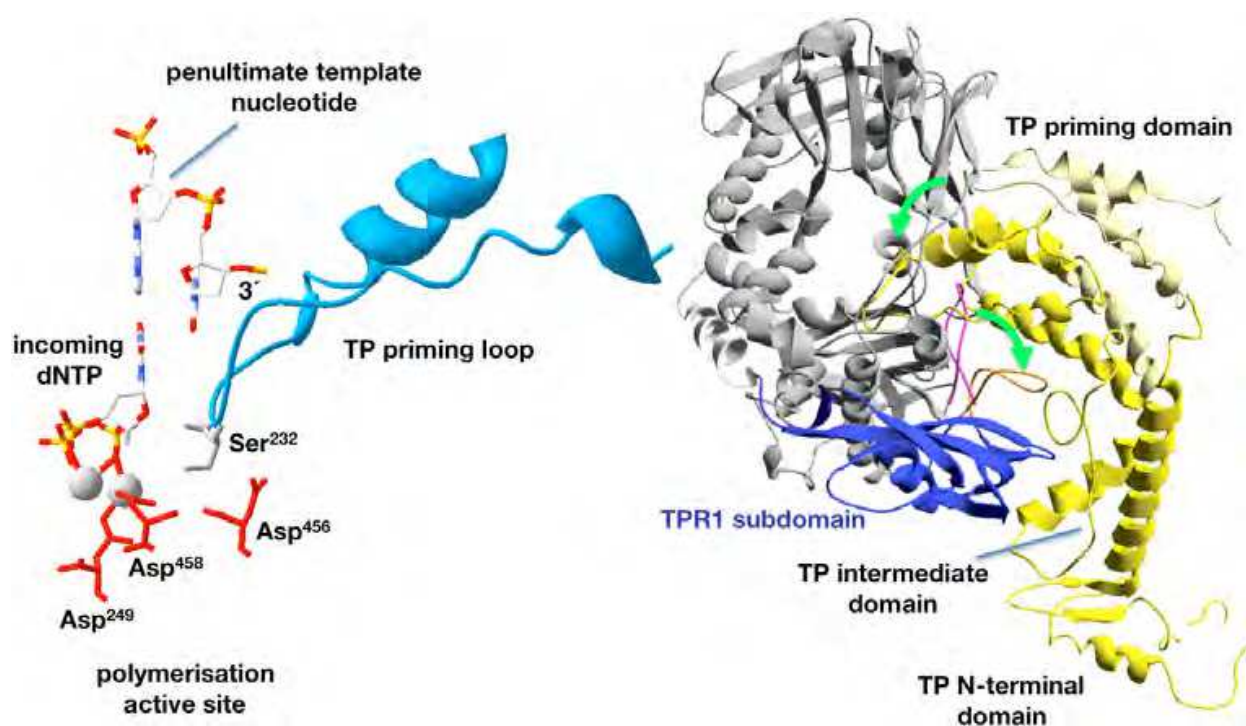


Fig. 4. Left, placement of TP priming residue Ser<sup>232</sup> (in grey) and penultimate template nucleotide at the  $\phi$ 29 DNApol active site (catalytic aspartates are shown in red). Right, flexible orientations of TPR1 loop in the apoenzyme (coloured in magenta) and its stable and moved out structural conformation shown in the DNA polymerase/TP complex (coloured in orange). TP is coloured in yellow. Green arrows indicate the suggested conformational changes of both, the DNA polymerase TPR1 loop and the TP priming domain to allow the formation of a stable heterodimer. Reproduced with permission from Pérez-Arnaiz, P., Longás, E., Villar, L., Lázaro, J.M., Salas, M. & de Vega, M. (2007). Involvement of phage  $\phi$ 29 DNA polymerase and terminal protein subdomains in conferring specificity during initiation of protein-primed DNA replication. *Nucleic Acids Research*. Vol. 35, No. 21, pp. 7061-7073.

its interactions with the polymerase during initiation (de Vega et al., 1998a; Kamtekar et al., 2006). This explains why DNA synthesis by the heterodimer cannot begin at internal sites of the phage genome, as the upstream 3' template would sterically clash with the TP (Kamtekar et al., 2006).

Our previous studies showed that  $\phi$ 29, GA-1 and Nf DNA polymerases display a great specificity for their corresponding primer TPs, as the heterologous systems did not give any detectable initiation product (González-Huici et al., 2000a; Longás et al., 2006). By means of chimerical proteins, constructed by swapping the priming domain of the related  $\phi$ 29 and GA-1 TPs, we showed that DNA polymerase can form catalytically active heterodimers exclusively with that chimerical TP containing the N-terminal and intermediate domains of the homologous TP, suggesting that the interaction between the polymerase TPR1 subdomain and the TP intermediate domain is the one main responsible for the specificity between both proteins (Pérez-Arnaiz et al., 2007).

In addition, the independent expression of the  $\phi$ 29 TP priming domain and intermediate plus N-terminal domains showed that the former can only prime initiation in the presence of the latter that assists the TP-dAMP formation most probably by inducing a conformational change in the DNA polymerase (Pérez-Arnaiz et al., 2007). The structure of the  $\phi$ 29 DNAPol forming a complex with the TP is very similar to that of the apo enzyme, the main conformational changes being restricted to TPR1 residues 304-315 (Kamtekar et al., 2006). Such residues form loops with a high degree of flexibility in the apo enzyme. By the contrary, the  $\phi$ 29 heterodimer structure shows that this loop moves out to allow the TP to access the polymerase active site. Altogether, these results led to propose a model for the DNA polymerase-TP interaction in which the TP intermediate domain would recognise specifically and interact with the DNA polymerase TPR1 subdomain. Such interaction would promote the change of the TPR1 loop from a flexible to the stable moved out conformation that now would allow the proper (prone to catalysis) placement of the TP priming domain into the DNA polymerase structure (Pérez-Arnaiz et al., 2007) (see Figure 4, right panel).

