

Highly Efficient DNA Synthesis by the Phage ϕ 29 DNA Polymerase

SYMMETRICAL MODE OF DNA REPLICATION*

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Luis Blanco, Antonio Bernad†, José M. Lázaro, Gil Martín§, Cristina Garmendia, and Margarita Salas¶

From the Centro de Biología Molecular (Consejo Superior de Investigaciones Científicas), Universidad Autónoma de Madrid, Canto Blanco, Madrid 28049, Spain

The results presented in this paper indicate that the ϕ 29 DNA polymerase is the only enzyme required for efficient synthesis of full length ϕ 29 DNA with the ϕ 29 terminal protein, the initiation primer, as the only additional protein requirement. Analysis of ϕ 29 DNA polymerase activity in various *in vitro* DNA replication systems indicates that two main reasons are responsible for the efficiency of this minimal system: 1) the ϕ 29 DNA polymerase is highly processive in the absence of any accessory protein; 2) the polymerase itself is able to produce strand displacement coupled to the polymerization process. Using primed M13 DNA as template, the ϕ 29 DNA polymerase is able to synthesize DNA chains greater than 70 kilobase pairs. Furthermore, conditions that increase the stability of secondary structure in the template do not affect the processivity and strand displacement ability of the enzyme. Thus, the catalytic properties of the ϕ 29 DNA polymerase are appropriate for a ϕ 29 DNA replication mechanism involving two replication origins, strand displacement and continuous synthesis of both strands. The enzymology of ϕ 29 DNA replication would support a symmetrical model of DNA replication.

Bacteriophage ϕ 29 from *Bacillus subtilis* contains a linear, double-stranded DNA, 19,285 bp¹ long (Vlček and Pačes, 1986), with the terminal protein p3 covalently linked to each 5'-end by a phosphodiester bond between the OH group of serine residue 232 and 5'-dAMP (Hermoso *et al.*, 1985). *In vivo* (Inciarte *et al.*, 1980; Harding and Ito, 1980; Sogo *et al.*, 1982) and *in vitro* (Peñalva and Salas, 1982; Shih *et al.*, 1982) studies have shown that ϕ 29 DNA replication occurs via a protein-primed mechanism where DNA synthesis starts non-simultaneously from either end of the linear DNA molecule with the covalent linkage of dAMP to a free molecule of the terminal protein; the subsequent elongation of the DNA chain occurs by a strand-displacement mechanism. Such a displace-

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† Fellow from Caja de Ahorros de Madrid.

‡ Fellow from the Spanish-French Mercure Program.

¶ To whom correspondence and reprint requests should be addressed.

¹The abbreviations used are: bp, base pairs; kb, kilobase pairs; SDS, sodium dodecyl sulfate.

ment mechanism would be best served by a highly processive DNA polymerase which could synthesize long DNA chains without dissociating from the template.

A DNA polymerase, the viral protein 2, has been shown by genetic and biochemical analysis to be essential for ϕ 29 DNA synthesis both *in vivo* and *in vitro* (Mellado *et al.*, 1980; Blanco *et al.*, 1983; Matsumoto *et al.*, 1983; Watabe *et al.*, 1983). The enzyme consists of a single polypeptide of $M_r = 66,520$ that has DNA polymerase and 3' → 5' exonuclease activities and, in addition, is able to catalyze the formation of the initiation complex between the terminal protein and dAMP (Blanco and Salas, 1984; Watabe *et al.*, 1984, a and b; Blanco and Salas, 1985a); this is further elongated to produce full length ϕ 29 DNA (Blanco and Salas, 1985b). Ammonium ions stimulate ϕ 29 DNA-protein p3 replication, mainly through formation of a stable complex between the terminal protein and the DNA polymerase (Blanco *et al.*, 1987). The viral protein p6 that specifically recognizes the ϕ 29 DNA ends (Prieto *et al.*, 1988) stimulates ϕ 29 DNA-protein p3 replication, but only when assayed near *in vivo* ionic strength conditions (Blanco *et al.*, 1986, 1988a).

In this paper, we describe the catalytic properties of the ϕ 29 DNA polymerase responsible for the efficient synthesis of full length ϕ 29 DNA. A symmetrical model of ϕ 29 DNA replication is also discussed. A preliminary account of some of these findings has been presented (Blanco *et al.*, 1988b).

