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Protein-primed DNA replication: a transition between two modes of priming by a unique DNA polymerase

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Phage ϕ29 from *Bacillus subtilis* is a paradigm of the protein-primed replication mechanism, in which a single-subunit DNA polymerase is involved in both the specific protein-primed initiation step and normal DNA elongation. To start ϕ29 DNA replication, the viral DNA polymerase must interact with a free molecule of the viral terminal protein (TP), to prime DNA synthesis once at each ϕ29 DNA end. The results shown in this paper demonstrate that the DNA polymerase–primer TP heterodimer is not dissociated immediately after initiation. On the contrary, there is a transition stage in which the DNA polymerase synthesizes a five nucleotide-long DNA molecule while complexed with the primer TP, undergoes some structural change during replication of nucleotides 6–9, and finally dissociates from the primer protein when nucleotide 10 is inserted onto the nascent DNA chain. This behaviour probably reflects the polymerase requirement for a DNA primer of a minimum length to efficiently catalyze DNA elongation. The significance of such a limiting transition stage is supported by the finding of abortive replication products consisting of the primer TP linked up to eight nucleotides, detected during *in vitro* replication of ϕ29 TP-DNA particularly under conditions that decrease the strand-displacement capacity of ϕ29 DNA polymerase.

Keywords: linear DNA replication/protein priming/sliding back/terminal protein

Introduction

Protein priming is one of the mechanisms found in evolution to overcome the problem of replicating the molecular ends of linear genomes. These DNA regions cannot be replicated via RNA priming, since the elimination of the primer at the DNA ends would result in gaps that could not be filled in by any DNA polymerase. In the protein priming alternative, the DNA polymerase initiates DNA synthesis using as primer a hydroxyl group provided by a Ser, Thr or Tyr residue of a specific protein. After this initiation reaction, the primer protein remains covalently linked to the 5′ end of the new DNA chain, and is referred to as terminal protein (TP). Since both strands are replicated continuously from both DNA ends, no further priming events are required. This mechanism may occur in a wide variety of genomes, including bacteriophages, linear plasmids, eukaryotic viruses (reviewed by Salas, 1991; Salas *et al.*, 1996) and perhaps some bacterial chromosomes (Chen, 1996).

The ϕ29 genome consists of a linear double-stranded DNA (dsDNA), 19 285 bp long, with a TP covalently linked to each 5′ end. To initiate replication, a histone-like viral protein forms a nucleoprotein complex with the origins of replication that probably contributes to the unwinding of the double helix at both DNA ends (Serrano *et al.*, 1990). Then, the DNA polymerase catalyses the addition of the first dAMP to the hydroxyl group provided by Ser232 of the TP. This protein-primed event occurs opposite to the second 3′ nucleotide of the template, and the initiation product (TP-dAMP) slides back one position in the DNA to recover the terminal nucleotide (Méndez *et al.*, 1992). After initiation, the same DNA polymerase replicates one of the DNA strands while displacing the other. The high processivity and strand displacement ability of ϕ29 DNA polymerase makes it possible to complete replication of the ϕ29 TP-containing genome (TP-DNA) in the absence of any helicase or accessory processivity factors (reviewed by Blanco and Salas, 1996).

The genes encoding the DNA polymerase and the primer TP are linked (Yoshikawa and Ito, 1982) and transcribed from the same promoter (Barthelemy *et al.*, 1986), and both proteins remain associated when purified from ϕ29-infected *Bacillus subtilis* cells (Watabe *et al.*, 1983; Matsumoto *et al.*, 1984). A physical interaction between ϕ29 TP and DNA polymerase was described initially by Blanco *et al.* (1987), based on the differential sedimentation of the free TP (31 kDa) and the heterodimeric DNA polymerase–TP complex (97 kDa), after ultracentrifugation in a glycerol gradient. Using this approach, and taking advantage of the fact that ϕ29 DNA polymerase is able to catalyse initiation *in vitro* in the absence of template DNA (Blanco *et al.*, 1992), it was shown that the initiation product, TP-dAMP, remains associated with the DNA polymerase (Blanco *et al.*, 1992).

It is likely that an efficient transition between protein-primed initiation and DNA elongation will involve the dissociation of the DNA polymerase–TP heterodimer. We have tried to characterize the step at which this dissociation occurs, using an *in vitro* ϕ29 DNA replication system and single-stranded oligonucleotides with the sequence of the ϕ29 replication origins, shown to be active templates for both TP-primed initiation and further elongation (Méndez *et al.*, 1992). Our results indicate that, under conditions that resemble an opened ϕ29 origin of replication, the primer TP and the DNA polymerase still form a complex after the synthesis of a five nucleotide long DNA molecule. Dissociation starts at nucleotide 6 and is achieved com-

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pletely after the incorporation of nucleotide 10. Interestingly, in a TP-independent reaction, we found that ϕ29 DNA polymerase requires a minimum DNA primer chain of six nucleotides to catalyse elongation efficiently. In addition, abortive replication products consisting of the primer TP linked up to eight nucleotides have been detected during *in vitro* replication of the natural ϕ29 TP-DNA by wild-type and several mutant derivatives of ϕ29 DNA polymerase. Based on these data, the different stages of the transition between TP-primed initiation and normal DNA elongation are discussed in the light of changes in the DNA polymerase–TP–DNA association.

Results

ϕ29 DNA polymerase and TP still form a complex after DNA-templated initiation and sliding back

To study the association–dissociation stages of the DNA polymerase–TP heterodimer during the first steps of ϕ29 DNA replication, single-stranded DNA (ssDNA) oligonucleotides corresponding to the template strand of the ϕ29 right or left DNA ends were used as templates in an *in vitro* replication assay carried out with purified TP and an exonuclease-deficient ϕ29 DNA polymerase. Then, the replication products were subjected to ultracentrifugation analysis in 15–30% glycerol gradients, under the conditions described in Materials and methods. The aim of this centrifugation step is to fractionate the replication products [TP-$(dNMP)_n$] that are still associated with the DNA polymerase (mol. wt ⩾97 kDa) from those already dissociated (mol. wt of the free TP, 31 kDa). The different fractions of the gradient are subjected to SDS–PAGE in conditions that allow the resolution of the different TP-$(dNMP)_n$ intermediates.

Oligonucleotide oriL(10) (sequence: 3′ TTTCATTCGG) was firstly used as template in an *in vitro* replication assay in the presence of [α-^{32}P]dATP as the only nucleotide, thus limiting replication to the three terminal T residues. The reaction products were subjected to ultracentrifugation in glycerol gradients and electrophoresis as described above. The Coomassie staining of the gel (Figure 1A) revealed that the DNA polymerase (67 kDa) and the TP (31 kDa) co-sedimented as a heterodimer [compare with the sedimentation of monomeric bovine serum albumin (BSA), a 66 kDa protein]. As shown in Figure 1B, not only the initiation product, TP-dAMP, but also TP-$(dAMP)_2$ and TP-$(dAMP)_3$ migrated in the glycerol gradient at the position corresponding to the DNA polymerase–TP heterodimer. Thus, neither DNA-templated protein-primed initiation nor the sliding-back step result in the dissociation of the DNA polymerase–primer TP complex. The same result was obtained with oligonucleotide oriR(12), corresponding to the ϕ29 right origin of replication (results not shown).