### 2.2.3 Recognition of replication origins by the DNA polymerase/TP heterodimer

The  $\phi$ 29 DNAPol/TP heterodimer recognises the replication origins at the genome ends (see Figure 1). Blunt-ended DNA fragments containing the left or right  $\phi$ 29 DNA ends, but not internal  $\phi$ 29 DNA fragments, were active as templates in *in vitro* initiation reactions (García et al., 1984; Gutiérrez et al., 1986a; Gutiérrez et al., 1986b). However, the activity was 6- to 10-fold lower than that obtained with TP-DNA (Gutiérrez, et al. 1986a; Gutiérrez et al., 1986b). These results indicated on the one hand, that specific DNA sequences located at the  $\phi$ 29 DNA ends are involved in origin recognition and on the other hand, that the parental TP is a major signal in the template for such a recognition, strongly suggesting that the heterodimer is recruited to the origin through interactions with the parental TP. In agreement with this, detection of initiation activity by using heterologous systems in which DNA polymerase, primer TP and TP-DNA came from  $\phi$ 29 and Nf related phages, showed that initiation was selectively enhanced when the DNA polymerase and the TP-DNA were from the same phage, implying a specific interaction between DNA polymerase and parental TP (González-Huici et al., 2000a). In line with this, a chimerical  $\phi$ 29 DNAPol containing the GA-1 DNA polymerase TPR1 subdomain was capable of catalysing the initiation reaction primed by GA-1 TP but solely in the presence of  $\phi$ 29 TP-DNA, supporting



the hypothesis that a major contribution to the parental TP recognition is carried out by the DNA polymerase (Pérez-Arnaiz et al., 2007). Similarly, mutations introduced at several TP-intermediate domain residues rendered TP mutants that could not support DNA replication when they acted as parental TP, indicating also a contribution of the primer TP in the specific recognition of the replication origins (Illana et al., 1998; Serna-Rico et al., 2000). Furthermore, measurement of the ability of the different DBPs coming from  $\phi$ 29, Nf and GA-1 bacteriophages to activate homologous and heterologous replication origins showed also a specific recognition of each nucleoprotein complex by the homologous DNA polymerase/TP heterodimer (Freire et al., 1996). The fact that  $\phi$ 29 DBP stimulates the initiation activity of the heterodimer formed by GA-1 primer TP and a chimerical  $\phi$ 29 DNAPol containing the TPR1 subdomain of GA-1 DNA polymerase to a similar extent as that of the  $\phi$ 29 heterodimer, favours the hypothesis of a main and specific recognition of the DBP by the DNA polymerase (Pérez-Arnaiz et al., 2007).

#### 2.2.4 A sliding-back mechanism for protein-primed DNA replication

As already indicated, the DNA ends of  $\phi$ 29 and those of the  $\phi$ 29-related phages have a reiteration of three nucleotides (3'-TTT... 5'). Once the replication origins are specifically recognised by the heterodimer formed by the DNA polymerase and the primer TP (Blanco et al., 1987; Freire et al., 1996; González-Huici et al., 2000a; González-Huici et al., 2000b; Pérez-Arnaiz et al., 2007), the DNA polymerase catalyses the formation of a phosphoester bond between the initiator dAMP and the hydroxyl group of Ser<sup>232</sup> of the TP (see Figure 1), a reaction directed by the second T at the 3' end of the template strand (Méndez et al., 1992) and performed by the same catalytic residues responsible for canonical polymerisation (Blanco & Salas, 1995, 1996). Modelling of an incoming dNTP and a template strand onto the  $\phi$ 29 DNAPol/primer TP complex shows that the priming Ser<sup>232</sup> of TP is placed at the catalytic site of the DNA polymerase in line to attack nucleophilically the  $\alpha$ -phosphate of the incoming nucleotide to form the phosphoester bond (Figure 4, left panel). The model also suggests that the 3' end of the template strand goes deep into the catalytic site of the DNA polymerase through the downstream template strand tunnel until it positions the penultimate 3' dTMP of the template strand at the catalytic site, allowing it to direct insertion of the initiator dAMP. To perform TP-DNA full-length synthesis, the TP-dAMP initiation product translocates backwards one position to recover the template information corresponding to the first 3'-T, the so-called sliding-back mechanism that requires a terminal repetition of 2 bp. This reiteration permits, prior to DNA elongation, the asymmetric translocation of the initiation product, TP-dAMP, to be paired with the first T residue (Méndez et al., 1992) (see scheme in Figure 5).

Our studies have shown how the sliding-back mechanism, or variations on it, seems to be a common feature of protein-priming systems to restore full-length DNA. Thus, in the case of the  $\phi$ 29-related phage GA-1, initiation also occurs at the 3' second nucleotide of the template (3'-TTT) and, to a lesser extent, at the third nucleotide (Illana et al., 1996). The  $\phi$ 29-related phage Nf and the *Streptococcus pneumoniae* phage Cp-1 initiate at the 3' third nucleotide of their terminal repetition (3'-TTT) (Longás et al., 2008; Martín et al., 1996b), whereas the *E. coli* phage PRD1 initiates at the fourth nucleotide (3'-CCCC) (Caldentey et al., 1993), requiring two and three consecutive sliding-back steps, respectively, to recover the DNA end information (stepwise sliding-back). The adenovirus genome ends present a more complex reiteration (3'-GTAGTA), the 3' fourth to six template positions directing the



formation of the TP-CAT initiation product. Thus, recovery of the 3' ends is performed by a single jump, after which TP-CAT is paired with the terminal 3'-GTA (jumping-back) (King & van der Vliet, 1994) (see scheme in Figure 5).