EXPERIMENTAL PROCEDURES

Materials

Nucleotides, DNA Fragments, and Proteins—Unlabeled nucleotides were purchased from Pharmacia P-L Biochemicals. [α -³²P] dATP (410 Ci/mmol) was obtained from Amersham International Plc. The *Hind*III L fragment, 273 bp long, from the right ϕ 29 DNA end, was isolated from proteinase K-treated ϕ 29 DNA (Inciarte *et al.*, 1976) by gel electrophoresis in 3.5% acrylamide gels and labeled with [α -³²P]dATP at the internal 3'-end with the Klenow fragment of *Escherichia coli* DNA polymerase I, obtained from New England Biolabs. When indicated, the *Hind*III L fragment was denatured by heating for 10 min in boiling water and quickly cooling in ice water. The ϕ 29 DNA polymerase and the ϕ 29 terminal protein were purified as described (Blanco and Salas, 1984; Prieto *et al.*, 1984). Rabbit anti-p2 serum was obtained by using highly purified protein p2 as antigen following conventional methods. The IgG fraction was purified by affinity chromatography on a protein A-Sepharose column as described (Chalon *et al.*, 1979; Hjelm *et al.*, 1972). The *E. coli* ssb and rep proteins were obtained from A. Kornberg (Stanford University), bacteriophage T4 gp32 from J. J. Toulmé (Muséum National D'Histoire Naturelle, Paris, France) and adenovirus DBP from P. van der Vliet (State University of Utrecht, The Netherlands).

Templates—The ϕ 29 DNA-protein p3 complex and M13mp19 single-stranded DNA were isolated as described (Peñalva and Salas, 1982; Heidecker *et al.*, 1980). The universal 17-mer oligonucleotide primer was obtained from Boehringer Mannheim. Hybridization of oligonucleotide primers to M13mp19 single-stranded DNA (100-fold

molar excess of primer) was in 17 mM Tris-HCl, pH 8.2, and 17 mM MgCl₂. The hybridization mixture was heated to 60 °C for 60 min, and allowed to cool to room temperature for 15 min.

Methods

Gel Retardation Assay—Protein p2 in the amount indicated in each case was incubated for 30 min at 0 °C in a final volume of 20 μ l of a buffer containing 12 mM Tris-HCl, pH 7.5, and 1 mM EDTA, with ³²P-labeled HindIII L fragment (~5000 cpm; 2.5 ng) from the right ϕ 29 DNA end, either native or heat-denatured. 20 mM (NH₄)₂SO₄ was added to the incubation mixture with native DNA. Protein p2-DNA complexes were resolved in low ionic strength polyacrylamide gels essentially as described (Carthew *et al.*, 1985). After electrophoresis, the gels were dried and autoradiographed.

Replication Assay with Phage ϕ 29 DNA-Protein p3 as Template—For ϕ 29 DNA-protein p3 replication, the incubation mixture contained, in 25 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM spermidine, 20 mM (NH₄)₂SO₄, ϕ 29 DNA-protein p3 (0.5 μ g), dCTP, dGTP, dTTP, [α -³²P]dATP, and purified proteins p2 and p3 at the concentration and amounts indicated in each case. After incubation for the indicated times at 30 °C, the reaction was stopped by adding 10 mM EDTA, 0.1% SDS, and the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. The Cerenkov radiation of the excluded fraction was counted. When indicated, the labeled DNA from the excluded fraction was treated with proteinase K and subjected to electrophoresis in neutral or alkaline agarose gels (McDonell *et al.*, 1977).

Replication Assay with Primed M13 DNA: Strand Displacement Assay—For M13 DNA replication, single-stranded M13mp19 DNA was hybridized with a 17-mer M13 oligonucleotide primer. The incubation mixture contained, in 10 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.5 μ g of primed M13 DNA, dGTP, dCTP, dTTP, [α -³²P]dATP, and either the ϕ 29 DNA polymerase or the Klenow fragment of the *E. coli* DNA polymerase I at the concentration and amounts indicated in each case. Different salt concentrations were also used as indicated. After incubation for the indicated times at 30 °C, the samples were processed and counted as described above and subjected to electrophoresis in alkaline 0.7% agarose gels alongside DNA length markers (1 kb ladder from Bethesda Research Laboratories). The DNA markers were detected with ethidium bromide, and the synthesized DNA was detected by autoradiography of the dried gels.