ϕ29 DNA polymerase is able to synthesize a nine nucleotide long DNA molecule still forming a heterodimer with the primer TP

Having established that ϕ29 TP and DNA polymerase remain complexed after the replication of the three terminal nucleotides, the next step was to determine the step at which this complex dissociates. This was addressed using the same approach as above, but performing the *in vitro* assay in the presence of the four nucleotides to allow complete replication of the template molecule oriL(10). As shown in Figure 2A, the replication intermediates up to TP-$(dNMP)_9$ migrated in the position corresponding to the heterodimeric complex (fractions 10–13). Interestingly, the products TP-$(dNMP)_6$ to TP-$(dNMP)_8$ showed a slightly slower sedimentation (fractions 12–15), suggesting that this change is the consequence of some structural modification in the heterodimer, when the polymerase is about to separate from the primer TP. In fact, the band corresponding to TP-$(dNMP)_{10}$ showed a drastic change in sedimentation towards the position of the free TP, suggesting that the DNA polymerase has dissociated from the TP and has been further released from the DNA (see scheme in Figure 2C).

However, it could be argued that the 'free' DNA polymerase remains linked to the DNA primer–template junction after dissociation from the primer TP. In that case, the intermediate mobility of the products TP-$(dNMP)_6$ to TP-$(dNMP)_9$ would correspond to molecular complexes in which the template DNA is acting as a 'bridge' between the TP and the DNA polymerase. Following this reasoning, dissociation of the DNA polymerase from the DNA would occur only when replication is completed [TP-$(dNMP)_{10}$]. An argument against this interpretation is that the salt concentration present in the sedimentation analysis (0.2 M NaCl) does not allow a stable interaction between the DNA polymerase and the DNA. However, to rule out this possibility unequivocally, a single-stranded oligonucleotide of slightly increased length [oriL(12)] was used as template for replication. If DNA polymerase–TP dissociation really occurred at position 10, all the replication products equal to or longer than TP-$(dNMP)_{10}$ would migrate at the 'free TP' position. On the contrary, if both proteins remain 'bridged' by the template after dissociation, only the final replication product [TP-$(dNMP)_{12}$] would migrate at the free TP position. The experiment (Figure 2B) showed that the three bands corresponding to TP-$(dNMP)_{10}$, TP-$(dNMP)_{11}$ and TP-$(dNMP)_{12}$ migrate as 'dissociated' molecules, suggesting that the sharp change in mobility between products TP-$(dNMP)_9$ and TP-$(dNMP)_{10}$ really reflects the dissociation of the DNA polymerase–primer TP heterodimer. Again, the products TP-dAMP to TP-$(dNMP)_5$ migrated in the position corresponding to the heterodimer, whereas the sedimentation of TP-$(dNMP)_6$ to TP-$(dNMP)_8$ was slightly slower, reinforcing the hypothesis that the protein–protein complex undergoes some structural change just before dissociation.

ϕ29 DNA polymerase requires a DNA primer of a minimum length of six nucleotides to catalyse polymerization efficiently

The experiments described above indicated that, after protein-primed initiation, the DNA polymerase remains complexed with the primer TP during the synthesis of at least 6–9 nucleotides. This could indicate the shortest dsDNA molecule that can be bound by ϕ29 DNA polymerase in a polymerization-competent conformation, i.e. with the primer terminus adequately positioned at the polymerization active site. To test this hypothesis in conditions not involving a protein-primed initiation step, the ϕ29 right origin of replication was simulated by

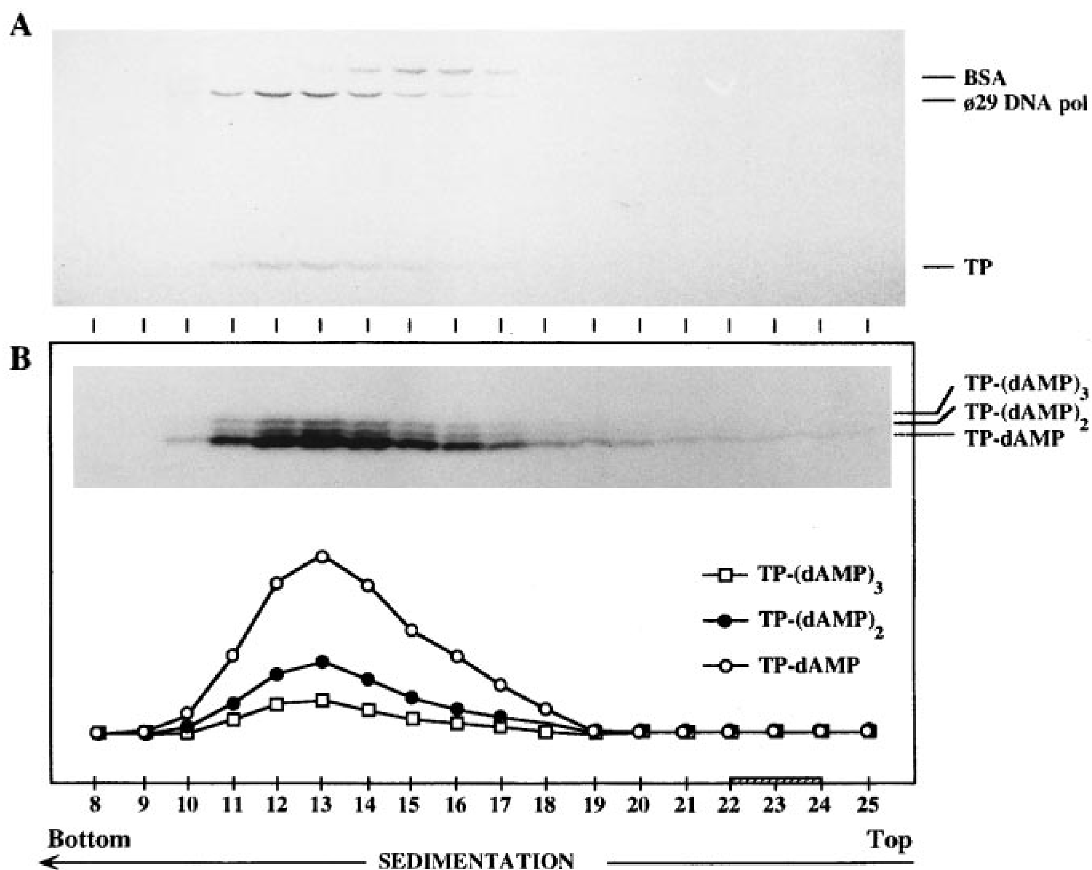

Fig. 1. Interaction between the primer TP and the DNA polymerase after the initiation reaction and the sliding-back step. *In vitro* replication of oligonucleotide oriL(10) was performed in a 15 min reaction as indicated in Materials and methods. dATP was the only nucleotide present in the reaction, thus limiting replication to the three first nucleotides. The replication products were subjected to sedimentation analysis in 15–30% glycerol gradients. After ultracentrifugation, the gradients were collected in 34 fractions and an aliquot of each fraction was analysed by SDS–PAGE. Fractions 1–7 are not shown because they do not contain either single or complexed protein species. (**A**) As shown by Coomassie staining of the gel, ϕ29 DNA polymerase and TP migrated in fractions 11–14, in the position expected for a heterodimer of ~97 kDa. BSA (66 kDa), loaded in the gradient as a control, migrated in fractions 14–17. (**B**) An aliquot of the same fractions as above was subjected to high resolution SDS–PAGE, under the conditions indicated in Materials and methods. The autoradiograph is included as an inset of the lower panel, showing the densitometric quantitation of the bands corresponding to TP-dAMP, TP-$(dAMP)_2$ and TP-$(dAMP)_3$. The shadowed area (fractions 22–24) represents the mobility of the free TP-dAMP product, as determined in independent experiments.