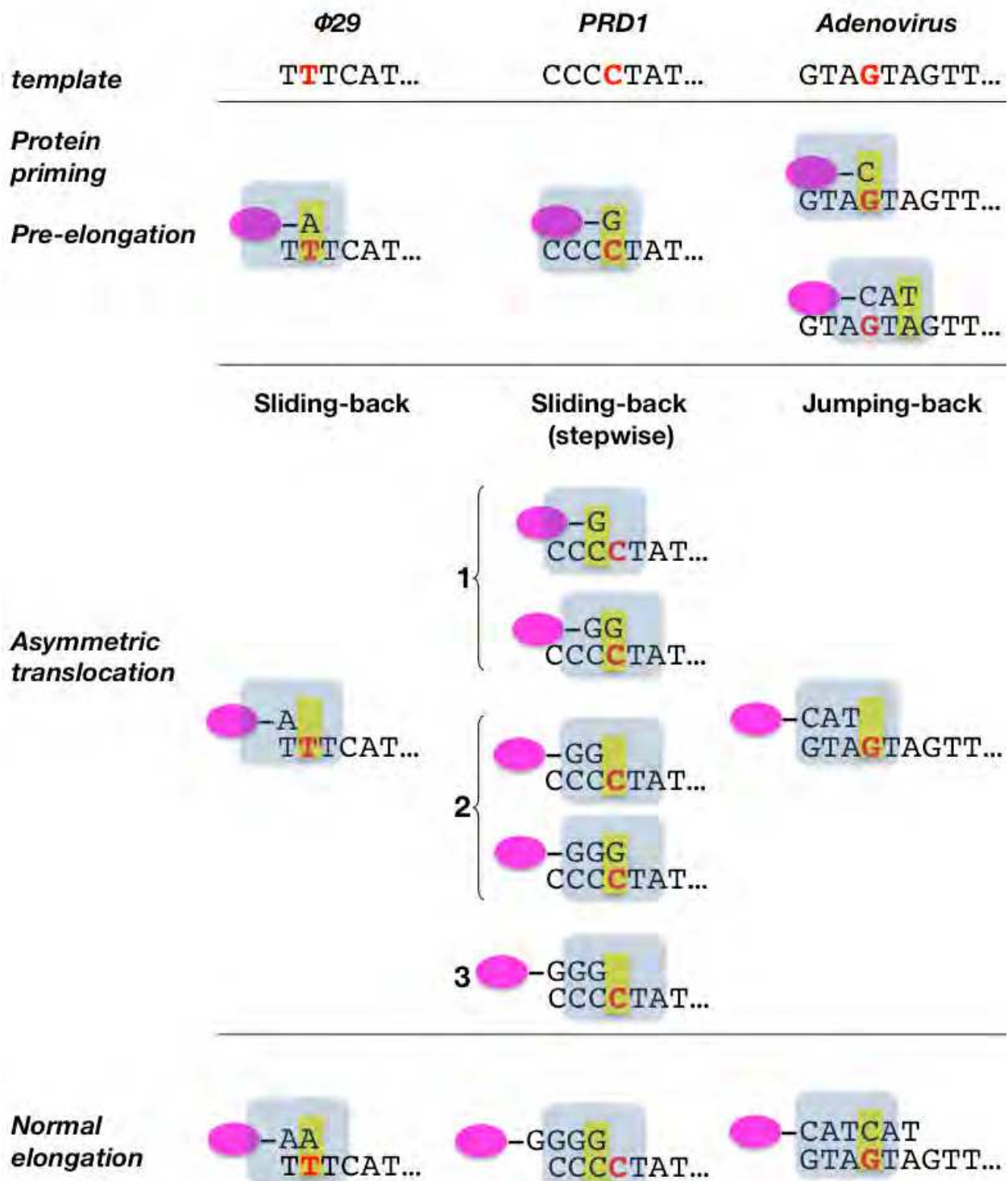


Fig. 5. Sliding-back (jumping-back) model for the transition from initiation to elongation. TP is represented as a pink oval and DNA polymerase as a grey square. The internal template nucleotide that directs the insertion of the initiator nucleotide is shown in bold red letter. Yellow box represents the catalytic active site of the DNA polymerase.

### 2.2.4.1 Why sliding-back during genome replication?

Protein-primed initiation can be predicted to be an inaccurate reaction. The insertion fidelity of protein-primed initiation in  $\phi$ 29 has been shown to be quite low, the insertion discrimination factor being about  $10^2$ . Even more, the 3'-5' exonuclease activity of  $\phi$ 29 DNAPol is unable to act on the TP-dNMP initiation complex, precluding the possibility that a wrong dNMP covalently linked to TP could be proofread (Esteban et al., 1993). If misincorporation during the initiation reaction takes place, a mismatch would be produced with the terminal 3'-T on the template after the sliding-back of the incorrect TP-dNMP complex, unfavouring the further elongation step. If an incorrect TP-dNMP product were elongated it would be corrected in the next round of replication, because it would not serve as a template (Esteban et al., 1993). Thus, the sliding-back and its variations are envisaged to increase the fidelity during the initiation reaction, as several base pairing checking steps have to occur before elongation of the initiation product takes place (King & van der Vliet, 1994; Méndez et al., 1992). The fact that other TP-containing genomes also contain some kind of sequence repetitions at their ends supports the hypothesis that the sliding-back type of mechanism could be a common feature of protein-primed replication systems (Méndez et al., 1992).

### 2.3 Transition from protein-primed to DNA-primed replication

Our functional analyses established that the  $\phi$ 29 DNAPol/primer TP heterodimer do not dissociate immediately after initiation or after sliding-back (Méndez et al., 1997). The same DNA polymerase molecule incorporates 5 nucleotides to the primer TP while is still complexed with the latter (initiation mode), undergoes some structural change during incorporation of nucleotides 6-9 (transition) and finally dissociates from the primer TP when nucleotide 10 is incorporated into the nascent DNA chain (elongation mode) (Méndez et al., 1997). These results probably reflect the polymerase requirement for a DNA primer of a minimum length to catalyse DNA elongation efficiently.