Challenger Template-Competition Assay—When indicated, a pre-elongation complex protein p2/protein p3-(dA)₃ was allowed to form in the presence of ϕ 29 DNA-protein p3 as template and dATP as the only nucleotide (taking into account that the DNA sequence at both 5'-ends is AAAGTA . . .), prior to adding the challenger DNA; the incubation mixture contained in 80 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM spermidine, 20 mM (NH₄)₂SO₄, ϕ 29 DNA-protein p3 (0.1 μ g), 20 μ M dATP, protein p3 (40 ng), and protein p2 (16 ng). After incubation for 10 min at 30 °C, the reaction mixture was completed up to 100 μ l by adding 0.25 μ M [α -³²P]dATP (5 μ Ci), dCTP, dGTP, and dTTP to a final concentration of 20 μ M, in the presence or absence of 10 μ g of single-stranded M13mp19 challenger DNA (200-fold molar excess). The reaction was continued at 30 °C, and, at the indicated times, samples of 25 μ l were withdrawn, stopped, and filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. The Cerenkov radiation of the excluded fraction was counted. As a control, ϕ 29 DNA-protein p3 replication was directly measured in the presence of the challenger DNA; in this case, the reaction mixture was as described before but containing both ϕ 29 DNA-protein p3 (0.1 μ g) as template and single-stranded M13mp19 (10 μ g) as challenger DNA.

Inhibition of ϕ 29 DNA Polymerase by Anti-p2 IgG—The ϕ 29 DNA polymerase p2 (48 ng) was preincubated for 30 min at 4 °C with either control or anti-p2 IgG (16 μ g), and then, replication of either ϕ 29 DNA-protein p3 or primed M13 DNA was carried out as described. Alternatively, to study the processivity of the ϕ 29 DNA polymerase, the control or anti-p2 IgG was added 1 min after the primed DNA replication reaction had started, and the length of the synthesized DNA was analyzed by alkaline agarose gel electrophoresis as described.

Helicase Assay—A restriction fragment (500 bp long), containing 325 nucleotides corresponding to the sequence of the ϕ 29 gene 2, was ³²P-labeled by filling in with the *E. coli* DNA pol I (Klenow fragment), heat-denatured and reannealed with a gene 2-containing recombinant M13mp19 single-stranded DNA to obtain a partially duplex M13 DNA substrate with a 5'-single-stranded tail (175 nucleotides long).

The hybrid, purified by Sepharose 4B chromatography, was used as substrate for the helicase assay, carried out essentially as described by Matson and George (1987), and monitored by agarose gel electrophoretic analysis of the ³²P-labeled material displacement.

RESULTS

High Level of ϕ 29 DNA-Protein p3 Synthesized in Vitro—To find out the efficiency of a double-stranded DNA replication system, one important point is to demonstrate that both DNA strands are fully replicated, and therefore that these newly synthesized strands can act as templates in the next round of replication. Using a minimal replication system based on ϕ 29 DNA-protein p3 as template, ϕ 29 DNA polymerase, and the terminal protein, unit length ϕ 29 DNA was synthesized (Fig. 1A). For efficient ϕ 29 DNA replication, the amount of ϕ 29 DNA-protein p3 synthesized *in vitro* should be higher than the initial template. Since under the conditions used the reaction reaches a plateau after 10 min of incubation, one-half of the reaction mixture was withdrawn for analysis after 15 min; the other half was made up to its final volume by a new addition of all components except the ϕ 29 DNA-protein p3 template. This was repeated six times, and the samples corresponding to each replication cycle, corrected by the dilution factor, were analyzed by electrophoresis in neutral agarose gels. Fig. 1B shows that, after six incubation cycles, ϕ 29 DNA-protein p3 increased to 14-fold the initial value. This exponential increase indicates a high efficiency of this minimal system in terms of both the number of initiation events and full elongation of both DNA strands.