hybridization of two oligonucleotides, the one corresponding to the primer strand being 5′-labelled. The 3′ end of the primer strand can be used either as a substrate for the 3′–5′ exonuclease activity of ϕ29 polymerase or as a primer for the synthetic activity of the enzyme. By adding ϕ29 DNA polymerase in the absence of dNTPs, the 3′–5′ exonuclease activity of the enzyme should generate a set of DNA primers of decreasing lengths, and later addition of the four dNTPs should lead to repolymerization of those primer strands that could still be used by the DNA polymerase. The result of this experiment is presented in Figure 3. The TP-free DNA molecule corresponding to the ϕ29 DNA right end was incubated with the DNA polymerase in the absence of dNTPs, using either Mg^{2+} (lane 2) or Mn^{2+} (lane 5) as metal activators. All the possible primers ranging from one to 28 nucleotides were generated. Upon addition of the four dNTPs, all the DNA primers were repolymerized except those shorter than six nucleotides, which were not used by the DNA polymerase (Figure 3, lanes 3 and 4, and 6 and 7). This is in agreement with the observation that ϕ29 DNA polymerase synthesizes a DNA chain of a minimum of six nucleotides before it starts to change from the initiation mode (complexed with the primer TP) to the elongation mode.

Mutational analysis of the ϕ29 DNA six terminal nucleotides

Six base pairs form an inverted terminal repeat (ITR) at both ϕ29 DNA ends. Having established that ϕ29 TP and DNA polymerase remain complexed during the synthesis of replication intermediates ranging from TP-(dAMP) to TP-$(dNMP)_5$ ('initiation mode' of the DNA polymerase), we asked whether mutations in this conserved ITR would affect the progression of ϕ29 DNA polymerase through this particular stage and/or the transition to the 'elongation mode'. To address this question, a mutational analysis of the ITR was carried out using ssDNA oligonucleotides corresponding to the ϕ29 DNA right end.

As a control, the efficiencies of initiation and replication (i.e. full-length synthesis) of oriR(12), a 12mer with the sequence of the ϕ29 DNA right origin of replication (template strand), were considered as 100% in each case. The individual importance of the six nucleotides forming the ITR for complete replication was analysed using 12mer oligonucleotides with mutations at each of the six terminal bases. Complete replication of a 12mer includes the three stages: 'initiation mode' (nucleotides 1–5), 'transition' (nucleotides 6–9) and 'elongation mode' (nucleotides 10–12). As shown in Table I, substitution of the first, second

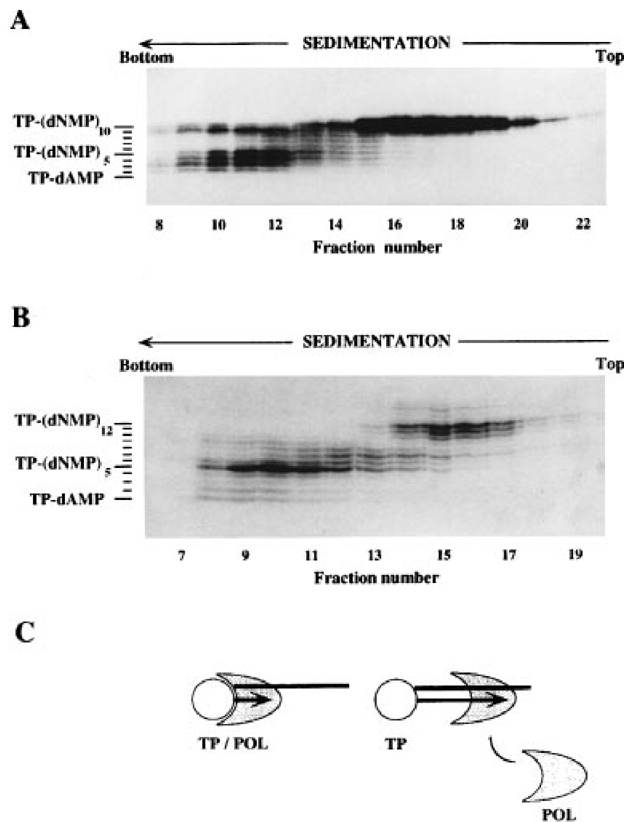

Fig. 2. Synthesis of a short DNA molecule before dissociation of the DNA polymerase–TP heterodimer. (**A**) Replication of oriL(10) was carried out in the presence of the four dNTPs, as indicated in Materials and methods. The sample was subjected to sedimentation analysis in a 15–30% glycerol gradient. After fractionation of the gradient, an aliquot of fractions 8–22 was analysed by high-resolution SDS–PAGE. Each band represents a different TP-$(dNMP)_n$ product, ranging from TP-$(dAMP)_2$ to TP-$(dNMP)_{10}$. The band corresponding to TP-dAMP was also detected in longer exposures. The different products up to TP-$(dNMP)_5$ migrated at fractions 10–12, at the mobility expected for the DNA polymerase–TP heterodimer. Products TP-$(dNMP)_6$ to TP-$(dNMP)_8$ showed a slightly slower mobility (fractions 12–14), and product TP-$(dNMP)_{10}$ migrated at fractions 16–18, indicating that it has dissociated from the DNA polymerase. The difference with respect to the theoretical mobility of the free TP is probably due to the molecular weight of the DNA molecule attached to the TP (10 bp = 6.5 kDa). (**B**) Same as above, but using oriL(12) as template. Since the amount of intermediate elongation products was higher, exposure time was shorter than in (A). Only products equal to or longer than TP-$(dNMP)_{10}$ migrated in the position corresponding to free TP molecules. (**C**) The primer TP and the DNA polymerase are tentatively depicted as a white circle and a shadowed figure, respectively.

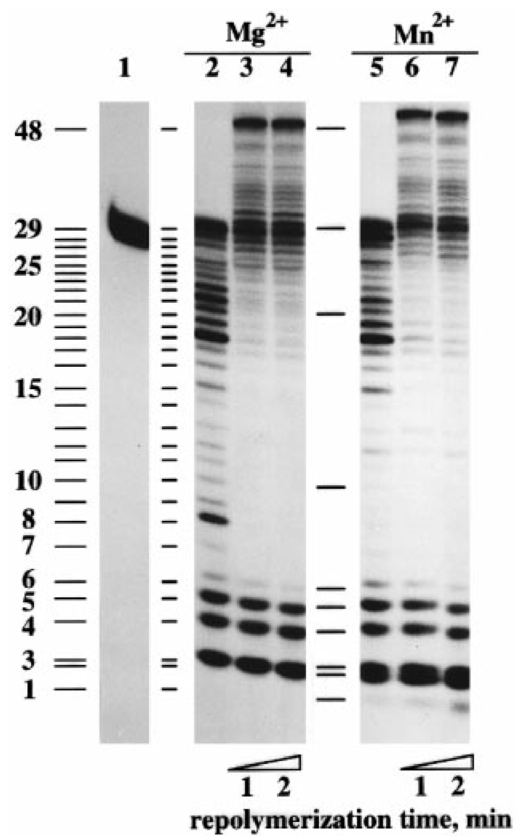

Fig. 3. 3′–5′ exonucleolysis followed by DNA polymerization in a TP-free ϕ29 right origin of replication. Oligonucleotide oriR(29)d was 5′-labelled and hybridized to oriR(48) to generate a primer–template structure with the sequence of the ϕ29 right end. In the absence of dNTPs, the action of the 3′–5′ exonuclease activity of ϕ29 DNA polymerase generates partially degraded primer molecules. The ability of the DNA polymerase to repolymerize primer chains of different lengths was assessed by further addition of the four dNTPs to the reaction mixture. Lane 1, mobility of the oriR(29)d/oriR(48) DNA molecule in a 7 M urea–20% polyacrylamide gel. Lanes 2 and 5, action of the exonuclease activity of ϕ29 DNA polymerase on oriR(29)d/oriR(48), using Mg^{2+} or Mn^{2+} as metal activators, respectively. Lanes 3 and 4: repolymerization of the different primers generated, in the presence of Mg^{2+}. Lanes 6 and 7: same as lanes 3 and 4, in the presence of Mn^{2+}.