We have shown that the strength of the  $\phi$ 29 DNA pol-primer TP interaction is differently contributed by the TP priming and intermediate domains (Pérez-Arnaiz et al., 2007), supporting the model proposed for the transition from the protein-primed initiation to the DNA-primed elongation modes (Kamtekar et al., 2006). Thus, the TP intermediate domain would be in a fixed orientation on the polymerase by means of stable contacts with the TPR1 subdomain. The weak interaction observed with the DNA polymerase would facilitate the TP priming domain to rotate following the helicoidal pathway as DNA is synthesized. The relative motion of the TP priming domain with respect to the fixed TP intermediate domain would be possible due to the flexibility of the hinge region that connects both domains. After incorporation of 6-7 nucleotides the proximity of the priming Ser to the hinge region would impede a further priming domain rotation, promoting complex dissociation (Kamtekar et al., 2006) (see Figure 6).

### 2.4 DNA-primed elongation

Once the initiation, sliding-back and transition steps have been fulfilled and  $\phi$ 29 DNAPol has separated from the primer TP, the DNA polymerase resumes TP-DNA replication; therefore, the same DNA polymerase molecule accounts for complete genome replication from a single binding event (Blanco et al., 1989). As mentioned before, the high stability of the  $\phi$ 29 DNAPol/DNA complex, by virtue of the "internal sliding-clamp-like" structure

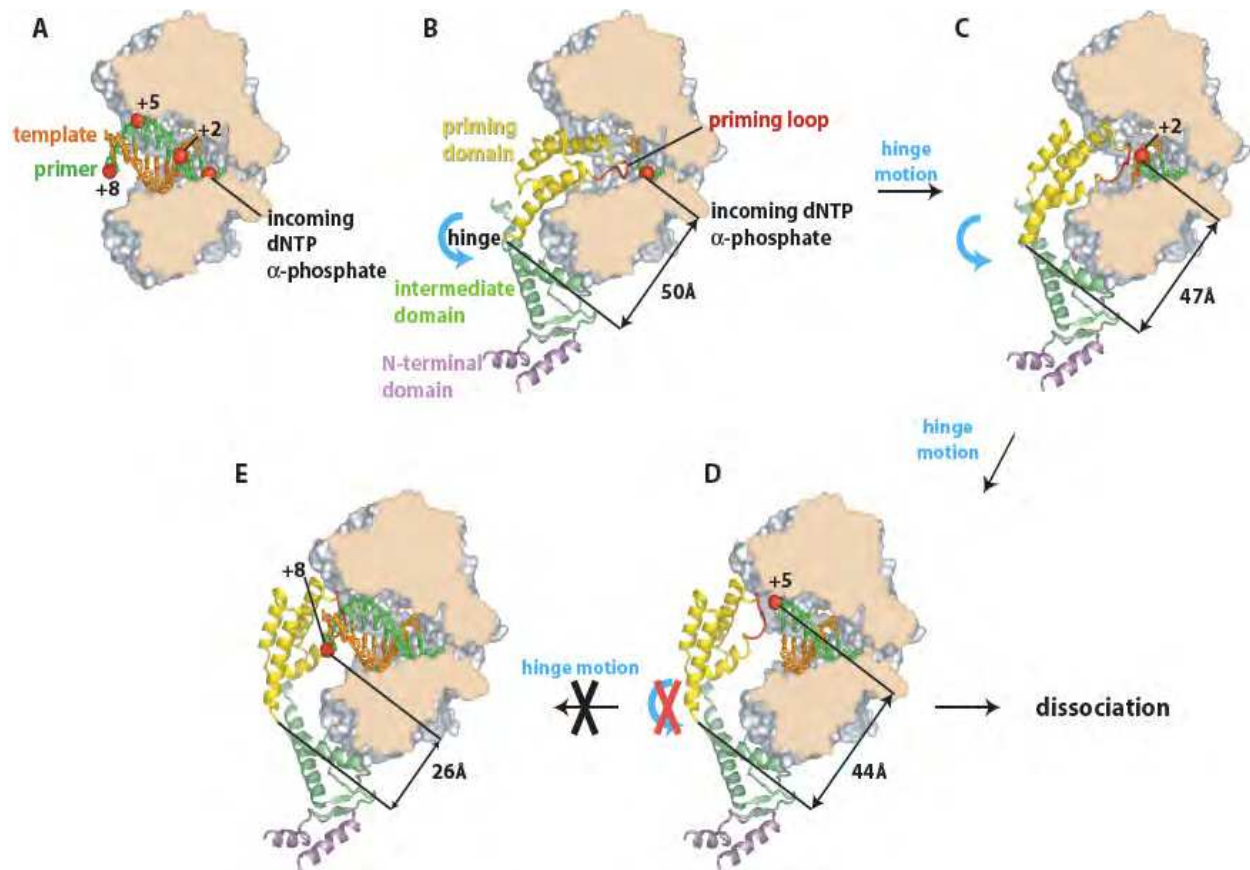


Fig. 6. A model for the transition from initiation of replication to elongation (see main text for details). Reproduced with permission from Kamtekar, S., Berman, A.J., Wang, J., Lázaro, J.M., de Vega, M., Blanco, L., Salas, M. & Steitz, T.A. (2006). The phi29 DNA polymerase:protein-primer structure suggests a model for the initiation to elongation transition. *EMBO J.* Vol. 25, No. 6, pp. 1335-1343.

formed by thumb, palm, TPR1 and TPR2 subdomains, allows the polymerase to perform complete DNA replication without the assistance of processivity factors, in contrast to most replicative DNA polymerases. In addition, the singular TPR2 subdomain enables the  $\phi$ 29 DNApol to couple polymerisation to the unwinding of the downstream dsDNA regions (strand displacement capacity) making unnecessary the intervention of a helicase-like protein (Blanco et al., 1989).