Processive Synthesis of ϕ 29 DNA-Protein p3 by the ϕ 29 DNA Polymerase—To test the processivity of the ϕ 29 DNA polymerase, the synthesis of full length ϕ 29 DNA was studied using increasing dilutions of the enzyme, and the labeled DNA synthesized *in vitro* was analyzed by alkaline agarose gel electrophoresis. In the conditions used (20 μ M dNTPs), the time needed for ϕ 29 DNA full length synthesis is about 8 min. A nonprocessive pattern of elongation should give rise to a decrease in the DNA size concomitant with the polymerase dilution. Fig. 2 shows that, whereas a 128-fold decrease in the primer terminus/DNA polymerase molar ratio decreased about 20-fold the amount of DNA synthesized, unit length ϕ 29 DNA was reached in all cases, indicating that the enzyme molecule that initiates replication elongates the DNA chain without dissociation. Furthermore, whereas the ϕ 29 DNA replication catalyzed by protein p2 could be completely inhibited when a 200-fold molar excess of single-stranded M13 DNA was simultaneously added to the natural template, the formation of a pre-elongation complex with the ϕ 29 DNA-protein p3 template (see "Experimental Procedures") was enough to prevent the inhibition by competitor DNA (Fig. 3). Taking into account the length of ϕ 29 DNA (19,285 bp), the above results indicate that the ϕ 29 DNA polymerase is a highly processive enzyme. The efficiency of this minimal system in terms of full length synthesis and processivity depends to a great extent on the strand displacement being coupled to the polymerization process. In fact, the results shown in Figs. 1 and 2 indicate that strand displacement is taking place with the ϕ 29 DNA polymerase p2 and the terminal protein p3 as the only proteins present in the *in vitro* ϕ 29 DNA replication system, suggesting that the ϕ 29 DNA polymerase has helicase-like activity. However, since the terminal protein was present, the possibility that the latter was providing the helicase activity had to be ruled out.

Strand Displacement by the ϕ 29 DNA Polymerase in the Replication of Primed M13 DNA—To determine whether the ϕ 29 DNA polymerase itself was able to produce strand displacement and was a processive enzyme in the absence of the

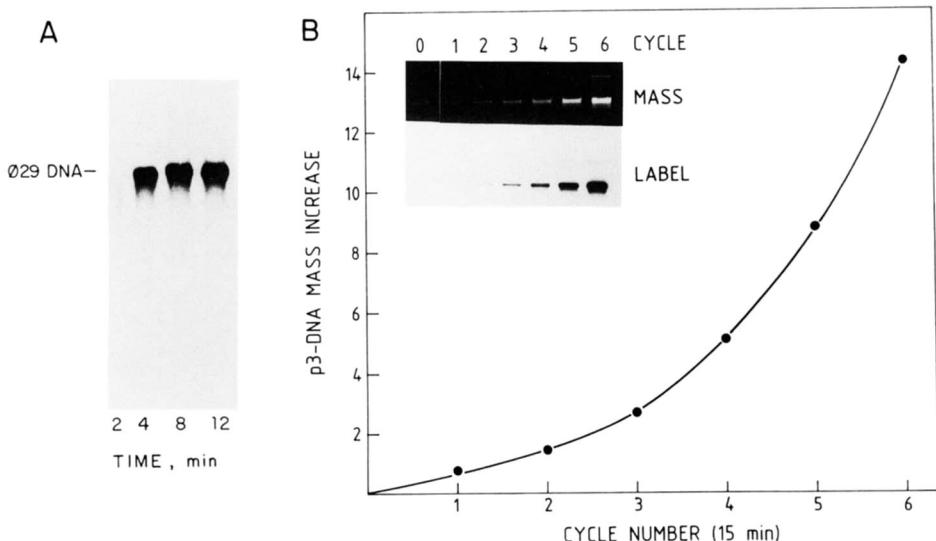


FIG. 1. High level synthesis of ϕ 29 DNA-protein p3 *in vitro*. The replication assay was carried out as described under “Experimental Procedures,” using 80 μ M concentration each of dCTP, dGTP, dTTP, and [α - 32 P]dATP (2 μ Ci), 21 ng of terminal protein (p3), 80 ng of ϕ 29 DNA polymerase (p2), and 0.5 μ g of ϕ 29 DNA-protein p3 as template. A, after incubation for the indicated times at 30 °C, the samples were processed and analyzed by alkaline agarose gel electrophoresis as described under “Experimental Procedures.” The amount of dNMP incorporated was, in nmol, 0.51 (2 min), 1.03 (4 min), 1.29 (8 min), and 1.4 (12 min). B, as it was preliminarily reported (Blanco *et al.*, 1988b), after incubation for 15 min at 30 °C, half of each sample was removed for further analysis, and the other half was completed up to the final volume with the corresponding amount of proteins p3 and p2 and reaction mixture; the reaction was allowed to continue for another 15 min, this process being repeated six times (cycles). The DNA mass present at the end of each cycle was analyzed by neutral agarose gel electrophoresis of the corresponding aliquots corrected by the dilution factor (cycle number). The DNA mass was detected by ethidium bromide staining, and then the gels were dried and autoradiographed. Quantitation was done by densitometry of the autoradiographs and stained bands.