and fourth nucleotides of oriR(12) significantly reduced the replication efficiency (from 17 to 30%). The low replication efficiency of oriR(12)T1C and oriR(12)T2C, lacking a terminal repetition of two nucleotides, could be explained by the impossibility of these templates sustaining the sliding-back step (Mendez *et al.*, 1992). We tested this in a limited elongation assay, carried out in the presence of the chain terminator ddTTP. Under these conditions, the longest product obtained was TP-$(dNMP)_5$, as expected from the wild-type template sequence, 3′-TTTCAT... (see Figure 4). The length of this product is precisely the upper limit corresponding to the initiation mode of ϕ29 DNA polymerase, prior to the transition stage. Substitution of the first (T1C) or second (T2C) nucleotides in the template

Table I. Mutational analysis of the ITR of ϕ29 DNA

Oligonucleotide	Sequence (3′–5′)	Initiation efficiency (%)	Replication efficiency (%)
oriR(12)	TTTCATCCCATG	100	100
oriR(12)T1C	<u>C</u>TTCATCCCATG	30	30
oriR(12)T2C	T<u>C</u>TCATCCCATG	110	25
oriR(12)T3C	TT<u>C</u>CATCCCATG	110	140
oriR(12)C4G	TTT<u>G</u>ATCCCATG	61	17
oriR(12)A5G	TTTC<u>G</u>TCCCATG	120	160
oriR(12)T6G	TTTCA<u>G</u>CCCATG	130	180

Oligonucleotides oriR(12) and mutant derivatives (single substitutions underlined in the sequence) were used as templates for both the initiation assay (TP-dAMP formation) and full replication assay, as indicated in Materials and methods. After SDS–PAGE, the bands corresponding to the TP-dAMP or full-length [TP-$(dNMP)_{12}$] products were quantitated using a Phosphorimager. The efficiency of replication was calculated as the ratio between 'fully replicated' molecules versus 'initiated' molecules. For both initiation and replication efficiencies, the values are expressed as a percentage of those corresponding to the wild-type oriR(12).

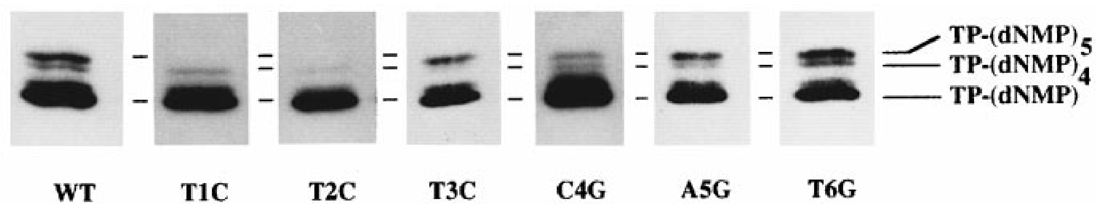

Fig. 4. Limited replication of oligonucleotides with mutations at the six terminal nucleotides. Oligonucleotide oriR(12) and oligonucleotides with single variations on the original sequence were used as templates for initiation and partial replication reactions, under the conditions described in Materials and methods. WT corresponds to oriR(12), T1C to oriR(12)T1C, and so on. After incubation, samples were subjected to high-resolution electrophoresis, that allows fractionation of the different TP-$(dNMP)_n$ products. Lanes corresponding to T1C, T2C and C4G were overexposed to visualize the longest elongation band.

reduced the efficiency of elongation during the initiation mode, and the longest product obtained lacked one nucleotide [TP-$(dNMP)_4$; Figure 4]. These results suggest that the sliding-back step, not allowed on templates T1C and T2C, is not absolutely required, but is very important for the next elongation steps preceding transition, subsequently affecting the replication efficiency. Substitution of the fourth nucleotide (C4G) of the template also reduced the efficiency of elongation during the initiation mode, although in this case TP-$(dNMP)_5$ can be detected as the longest product and, therefore, the defective elongation cannot be attributed to the sliding-back step. As expected from their normal replication efficiency (Table I), templates with substitutions at positions 3, 5 and 6 did not affect the efficiency of elongation during the initiation mode, leading to TP-$(dNMP)_5$ as the longest product. The replication efficiencies observed under these limited conditions (see Figure 4) correlate well with those of full-length replication indicated in Table I. Therefore, it appears that the role of the template ITR is restricted to events occurring during the initiation mode of ϕ29 DNA polymerase.

In vitro accumulation of abortive replication products

The experiments described so far have been performed using ssDNA oligonucleotides as templates, whereas the natural ϕ29 origins are TP-containing dsDNA molecules. We investigated whether transition from initiation to elongation would represent a limiting step in the complete replication origins. As shown in Figure 5, during *in vitro* replication of ϕ29 TP-DNA, abortive products corresponding to replication intermediates of different lengths could be detected in the presence of Mg^{2+} or Mn^{2+} as activating metal ions. When using the wild-type ϕ29 DNA polymerase, mainly TP-dAMP and TP-$(dAMP)_2$ were detected, although a faint band corresponding to TP-$(dNMP)_8$ could be also detected in the presence of Mn^{2+}. It should be taken into account that the abortive replication products could be degraded by the 3′–5′ exonuclease activity of the DNA polymerase, able to excise all the nucleotides of the nascent chain with the exception of the one linked to the TP (Esteban *et al.*, 1993). For a complete analysis of the abortive replication products, DNA polymerases devoid of exonuclease activity should be used in order to analyse the precise length of the 'stopped' molecules. Two different exonuclease-deficient DNA polymerases were used (Figure 5): N62D, essentially devoid of 3′–5′ exonuclease activity, but retaining the strand displacement ability (de Vega *et al.*, 1996); and D12A/D66A, a double mutant that eliminates both 3′–5′ exonuclease and strand

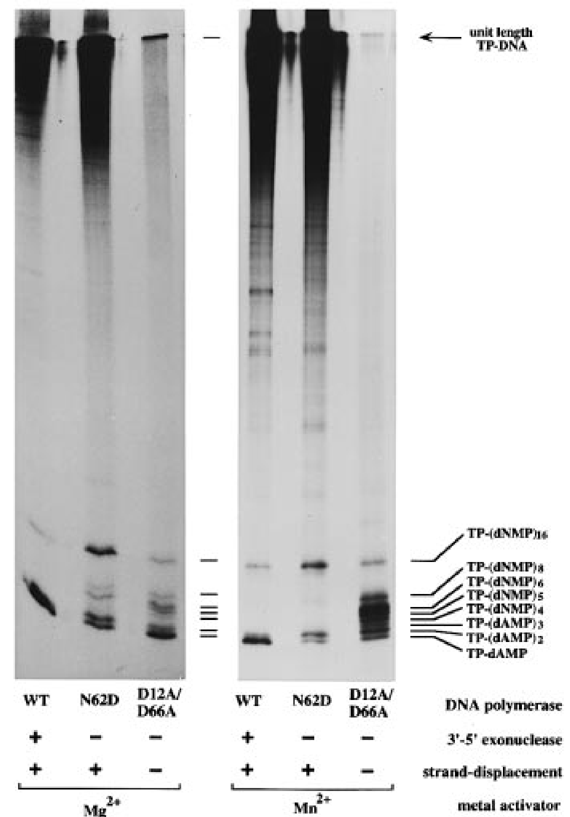

Fig. 5. Accumulation of abortive replication products *in vitro*. Replication of TP-DNA using either wild-type, N62D or D12A/D66A mutant DNA polymerases was carried out as indicated in Materials and methods, using either Mg^{2+} or Mn^{2+} as metal activators. Samples were analysed by high-resolution SDS–PAGE. The different transition products detected are indicated. Completely replicated molecules remain at the interphase with the stacking gel.