As mentioned above, since replication starts at both  $\phi$ 29 DNA ends and is coupled to strand displacement, this results in the generation of so-called type-I replication intermediates (see Figure 1). The ssDNA stretches generated are bound by the viral SSB, essential for elongation of replication *in vivo* (Mellado et al., 1980). Binding of  $\phi$ 29 SSB to  $\phi$ 29 DNA replicative intermediates has been demonstrated to occur *in vitro* (Gutiérrez et al., 1991b). The protein binds in a cooperative way (Soengas, et al., 1994) stimulating dNMP incorporation during  $\phi$ 29 DNA replication (Gutiérrez et al., 1991a), and increasing the elongation rate, mainly when  $\phi$ 29 DNApol mutants impaired in strand displacement are used, probably due to the helix destabilising activity of the  $\phi$ 29 SSB (Soengas et al., 1995). When the two converging DNA polymerases merge, a type-I replication intermediate becomes physically separated into two type-II replication intermediates (Gutiérrez et al., 1991b; Inciarte et al., 1980). Continuous elongation by the DNA polymerase completes replication of the parental strand.

### 2.5 Termination of TP-DNA replication

For termination of genome replication, the  $\phi$ 29 DNApol encounters a covalently linked TP molecule (see Figure 7, upper panel), terminating DNA replication by an as yet unknown mechanism. As already indicated, the TPR2 insertion of the  $\phi$ 29 DNApol, together with the exonuclease domain and the fingers and palm subdomains form a downstream tunnel whose narrow dimensions precludes the passage of a dsDNA through it. Considering that the terminal base of the template is covalently linked to the Ser<sup>232</sup> of TP, the priming loop of the parental TP has to enter the downstream template-binding tunnel of  $\phi$ 29 DNApol to allow the last 5'-dAMP (covalently linked to the parental TP) to be replicated. However, the dimensions of such a tunnel do not allow the TP to pass through it to reach the catalytic active site, so a disruption of the interactions of TPR2 with the exonuclease domain and the thumb subdomain of the polymerase is required to get an opened tunnel (see Figure 7, lower

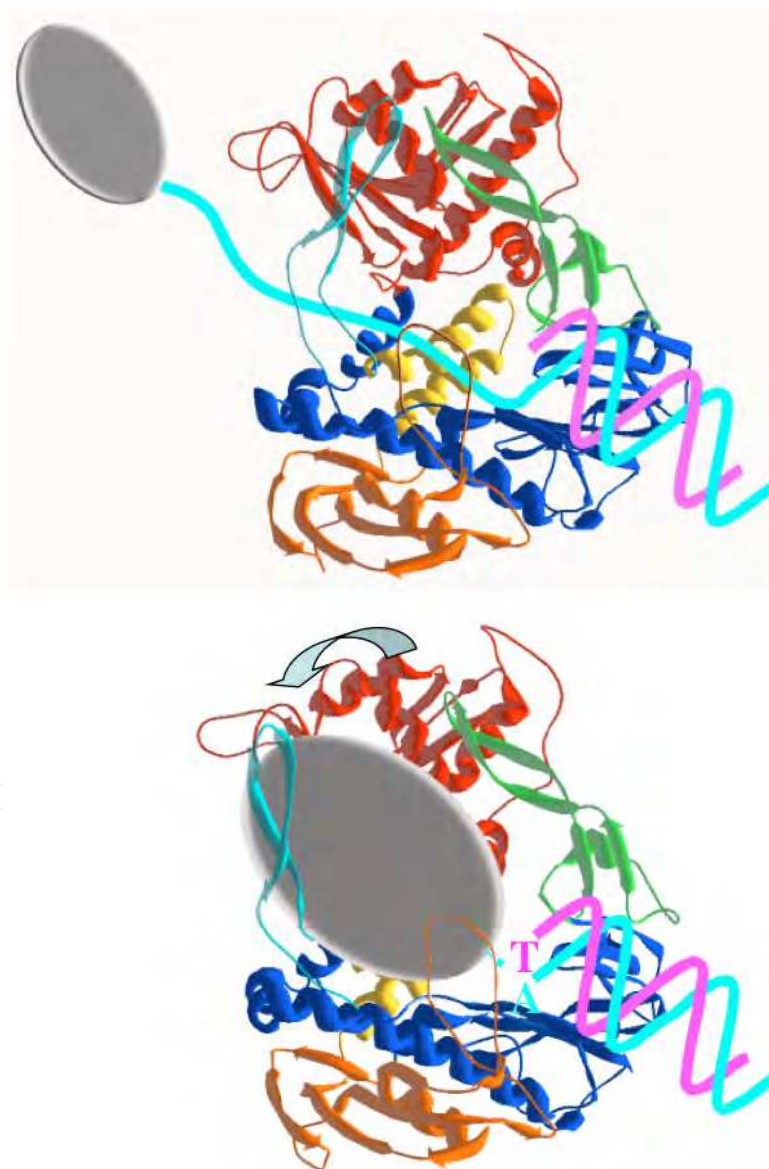


Fig. 7. Hypothetical partial opening of the TPR2 and thumb subdomains to allow the last 5'-template nucleotide to reach the polymerisation active site of the DNA polymerase. Template and primer strands are coloured in blue and magenta, respectively.



panel). By using primer/5'-streptavidine-bound template DNA to mimic the parental TP, we showed that all but 4 terminal nucleotides were replicated by the  $\phi$ 29 DNApol (de Vega et al., 1999). This situation could be parallel to the physiological one, in which  $\phi$ 29 DNApol has to finish the replication of a DNA template strand that contains a TP molecule covalently linked at its 5' end. It is tempting to speculate that during termination of replication, a specific interaction between DNA polymerase and TP is required to allow synthesis of the terminal nucleotide.

### 2.6 Terminal protein-primed DNA amplification

By using appropriate amounts of the four  $\phi$ 29 DNA replication proteins described above, primer TP, DNA polymerase, the double-stranded DNA-binding protein, and the single-stranded DNA-binding protein, we were able to amplify limited amounts of the  $\phi$ 29 TP-DNA molecule by three orders of magnitude after 1 hr of incubation at 30 °C. Moreover, the quality of the amplified DNA was demonstrated by transfection experiments, in which infectivity of the synthetic (amplified)  $\phi$ 29 TP-DNA, measured as the ability to produce phage particles, was identical to that of the natural  $\phi$ 29 TP-DNA obtained from virions (Blanco et al., 1994), leading us to establish some of the requisites for the development of isothermal DNA amplification strategies based on the  $\phi$ 29 DNA replication machinery to amplify very large (> 70 kb) segments of exogenous DNA.