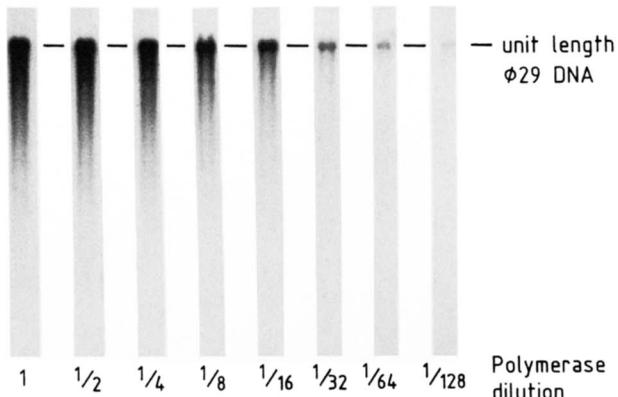


FIG. 2. Processive synthesis of ϕ 29 DNA-protein p3 by the ϕ 29 DNA polymerase. The replication assay was carried out as described under “Experimental Procedures,” using 20 μ M concentration each of dCTP, dGTP, dTTP, and [α - 32 P]dATP (1 μ Ci), 21 ng of terminal protein (p3), 0.5 μ g of ϕ 29-DNA-protein p3 as template, and the indicated amounts of ϕ 29 DNA polymerase p2; dilution 1 corresponds to 160 ng of ϕ 29 DNA polymerase (primer terminus/DNA polymerase ratio = 1/32). After incubation for 10 min at 30 °C, the samples were processed and analyzed in alkaline agarose gels as described under “Experimental Procedures.” The amount of dNMP incorporated was, in nmol, 1.49, 1.45, 1.02, 0.8, 0.63, 0.23, 0.14, and 0.078 for dilutions 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128, respectively. A preliminary account of these results has been given (Blanco *et al.*, 1988b).

terminal protein, replication of singly primed M13 DNA was studied. For comparison, the Klenow fragment of the *E. coli* DNA polymerase I was also used. The alkaline analysis of the *in vitro* labeled DNA shown in Fig. 4 indicates that the Klenow enzyme stopped synthesis after 20 min, when the M13 DNA circle was completed. On the contrary, the ϕ 29 DNA polymerase was able to replicate M13 DNA in 5 min,

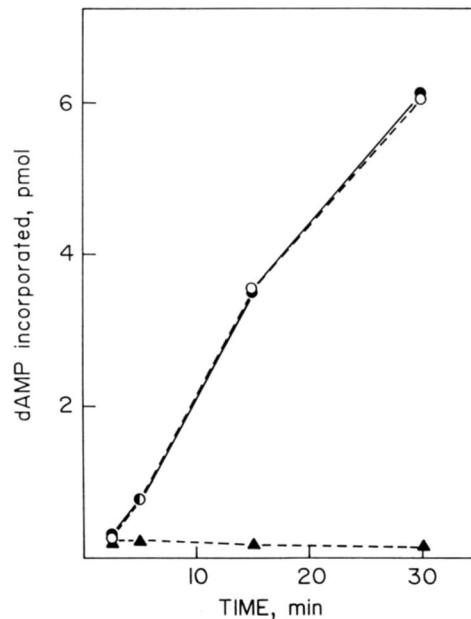


FIG. 3. Challenger template-competition assay. A pre-elongation complex protein p2/protein p3-(dA)₃ was allowed to be formed in the presence of ϕ 29 DNA-protein p3 as template (see “Experimental Procedures”), and the subsequent elongation was carried out either in the absence (○—○) or in the presence (●—●) of 200-fold molar excess of single-stranded M13 challenger DNA. As a control, the challenger DNA was added simultaneously to the ϕ 29 DNA-protein p3 template (▲—▲).

proceeding even further through strand displacement. In fact, at longer incubation times, higher amounts of longer DNA was synthesized, the length of the DNA produced after 40 min being greater than 70 kb, which indicated that more than