displacement, the latter defect impeding the synthesis of full-length ϕ29 DNA molecules (Bernad *et al.*, 1989; Soengas *et al.*, 1992). During replication of natural TP-DNA with both mutant DNA polymerases, it was possible to detect the accumulation of replication products not seen with the wild-type enzyme. When Mg^{2+} was used as metal activator, both mutant DNA polymerases produced stops at TP-$(dNMP)_6$ and TP-$(dNMP)_8$, probably reflecting the molecules aborted during the transition from the 'initiation mode' to the 'elongation mode' of ϕ29 DNA replication. Two other stops corresponding to TP-$(dNMP)_4$ and TP-$(dNMP)_5$ were only obtained with mutant D12A/D66A, that lacks strand displacement capacity (see Figure 5). When Mn^{2+} was used, a metal that gives an

optimal activation of the initiation reaction but reduces the efficiency of processive synthesis by ϕ29 DNA polymerase (Esteban *et al.*, 1992), mutant N62D showed only a faint band corresponding to TP-$(dNMP)_8$, as in the case of the wild-type enzyme. Interestingly, mutant D12A/D66A showed a large accumulation of products corresponding to TP-$(dNMP)_3$ to TP-$(dNMP)_6$ and, therefore, a poor proportion of molecules proceeding through the transition step (see Figure 5). In all cases, mutant and wild-type enzymes produced an extra abortive product corresponding to TP-$(dNMP)_{16}$. This stop, previously described during replication of TP-DNA with exonuclease-deficient mutant polymerases (Esteban *et al.*, 1994), occurs after the transition event described herein.

Discussion

The process of ϕ29 TP-DNA replication is achieved by the sequential usage of two modes of priming. For initiation, ϕ29 DNA polymerase forms a complex with a free molecule of the TP and the resultant heterodimer recognizes the origin of replication. The parental TP may contribute, by protein–protein interactions, to recruit the heterodimer to the origin. After incorporation of the initiating dAMP residue to the primer TP, the same DNA polymerase starts elongation of the nascent DNA chain, using as primer the 3′ hydroxyl group of the initiation product, TP-dAMP. In this work, we have focused on the transition between protein-primed initiation and DNA-primed elongation. Initiation of ϕ29 DNA replication occurs at the second 3′ nucleotide of the template, perhaps because the 3′-terminal nucleotide of the template DNA is involved in some interaction with the primer TP (mutation of the first nucleotide of the template resulted in a decreased efficiency of initiation). The first nucleotide is then recovered by a sliding-back of the initiation product, TP-dAMP. Protein-primed initiation of DNA replication in other systems also occurs at an internal position, close to the DNA end: the second nucleotide in *B.subtilis* phage GA-1 (Illana *et al.*, 1996), the third in *Streptococcus pneumoniae* phage Cp-1 (Martın *et al.*, 1996) and the fourth in *Escherichia coli* phage PRD1 (Caldentey *et al.*, 1993) and also in human adenovirus (King and Van der Vliet, 1994). In all these cases, different variations of the sliding-back mechanism ensure the maintenance of the DNA termini.

We have presented evidence that neither initiation nor sliding-back result in the dissociation of the DNA polymerase–primer TP heterodimer. Since the first and second dAMP residues are incorporated opposite to the same position of the template, sliding-back has to occur without translocation of the enzyme along the DNA. This could be explained if residue Ser232 in the TP (donor of the initial hydroxyl group) is located in a flexible domain that is retracted backwards during the sliding-back translocation (site-directed mutagenesis in several residues of ϕ29 TP is underway to assess this possibility). ϕ29 DNA polymerase and TP separate only when a 10 nucleotide long DNA primer chain has been synthesized. After sliding back, however, every nucleotide addition will require a translocation of the enzyme along the template. Our mutational analysis suggests that the ITR sequence (3′-TTTCAT), particularly the first and second (involved in sliding back), and fourth template nucleotides, are important for the first translocation steps following TP-primed initiation. The incorporation of new dNMPs to the nascent chain while the primer TP and DNA polymerase are still together will indeed introduce an increasing strain into the polymerization domain of the enzyme. According to the model depicted in Figure 6, this strain probably reaches a maximum when 5–6 nucleotides have been incorporated and induces some conformational change in the DNA polymerase during incorporation of nucleotides 7–9. This progressive change, detected as a difference in the sedimentation of the corresponding protein–protein complexes, could progressively reduce the molecular strain and result in the complete dissociation of the DNA polymerase from the TP to start normal DNA elongation. These three different steps could be referred to as the 'initiation mode', 'transition' and 'elongation mode' stages of ϕ29 DNA polymerase (Figure 6). Since the three-dimensional structure of ϕ29 DNA polymerase, or of any other protein-priming DNA polymerase, is unknown to date, the structural bases for a conformational change during the transition step are unclear at present. However, protein-priming DNA polymerases show several differences with respect to other families of DNA polymerases: first, their structural adaptation to use a protein as primer during initiation; second, their intrinsic high processivity, suggesting that during the DNA elongation mode some specific region of the DNA polymerase may wrap around the double helix and hold the enzyme on the DNA; and third, at least in the case of DNA polymerases from phages ϕ29 (Blanco *et al.*, 1989) and PRD1 (Caldentey *et al.*, 1992), strand displacement ability coupled to DNA polymerization. Taking into account all that, it seems quite possible that ϕ29 DNA polymerase undergoes a conformational change during the transition from initiation to elongation. Other approaches to assess directly the conformational change of the DNA polymerase during the transition (i.e. protease sensitivity, fluorometry, etc.) are technically difficult to undertake due to the fact that two proteins are required for the initiation step (TP and DNA polymerase), whereas only one of them is involved in DNA elongation (DNA polymerase).

There is an interesting parallelism between the mechanism described here for protein-primed initiation and the common mechanism of initiating DNA synthesis by the sequential use of a primase and a DNA polymerase. In several of the primases studied so far, the length of the primer synthesized by the primase is precisely 10–11 nucleotides, and then the DNA polymerase starts elongation (reviewed by Griep, 1995). We have shown, in a TP-independent reaction, that ϕ29 DNA polymerase requires a minimum length of 6 bp to catalyse polymerization efficiently. This minimum length increases up to 10 nucleotides when the TP is present. ϕ29 DNA polymerase, while still complexed with the TP, is somehow acting as a primase, prior to its highly processive mode of synthesis.