### 3. *In vivo* compartmentalisation of $\phi$ 29 DNA replication

It is well established that replication of phage genomes occurs at specific intracellular locations by the use of large organising structures that bring together replication factors to enhance the efficiency of the replication process. Some lines of evidence support that replication of phage DNA takes place in close association with the bacterial membrane (Firshein, 1989; Mosig & Macdonald, 1986; Siegel & Schaechter, 1973). Our recent investigations have also given evidences concerning compartmentalization of phage  $\phi$ 29 DNA replication in *B. subtilis* cells.

We have shown that  $\phi$ 29 TP binds to dsDNA through its N-terminal domain in a non-sequence dependent way, both *in vitro* and *in vivo* (Muñoz-Espín et al., 2010; Zaballos & Salas, 1989). This capacity enables the parental TP, and therefore the viral TP-DNA, to associate with the bacterial nucleoid early after injection of the  $\phi$ 29 genome (see scheme in Figure 8) where the *B. subtilis* RNA polymerase is also located (Muñoz-Espín et al., 2010). There, synthesis of the  $\phi$ 29 early proteins DNA polymerase, primer TP, SSB and DBP, essential for *in vivo*  $\phi$ 29 DNA replication takes place. Once synthesized, primer TP binds the bacterial chromosome and recruits the DNA polymerase to form the heterodimer that will recognise TP-DNA replication origins. At this stage, replication of TP-DNA will start from both terminal origins giving rise to the replicative intermediates type I and II, as it has been observed by electron microscopy (Harding & Ito, 1980; Inciarte et al., 1980; Sogo et al., 1982) (see scheme in Figure 8). By fluorescence microscopy, we have shown that at middle infection times, the DNA polymerase, TP, and viral TP-DNA are reorganised adopting a peripheral helix-like distribution toward the poles of the cell (Muñoz-Espín et al., 2009; Muñoz-Espín et al., 2010). Although the pathway followed by the  $\phi$ 29 replicative machinery from the nucleoid to bacterial peripheral regions remains to be determined, it has been proposed that it could travel associated to the replication of bacterial chromosome (Muñoz-Espín et al., 2010), as

newly synthesized bacterial DNA is translocated towards the cell poles *via* a helical structure (Berlatzky et al., 2008).

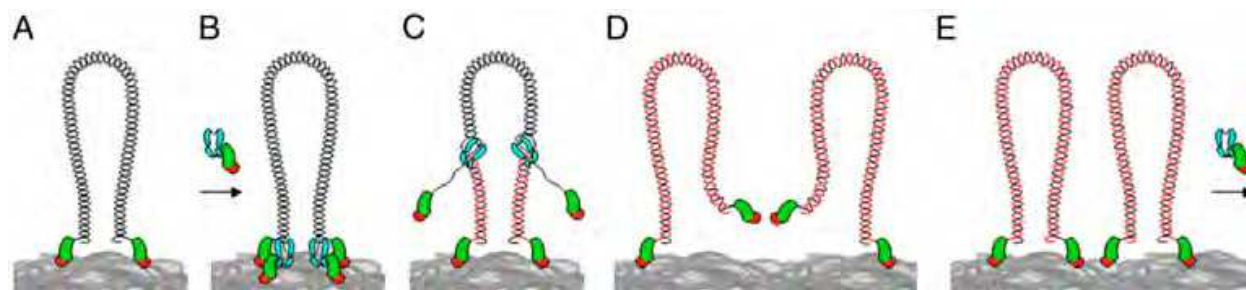


Fig. 8. Model of nucleoid-associated early  $\phi$ 29 DNA replication organised by the TP. (A) Attachment of  $\phi$ 29 TP-DNA to the bacterial nucleoid surface (gray mass at bottom) through the N-terminal domain (red) of the parental TPs (red and green). (B) Recruitment of the  $\phi$ 29 DNAPol/primer TP heterodimer to the replication origins of TP-DNA. (C) Processive elongation of the nascent DNA strands (red lines) coupled to strand displacement. (D and E) Once DNA replication is completed, two  $\phi$ 29 TP-DNA molecules are ready for another round of replication. For simplicity, other viral proteins involved in DNA replication are not drawn. Reproduced with permission from Muñoz-Espín, D., Holguera, I., Ballesteros-Plaza, D., Carballido-López, R. & Salas, M. (2010). Viral terminal protein directs early organisation of phage DNA replication at the bacterial nucleoid. *Proceedings of the National Academy of Sciences of USA*. Vol. 107, No. 38, pp. 16548-16553.

The  $\phi$ 29 membrane protein p16.7 has a non-sequence specific DNA binding capacity (Meijer et al., 2001) that enables it to interact with the  $\phi$ 29 replication origins through recognition of the parental TP (Serna-Rico et al., 2003). This protein also shows a helix-like pattern at the membrane of infected cells, most probably being involved in the compartmentalization of *in vivo* membrane-associated  $\phi$ 29 DNA replication through a direct contact with TP-DNA, organising the viral replicating intermediates at numerous peripheral locations (Albert et al., 2005; Muñoz-Espín et al., 2009) (see Figure 9). Different experimental approaches have demonstrated that protein p16.7 interacts directly with the *B. subtilis* actin-like cytoskeleton protein MreB (Muñoz-Espín et al., 2009). This protein forms helix-like filamentous structures *in vivo* essential for the control of the bacterial rod-shaped morphology (Jones et al., 2001), suggesting that MreB would contribute to efficient  $\phi$ 29 DNA replication by recruiting protein p16.7 to the appropriate sites at the cell membrane allowing simultaneous replication of multiple templates at numerous peripheral locations. Further evidence is the finding that  $\phi$ 29 DNA replication is severely affected in  $\Delta$ MreB cytoskeleton mutants (Muñoz-Espín et al., 2009).