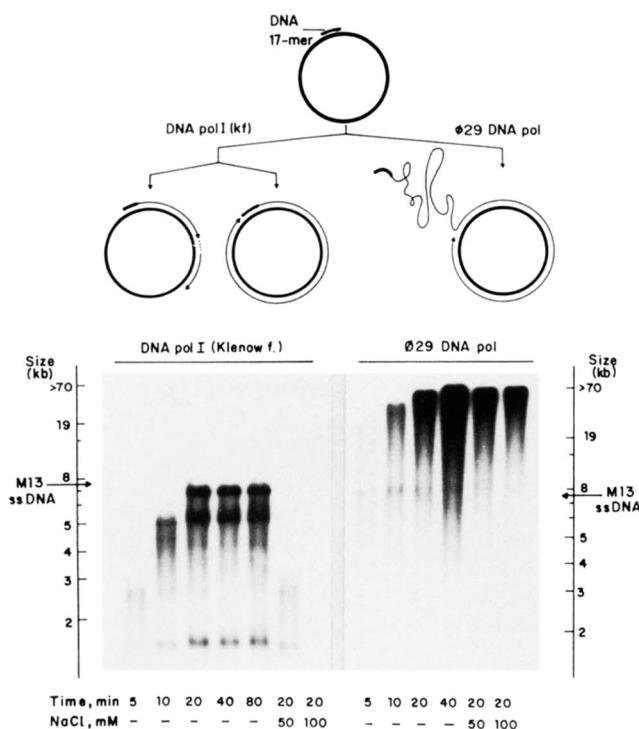


FIG. 4. Strand displacement-associated replication of singly primed M13 DNA by the ϕ 29 DNA polymerase. The incubation mixture was as described under "Experimental Procedures" and contained 0.4 mM concentration each of dCTP, dGTP, dTTP, and [α - 32 P]dATP (5 μ Ci), 0.5 μ g of primed M13 DNA, and either 160 ng of the ϕ 29 DNA polymerase or 2.5 units of the Klenow enzyme. When indicated, 50 mM or 100 mM NaCl was added to the background value of 10 mM. After incubation at 30 °C for the times indicated, the samples were processed and analyzed by alkaline agarose gel electrophoresis alongside DNA length markers, as described under "Experimental Procedures." A preliminary account of these results has been given (Blanco *et al.*, 1988b). The figure shows a scheme of the replicating DNA molecules expected and the autoradiogram of the products of replication. The amount of dNMP incorporated was, in nmol, 0.06 (5 min), 0.14 (10 min), 0.21 (20 min), 0.20 (40 min), 0.22 (80 min), 0.07 (20 min/50 mM NaCl), and 0.02 (20 min/100 mM NaCl) for the Klenow enzyme, and 0.08 (5 min), 0.14 (10 min), 0.28 (20 min), 0.47 (40 min), 0.28 (20 min/50 mM NaCl), and 0.22 (20 min/100 mM NaCl) for the ϕ 29 DNA polymerase.

10-fold the length of M13 DNA had been synthesized. On the other hand, the Klenow enzyme produced stops at specific sites during M13 replication, whereas no such stops, except for the full length M13 DNA position, were seen with the ϕ 29 DNA polymerase.

In addition, when protein p2 was preincubated for 10 min at 4 °C with anti-p2 IgG (see "Experimental Procedures"), almost complete inhibition of primed M13 DNA replication occurred (see Table II). However, when anti-p2 IgG was added 1 min after the reaction was started, the inhibition was drastically reduced (result not shown), indicating that the ϕ 29 DNA polymerase molecule that initiates synthesis does not dissociate from the template during the primed M13 elongation process.

All these results clearly demonstrate that the ϕ 29 DNA polymerase by itself is able to produce strand displacement and is a highly processive enzyme in the absence of the ϕ 29 terminal protein.

Requirements for the Strand Displacement Mechanism of DNA Synthesis Carried Out by the ϕ 29 DNA Polymerase—The effect of increasing the ionic strength and decreasing the temperature, conditions that increase the stability of secondary structures in the template, was studied in the M13 strand

displacement assay. Fig. 4 shows that an increase of ionic strength up to 100 mM NaCl had little effect on the M13 replication by the ϕ 29 DNA polymerase, whereas replication by the Klenow enzyme was sensitive even to 50 mM NaCl. On the other hand, when the primed M13 DNA replication was carried out at 0 °C, the low temperature did not affect the strand displacement ability of the ϕ 29 DNA polymerase, although the elongation rate was reduced (not shown).