On the other hand, at the beginning of transcription, monomeric and multimeric RNA polymerases go through an initial abortive phase in which transcripts are synthesized and released from the ternary complex before the polymerase enters fully processive transcription (Eick *et al.*, 1994). It has been shown that T7 monomeric RNA polymerase undergoes a conformational change during the

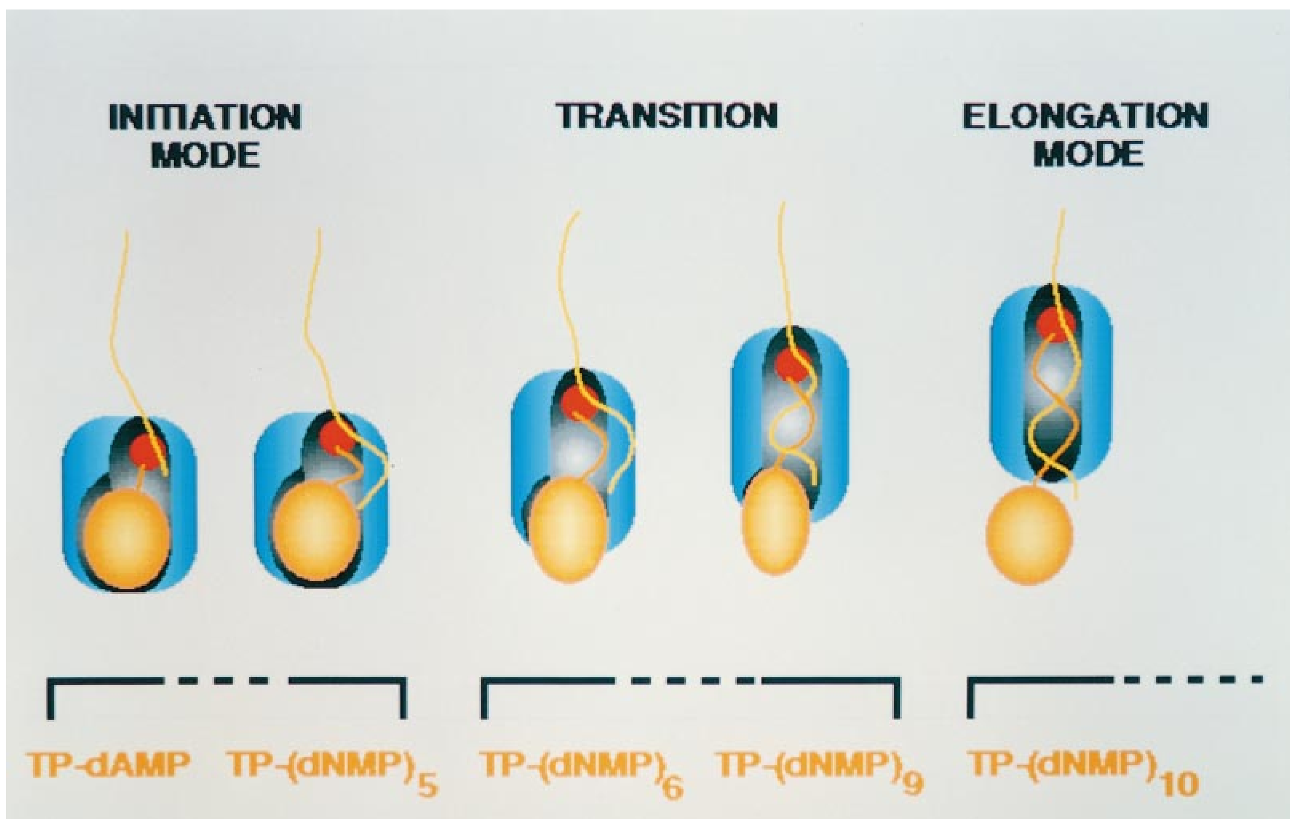

Fig. 6. Model for the transition from initiation to elongation during protein-primed DNA replication. The area represented in blue corresponds to ϕ29 DNA polymerase, with binding clefts for both TP (indicated by an orange sphere) and DNA. The polymerization active site is indicated by a red sphere. The conformational change corresponding to the transition stage from the initiation mode to the elongation mode is tentatively represented by a dimensional change of both TP and ϕ29 DNA polymerase. The different products synthesized by ϕ29 DNA polymerase as the limits of each of these stages are indicated at the bottom of the figure.

transition from the abortive to the processive phase, and the switch between both conformations is probably mediated by an interaction between the nascent chain and a polynucleotide-binding site located at least partly in the N-terminal domain of the enzyme (Sousa *et al.*, 1992). The length of synthesized RNA that triggers the transition from abortive to processive transcription is precisely nine nucleotides (Bonner *et al.*, 1992). Despite the obvious differences between the processes of replication and transcription, the overall three-dimensional structure of T7 RNA polymerase (Sousa *et al.*, 1992) is similar to that of the polymerization domain of DNA-dependent DNA polymerases (see reviews by Joyce and Steitz, 1994; Sousa, 1996), suggesting that the mechanistic problems associated with '*de novo*' nucleic acid synthesis could be similar in the case of T7 RNA polymerase and ϕ29 DNA polymerase.

During the structure–function analysis of ϕ29 DNA polymerase, several mutations in conserved residues of the C-terminal domain (responsible for the synthetic activities) have been described that, while having little effect in initiation, impaired the normal progression to the DNA elongation stage of ϕ29 DNA replication. This is the case for mutations in residues Asn387, that is necessary for DNA binding (Blasco *et al.*, 1993), and Lys498 and Tyr500, required for efficient stabilization of the primer terminus at the polymerization active site (Blasco *et al.*, 1995). Very recent studies in our laboratory suggest that several mutations in the 'YxGG' motif, involved in the communication between the polymerization and the exonuclease domains of ϕ29 DNA polymerase (Truniger *et al.*, 1996), may be also affected in transition (V.Truniger, L.Blanco and M.Salas, in preparation). Regarding mutations at the N-terminal domain (containing the 3′–5′ exonuclease active site), those exonuclease-deficient DNA polymerases that are also affected in strand displacement are able to reach positions 6, 8 and 16, but a significant proportion of nascent molecules abort at positions 4 and 5, not progressing into the conformational change described here as the transition preceding the elongation mode. This suggests that a proper strand displacement capacity may be required to facilitate transition, perhaps by the establishment of the initial interactions with the nascent displaced strand. This could also be related to the fact that T7 RNA polymerase, when engaged in abortive transcription, protects the coding but not the non-coding strand from DNase I digestion, but when engaged in processive transcription both strands are protected (Basu and Maitra, 1986; Shi *et al.*, 1988).

Using the natural ϕ29 TP-DNA molecule, it has been demonstrated that ϕ29 protein p6 stimulates both the initiation step and also the transition step from initiation to elongation (Blanco *et al.*, 1986, 1988) catalysed by the wild-type ϕ29 DNA polymerase. Interestingly, the pattern of the short elongation products obtained with the exo/strand displacement minus mutants of ϕ29 DNA polymerase on the natural ϕ29 TP-DNA molecule is modified by addition of protein p6 (Esteban *et al.*, 1994). On the

basis of the results described here, this change in pattern is interpreted as a difficulty in reaching positions 5 and 6, making the defective transition phenotype of those mutants more drastic. Since ϕ29 protein p6 is a dsDNA-binding protein that requires ~100 bp of the terminal ϕ29 DNA sequences, a more detailed analysis of its role at the transition stage cannot be assessed using single-stranded oligonucleotides as templates.