$\phi$ 29 gene 1 codes for a small protein (p1) that assembles into long protofilaments forming bidimensional sheets (Bravo & Salas, 1998) in association with the bacterial membrane *in vivo* (Serrano-Heras et al., 2003). Cell fractionation studies indicated that protein p1 is membrane-associated both during synthesis of  $\phi$ 29 DNA and after blocking  $\phi$ 29 DNA replication (Bravo & Salas, 1997). Membrane-association of p1 also occurs in the absence of other viral components, suggesting that protein p1 contacts the bacterial membrane directly (Bravo & Salas, 1997; Serrano-Heras et al., 2003). Phage  $\phi$ 29 DNA replication was shown to be significantly reduced when non-suppressor *B. subtilis* cells were infected with mutant phage *sus1*(629) at 37°C (Bravo & Salas, 1997; Prieto et al., 1988). In addition, protein p1 was

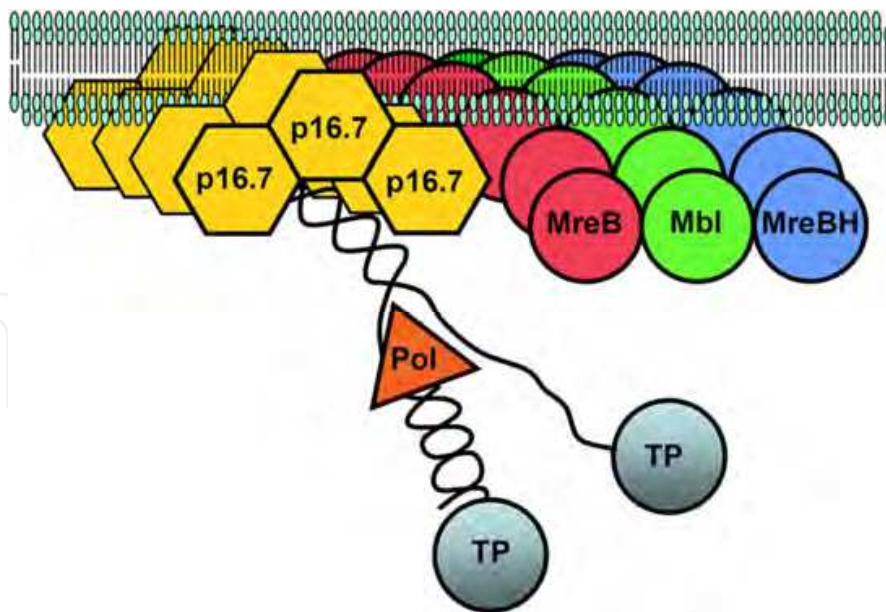


Fig. 9. Model of membrane-associated late  $\phi 29$  DNA replication organised by the MreB cytoskeleton. MreB, Mbl, and MreBH are shown to form a putative triple helical structure closely associated with the inner surface of the membrane. Each dimeric unit of protein p16.7 is represented by a yellow hexagon. The tridimeric p16.7 units form oligomers in a helix-like localisation at the cell membrane. For simplicity, other viral proteins involved in DNA replication are not drawn. Reproduced with permission from Muñoz-Espín, D., Daniel, R., Kawai, Y., Carballido-López, R., Castilla-Llorente, V., Errington, J., Meijer, WJ & Salas, M. (2009). The actin-like MreB cytoskeleton organises viral DNA replication in bacteria. *Proceedings of the National Academy of Sciences of USA*. Vol. 106, No. 32, pp. 13347-13352.

also shown to interact with the viral TP *in vitro* (Bravo et al., 2000). These results suggest that protein p1 is a component of a membrane-associated structure which would play, in addition to p16.7, a role in the organisation of  $\phi 29$  DNA replication by providing an anchoring site for the replication machinery.

#### 4. $\phi 29$ codes for a *B. subtilis* uracil-DNA glycosylase inhibitor.

Downstream to  $\phi 29$  gene 1 there is an open reading frame (ORF56) encoding an acidic protein of 56 amino acids (protein p56) that shows a dimeric state in solution and that accumulates throughout the  $\phi 29$  infective cycle (Serrano-Heras et al., 2006). Chemical crosslinking assays showed that viral p56 interacts, both during the infective process and in the absence of viral components, with *B. subtilis* uracil-DNA glycosylase (UDG), a key enzyme that eliminates uracil residues from the DNA during the Base Excision Repair (BER) pathway (Serrano-Heras et al., 2006). *In vitro* assays demonstrated that such an interaction inhibits UDG. In agreement with this, extracts from  $\phi 29$ -infected cells showed a dramatic drop in bacterial UDG activity, in contrast to the nearly 90% of the activity that remained after incubation with extracts from non-infected cells.

##### 4.1 Role of protein p56 in $\phi 29$ DNA replication

Inhibition of the cellular UDG activity after phage infection was established previously in two systems. The first was the inhibition of the host UDG after infection of *B. subtilis* with

the uracil-containing phage PBS2 (Friedberg et al., 1975). In this case, inhibition of cellular UDG was crucial to prevent the elimination of uracils from the viral genome. Similarly, bacteriophage T5 inhibits *E. coli* UDG, although the physiological role of this inhibition is still unclear, as its genome does not contain uracils (Warner et al., 1980).

Both, *in vitro* and *in vivo* assays have recently demonstrated the ability of  $\phi$ 29 DNApol to insert, extend and use as template dUMP residues with nearly the same efficiency that dTMP to give full-length DNA (Serrano-Heras et al., 2008). Whereas removal by the UDG of the uracil residues incorporated into the phage genome caused a drastic reduction in the efficiency of  $\phi$ 29 DNA replication, as  $\phi$ 29 DNApol is unable to use as template the resulting abasic sites (de Vega & Salas, unpublished results), such an inhibitory effect caused in replication by UDG was counteracted by the addition (*in vitro*) or expression (*in vivo*) of protein p56 (Serrano-Heras et al., 2008).