Protein-primed initiation seems to be a hazardous, error-prone step during ϕ29 DNA replication. On one hand, the insertion fidelity of ϕ29 DNA polymerase during initiation is much lower than the insertion fidelity during elongation (Esteban *et al.*, 1993). Although the sliding-back mechanism could represent a non-exonucleolytic editing step for initiation (Mendez *et al.*, 1992), incorrect initiation products can be elongated with a low discrimination rate (Esteban *et al.*, 1993). All these arguments predict a high frequency of mutation at the origin region. Despite this fact, the terminal sequence of ϕ29 DNA (3′-TTTCAT..) is conserved, not only at both ϕ29 DNA ends, but also in a family of phages related to ϕ29. It is tempting to speculate that all the specific molecular rearrangements occurring during transition from initiation to elongation function as a replication checkpoint: only when the first six nucleotides of the nascent DNA molecule have been replicated faithfully can the DNA polymerase undergo the structural change from the protein-priming to the DNA-priming modes, and the nascent chain is elongated to reach full-length DNA. This possibility would explain both the strict conservation of the ϕ29 ITR and the abortive products arising during the transition from initiation to elongation. In this sense, it is worth noting that ϕ29 DNA polymerase can be purified from ϕ29-infected *B.subtilis* cells complexed to short TP-$(dNMP)_n$ products (J.M. Lazaro and M.Salas, unpublished observations), suggesting that transition is a limiting step also during *in vivo* ϕ29 DNA replication.

Materials and methods

Nucleotides, DNAs and proteins

[α-^{32}P]dATP (400 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham International plc. Unlabelled nucleotides were from Pharmacia P-L Biochemicals. TP-DNA was obtained as described (Penalva and Salas, 1982). oriR(12), oriR(29), oriR(48), oriL(10) and oriL(12) are synthetic oligonucleotides with the sequence of the template strand of ϕ29 DNA right (R) or left (L) end. The number in parenthesis indicates the length of the oligonucleotide. To identify oligonucleotides with single variations from the wild-type sequence, a three-symbol code is included in the name [e.g. oriR(12)T1C]; the first letter indicates the original, wild-type nucleotide, the number marks its position from the 3′ end of the molecule and the second letter indicates the nucleotide introduced. Oligonucleotide oriR(29)d corresponds to the displaced strand of ϕ29 DNA right origin of replication. T4 polynucleotide kinase was obtained from New England Biolabs. Wild-type and 3′–5′ exonuclease-deficient (D12A/D66A; N62D) ϕ29 DNA polymerases were purified essentially as described (Lazaro *et al.*, 1995). ϕ29 TP was purified as described by Zaballos *et al.* (1989).

In vitro protein-primed initiation (TP-dAMP formation), limited elongation and full replication of oligonucleotides

The incubation mixture in a typical initiation assay contained, in 25 μl, 50 mM Tris–HCl, pH 7.5, 1 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 4% glycerol, 0.1 mg/ml BSA, 0.25 μM [α-^{32}P]dATP (2.5 μCi), 0.2 μg of the indicated oligonucleotide as template and the indicated amounts of purified TP and exonuclease-deficient D12A/D66A ϕ29 DNA polymerase. The limited elongation experiment was carried out essentially in the same form, but in the presence of 5 μM dATP, 5 μM dGTP and 100 μM ddTTP [for oligonucleotides oriR(12), oriR(12)T1C, oriR(12)T2C, oriR(12)T3C and oriR(12)T6G]; 5 μM dATP, 5 μM dCTP and 100 μM ddTTP [for oligonucleotide oriR(12)C4G]; 5 μM dATP, 5 μM dGTP and 100 μM ddCTP [for oligonucleotide oriR(12)A5G]; in the case of oriR(12)T2C, [α-^{32}P]dGTP was used instead of [α-^{32}P]dATP. Full replication of the template oligonucleotides was assayed by adding 20 μM of the four dNTPs. In all cases, after incubation for the indicated time at 30°C, the reactions were stopped by adding EDTA to 10 mM. Samples were filtered in Sephadex G-50 spin columns in the presence of 0.1% SDS, and the excluded volume was analysed by SDS–PAGE and autoradiography. Quantitation was done by densitometric analysis of the autoradiograms.

Analysis of the interaction between TP and DNA polymerase

In vitro reactions were performed as indicated above, but in 100 μl, in the presence of 5 μg of exonuclease-deficient D12A/D66A ϕ29 DNA polymerase (85 pmol), 5 μg of TP (160 pmol) and 1 μg of the corresponding template oligonucleotide (260 pmol). When indicated, elongation was limited to the three first nucleotides by adding 5 μM dATP as the only nucleotide to the reaction. For complete replication, 20 μM of the four dNTPs was added. The different products of the corresponding *in vitro* replication reactions were loaded in 15–30% glycerol gradients (4 ml), in the presence of 50 mM Tris–HCl, pH 7.5, 20 mM ammonium sulfate, 0.2 M NaCl, 1 mM EDTA, 0.05 mg/ml BSA and 7 mM 2-mercaptoethanol. Loaded gradients were subjected to ultracentrifugation in a Kontron rotor TST 60.4 (25 h at 58 000 r.p.m.) or a Beckman rotor SW 65 (24 h at 62 000 r.p.m.) at 4°C. The gradients were then fractionated and subjected to high resolution SDS–PAGE (360×280×0.5 mm).

Determination of the minimum DNA primer length required for polymerization (degradation/repolymerization assay)

The incubation mixture contained, in 10 μl, 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 0.15 ng of the DNA molecule [5′-labelled-oriR(29)d/oriR(48)] and 20 ng of wild-type ϕ29 DNA polymerase. Samples were incubated for 10 min at 20°C. One sample was stopped by addition of EDTA to 10 mM, and the others were incubated for the indicated times at 30°C after the addition of 1 μM dNTPs to allow repolymerization. The reactions were stopped by adding EDTA up to 10 mM. The products of the exonuclease and the (exonuclease + repolymerization) reactions were subjected to electrophoresis in 7 M urea–20% polyacrylamide gels. After electrophoresis, the gels were autoradiographed.

TP-DNA replication

The incubation mixture contained, in 25 μl, 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 20 mM ammonium sulfate, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 0.5 μg of TP-DNA, 20 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (1 μCi), 100 ng of purified TP and 200 ng of the indicated DNA polymerase. After incubation for 15 min at 30°C, the reactions were stopped by adding EDTA up to 10 mM and SDS up to 0.1%, and the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume was subjected to electrophoresis in high-resolution SDS–12% polyacrylamide gels. After electrophoresis, the gels were dried and autoradiographed.

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References

Barthelemy,I., Salas,M. and Mellado,R.P. (1986) *In vivo* transcription of bacteriophage ϕ29 DNA: transcription initiation sites. *J. Virol.*, **60**, 874–879.

Basu,S. and Maitra,U. (1986) Specific binding of monomeric bacteriophage T3 and T7 RNA polymerases to their respective cognate

promoters requires the initiating ribonucleoside triphosphate (GTP). *J. Mol. Biol.*, **190**, 425–437.

Bernad,A., Blanco,L., Lazaro,J.M., Martın,G. and Salas,M. (1989) A conserved 3′-5′ exoonuclease active site in prokaryotic and eukaryotic DNA polymerases. *Cell*, **59**, 219–228.

Blanco,L. and Salas,M. (1996) Relating structure to function in φ29 DNA polymerase. *J. Biol. Chem.*, **271**, 8509–8512.

Blanco,L., Gutierrez,J., Lazaro,J.M., Bernad,A. and Salas,M. (1986) Replication of phage φ29 DNA *in vitro*: Role of the viral protein p6 in initiation and elongation. *Nucleic Acids Res.*, **14**, 4923–4937.

Blanco,L., Prieto,I., Gutierrez,J., Bernad,A., Lazaro,J.M., Hermoso,J.M. and Salas,M. (1987) Effect of NH_4^+ ions on φ29 DNA–protein p3 replication: formation of a complex between the terminal protein and the DNA polymerase. *J. Virol.*, **61**, 3983–3991.

Blanco,L., Bernad,A. and Salas,M. (1988) Transition from initiation to elongation in the protein-primed φ29 DNA replication. Salt-dependent stimulation by the viral protein p6. *J. Virol.*, **62**, 4167–4172.

Blanco,L., Bernad,A., Lazaro,J.M., Martın,G., Garmendia,C. and Salas,M. (1989) Highly efficient DNA synthesis by the phage φ29 DNA polymerase. Symmetrical mode of DNA replication. *J. Biol. Chem.*, **264**, 8935–8940.

Blanco,L., Bernad,A., Esteban,J.A. and Salas,M. (1992) DNA-independent deoxynucleotidylation of the φ29 terminal protein by the φ29 DNA polymerase. *J. Biol. Chem.*, **267**, 1225–1230.

Blasco,M.A., Lazaro,J.M., Blanco,L. and Salas,M. (1993) φ29 DNA polymerase active site. The conserved amino acid motif 'Kx_3NSxYG' is involved in template–primer binding and dNTP selection. *J. Biol. Chem.*, **268**, 16763–16770.

Blasco,M.A., Mendez,J., Lazaro,J.M., Blanco,L. and Salas,M. (1995) Primer-terminus stabilization at the φ29 DNA polymerase active site: mutational analysis of conserved motif KxY. *J. Biol. Chem.*, **270**, 2735–2740.

Bonner,G., Patra,D., Lafer,E.M. and Sousa,R. (1992) Mutations in T7 RNA polymerase that support the proposal for a common polymerase active site structure. *EMBO J.*, **11**, 3765–3775.

Caldentey,J., Blanco,L., Savilahti,H., Bamford,D.H. and Salas,M. (1992) *In vitro* replication of bacteriophage PRD1 DNA. Metal activation of protein-primed initiation and DNA elongation. *Nucleic Acids Res.*, **20**, 3971–3976.

Caldentey,J., Blanco,L., Bamford,D.H. and Salas,M. (1993) *In vitro* replication of bacteriophage PRD1 DNA. Characterization of the protein-primed initiation site. *Nucleic Acids Res.*, **21**, 3725–3730.

Chen,C.W. (1996) Complications and implications of linear bacterial chromosomes. *Trends Genet.*, **12**, 192–196.

de Vega,M., Lazaro,J.M., Salas,M. and Blanco,L. (1996) Primer-terminus stabilization at the 3′–5′ exonuclease active site of φ29 DNA polymerase. Involvement of two amino acid residues highly conserved in proofreading DNA polymerases. *EMBO J.*, **15**, 1182–1192.

Eick,D., Wedel,A. and Heumann,H. (1994) From initiation to elongation: comparison of transcription by prokaryotic and eukaryotic RNA polymerases. *Trends Genet.*, **10**, 292–296.

Esteban,J.A., Bernad,A., Salas,M. and Blanco,L. (1992) Metal activation of synthetic and degradative activities of φ29 DNA polymerase, a model enzyme for protein-primed DNA replication. *Biochemistry*, **31**, 350–359.

Esteban,J.A., Salas,M. and Blanco,L. (1993) Fidelity of φ29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. *J. Biol. Chem.*, **268**, 2719–2726.

Esteban,J.A., Soengas,M.S., Salas,M. and Blanco,L. (1994) 3′→5′ exonuclease active site of φ29 DNA polymerase. Evidence favoring a metal-ion-assisted reaction mechanism. *J. Biol. Chem.*, **269**, 31946–31954.

Griep,M.A. (1995) Primase structure and function. *Indian J. Biochem.*, **32**, 171–178.

Illana,B., Blanco,L. and Salas,M. (1996) Functional characterization of the genes coding for the terminal protein and DNA polymerase from bacteriophage GA-1. Evidence for a sliding-back mechanism during protein-primed GA-1 DNA replication. *J. Mol. Biol.*, **264**, 453–464.

Joyce,C.M. and Steitz,T.A. (1994) Function and structure relationships in DNA polymerases. *Annu. Rev. Biochem.*, **63**, 777–822.

King,A. and Van der Vliet,P.C. (1994) A precursor terminal protein–trinucleotide intermediate during initiation of adenovirus DNA replication: regeneration of molecular ends *in vitro* by a jumping back mechanism. *EMBO J.*, **13**, 5786–5792.

Lazaro,J.M., Blanco,L. and Salas,M. (1995) Purification of bacteriophage φ29 DNA polymerase. *Methods Enzymol.*, **262**, 42–49.

Martın,A.C., Blanco,L., Garcıa,P., Salas,M. and Mendez,J. (1996) Protein-primed initiation of pneumococcal phage Cp-1 DNA replication occurs at the third 3′ nucleotide of the linear template: a stepwise sliding-back mechanism. *J. Mol. Biol.*, **260**, 369–377.

Matsumoto,K., Saito,T., Kim,C.I., Ando,T. and Hirokawa,H. (1984) Bacteriophage phi 29 DNA replication *in vitro*: participation of the terminal protein and the gene 2 product in elongation. *Mol. Gen. Genet.*, **196**, 381–386.

Mendez,J., Blanco,L., Esteban,J.A., Bernad,A. and Salas,M. (1992) Initiation of φ29 DNA replication occurs at the second 3′ nucleotide of the linear template: a sliding-back mechanism for protein-primed DNA replication. *Proc. Natl Acad. Sci. USA*, **89**, 9579–9583.

Salas,M. (1991) Protein-priming of DNA replication. *Annu. Rev. Biochem.*, **60**, 39–71.

Salas,M., Miller,J.T., Leis,J. and DePamphilis,M.L. (1996) Mechanisms for priming DNA synthesis. In DePamphilis,M.L. (ed.), *DNA Replication in Eukaryotic Cells.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 131–176.

Serrano,M., Salas,M. and Hermoso,J.M. (1990) A novel nucleoprotein complex at a replication origin. *Science*, **248**, 1012–1016.

Shi,Y., Gamper,H. and Hearst,J.E. (1988) Interaction of T7 RNA polymerase with DNA in an elongation complex arrested at a specific psoralen adduct site. *J. Biol. Chem.*, **263**, 527–534.

Soengas,M.S., Esteban,J.A., Lazaro,J.M., Bernad,A., Blasco,M.A., Salas,M. and Blanco,L. (1992) Site-directed mutagenesis at the Exo III motif of φ29 DNA polymerase. Overlapping structural domains for the 3′→5′ exonuclease and strand-displacement activities. *EMBO J.*, **11**, 4227–4237.

Sousa,R. (1996) Structural and mechanistic relationships between nucleic acid polymerases. *Trends Biochem. Sci.*, **21**, 186–190.

Sousa,R., Patra,D. and Lafer,E.M. (1992) Model for the mechanism of bacteriophage T7 RNAP transcription initiation and termination. *J. Mol. Biol.*, **224**, 319–334.

Truniger,V., Lazaro,J.M., Salas,M. and Blanco,L. (1996) A DNA binding motif coordinating synthesis and degradation in proofreading DNA polymerases. *EMBO J.*, **15**, 3430–3441.

Watabe,K., Shih,M.F. and Ito,J. (1983) *In vitro* replication of bacteriophage phi29 DNA. *Proc. Natl Acad. Sci. USA*, **80**, 4248–4252.

Yoshikawa,H. and Ito,J. (1982) Terminal proteins and short inverted terminal repeats of the small *Bacillus* bacteriophage genomes. *Proc. Natl Acad. Sci. USA*, **78**, 1336–1340.

Zaballos,A., Lazaro,J.M., Mendez,E., Mellado,R.P. and Salas,M. (1989) Effects of internal deletions on the priming activity of the phage φ29 terminal protein. *Gene*, **83**, 187–195.

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