As in the case of bacteriophage T5,  $\phi$ 29 TP-DNA does not contain uracils, then, why does phage  $\phi$ 29 synthesize a UDG inhibitor? It has been suggested that this inhibition is related to the mechanism of  $\phi$ 29 DNA replication. As illustrated in Figure 1, during  $\phi$ 29 TP-DNA replication replicative intermediates (type-I and type-II) with long stretches of single-stranded DNA are generated. The presence of uracil in the replicative intermediates could recruit components of the cellular BER pathway, such as UDGs and AP endonucleases. Further removal of the uracil moiety would render an abasic site that cannot be used as template by the  $\phi$ 29 DNApol. By the contrary, the AP site will be further recognised by the cellular AP endonuclease that would introduce a nick into the phosphodiester backbone with accompanying loss of the terminal DNA region, giving rise to shorter viral DNA molecules lacking one parental TP. Therefore, the action of the cellular UDG on single-stranded DNA regions of the  $\phi$ 29 replicative intermediates would be harmful for viral replication (Serrano-Heras et al., 2008).

## 5. Conclusions and future research

The availability of an efficient *in vitro*  $\phi$ 29 TP-DNA replication system, as well as the biochemical characterisation of the different proteins involved, have greatly contributed to lay the foundations of the different steps in the protein-priming mechanism of DNA replication. In this, a specific DNA polymerase catalyses the formation of a phosphoester bond between the initiator dNMP and the OH group of a specific residue in the TP. *In vitro* systems have been also developed for the replication of adenovirus and bacteriophages GA1, Nf, PRD1 and Cp1, showing that they use a similar protein-priming mechanism for the initiation of replication. Thus, the mechanism of initiation of  $\phi$ 29 DNA replication can be extrapolated to other organisms containing a TP covalently linked to the 5'-ends of their genomes, as human hepatitis B virus, mitochondrial plasmids, linear chromosomes and plasmids of *Streptomyces*, as well as several virus infecting Archaea, as halovirus.

Despite of the advances in the knowledge of the protein-priming process, several questions regarding the initiation at internal positions of the TP-containing genomes remain to be elucidated, as the basis for the specificity for the templating nucleotide. In this respect, the use of chimerical TPs, constructed by swapping the priming domains of the related  $\phi$ 29 and Nf proteins, allowed us to conclude that this domain is one of the structural determinants that dictates the internal 3' nucleotide used as template during initiation. On the other hand, the backwards motion of the primer TP with respect to the fixed template molecule, implies a breakage of the pair TP-A:T (in the case of bacteriophages  $\phi$ 29, Cp1, Nf and GA-1) or, most



drastically the triple base pairing TP-CAT:GTA, as in the case of adenovirus. This energetically unfavoured step should be explained by a power stroke mechanism, by which the energy released after dissociation of the pyrophosphate could drive the backwards movement of the TP-dNMP initiation product with respect to the DNA polymerase and template strand with the consequent correct base pairing with the preceding 3' nucleotide, to reach a more energetically favoured situation. The elucidation of the conformational changes that govern the sliding-back mechanism will give the clues to understand such a special way to initiate genome replication.

Termination of replication of TP-containing genomes is still an unresolved matter. We have shown that protein-primed DNA polymerases exhibit an exquisite specificity for their TP counterparts during the initiation step of TP-DNA replication. Based on the  $\phi 29$  DNApol structure, it seems obvious that to let the last 5'dAMP to be copied during termination, at least the parental TP priming loop of the template strand has to access the catalytic site of the polymerase following the downstream template tunnel pathway. The question that arises is whether there is also specificity between the DNA polymerase and the parental TP during termination, and how the DNA polymerase performs this step.

We are starting to understand how replication of  $\phi 29$  is organised and compartmentalised into the bacterium, acting in concert with cellular factors to increase the efficiency of this biological process. Thus, early after  $\phi 29$  infection TP-DNA replication takes place at the bacterial nucleoid by means of a non-specific interaction between the parental TP and the bacterial chromosome. Later during infection, the  $\phi 29$  DNA replication machinery is organised in peripheral helix-like structures through an interaction of the phage protein p16.7, which recognises the dsDNA of replicating TP-DNA molecules, with the actin-like MreB cytoskeleton.

In addition, phage  $\phi 29$  protein p56 is essential in the viral DNA replication cycle since it prevents the impairment caused by the host UDG. Inhibition of UDG has been proposed to be a defence mechanism developed by  $\phi 29$  to prevent formation of abortive replicative intermediates. This is the first case reported of an UDG inhibitor encoded by a non-uracil containing DNA.

The mechanistic details of how *B. subtilis* MreB is specifically employed by phage  $\phi 29$ , and how they are temporally and spatially organized may be main directions for future experiments. Since bacteriophages contain genomes with a limited size due to their small dimensions, it is expected that new bacterial proteins interacting with viral components may be discovered. A major challenge is to identify these novel targets that might be used by bacteriophages to optimize the production of high numbers of progeny.

## 6. Acknowledgements

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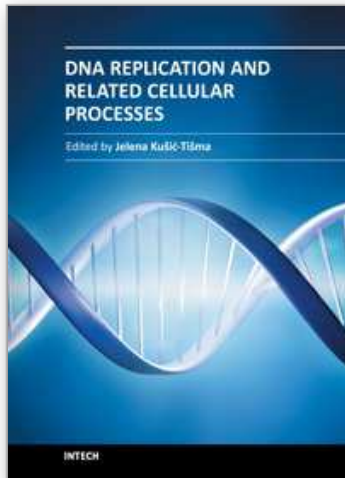
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## **DNA Replication and Related Cellular Processes**

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Since the discovery of the DNA structure researchers have been highly interested in the molecular basis of genome inheritance. This book covers a wide range of aspects and issues related to the field of DNA replication. The association between genome replication, repair and recombination is also addressed, as well as summaries of recent work of the replication cycles of prokaryotic and eukaryotic viruses. The reader will gain an overview of our current understanding of DNA replication and related cellular processes, and useful resources for further reading.

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