Tables I and II summarize the requirements of both ϕ 29 DNA-protein p3 and primed M13 DNA for strand displacement replication using the ϕ 29 protein p2 as the only DNA polymerase. The main differences between the two templates are the need for a terminal protein as primer in the ϕ 29 DNA-protein p3 system, the stimulation by viral protein p6 and the stronger requirement for NH₄⁺ ions. Ammonium ions were shown to be required for formation of a stable complex between the terminal protein and the ϕ 29 DNA polymerase required in the initiation step of ϕ 29 DNA-protein p3 repli-

TABLE I
Requirements for the efficient replication of phage ϕ 29 DNA-protein p3 with purified DNA polymerase p2 and terminal protein p3

The replication assay was carried out as described under "Experimental Procedures," using 20 μ M concentration each of dCTP, dGTP, dTTP, and [α - 32 P]dATP (1 μ Ci), 15 ng of terminal protein p3, 47 ng of ϕ 29 DNA polymerase p2, and 0.5 μ g of ϕ 29 DNA-protein p3 as template. ϕ 29 protein p6 (2 μ g) and other components were added or omitted as indicated. After incubation for 5 min at 30 °C, the samples were processed as described under "Experimental Procedures." The value of 100% represents the incorporation of 129 pmol of dNMP.

Component omitted or added	ϕ 29 DNA-protein p3 replication
	%
None	100
-Terminal protein	1
- ϕ 29 DNA polymerase	<0.1
- ϕ 29 DNA-protein p3	0.1
-MgCl ₂	0.8
-Ammonium sulfate	3.8
+IgG α - ϕ 29 DNA polymerase	0.2
+rNTPs, 0.5 mM	35.6
+ ϕ 29 protein p6	150
+NaCl, 100 mM	10.2
+ ϕ 29 protein p6 + NaCl, 100 mM	100

TABLE II
Requirements for the strand displacement-associated replication of primed M13 DNA by the ϕ 29 DNA polymerase

The replication assay was carried out as described under "Experimental Procedures," using 80 μ M concentration each of dCTP, dGTP, dTTP, and [α - 32 P]dATP (1 μ Ci), 47 ng of ϕ 29 DNA polymerase, and 0.5 μ g of primed M13 DNA as template. ϕ 29 terminal protein p3 (15 ng), ϕ 29 protein p6 (2 μ g), and other components were added or omitted as indicated. After incubation for 10 min at 30 °C, the samples were processed as described under "Experimental Procedures." The value of relative activity 100% represents the incorporation of 123 pmol of dNMP.

Component omitted or added	Primed M13 DNA replication
	%
None	100
- ϕ 29 DNA polymerase	0.6
-Primed M13	0.6
-MgCl ₂	1.4
+IgG α - ϕ 29 DNA polymerase	<0.6
+Terminal protein	69.3
+Ammonium sulfate	229.2
+rNTPs, 0.5 mM	34.0
+ ϕ 29 protein p6	80.4
+NaCl, 100 mM	69.4
+ ϕ 29 protein p6 + NaCl, 100 mM	37.6

cation. In addition, they stimulated the rate of elongation about 2.5-fold (Blanco *et al.*, 1987). Ammonium ions stimulated the replication of primed M13 DNA about 2.3-fold (see Table II), due to an increase in the rate of elongation (not shown). The ϕ 29 protein p6, that strongly stimulates ϕ 29 DNA-protein p3 replication at high NaCl concentration by preventing the salt inhibition (see Table I) and acts at the transition step from initiation to elongation (Blanco *et al.*, 1988a), was unable to stimulate primed M13 DNA synthesis either in the absence or in the presence of 100 mM NaCl (Table II). These results indicate that the protein p6 effect is dependent on the presence of specific ϕ 29 DNA sequences. Furthermore, addition of different DNA binding proteins as *E. coli* ssb, bacteriophage T4 gp32, adenovirus DBP, or the *E. coli* helicase rep did not stimulate DNA synthesis by ϕ 29 DNA polymerase with either ϕ 29 DNA-protein p3 or primed M13 DNA (Blanco *et al.*, 1988a and results not shown).

No helicase activity, tested as described under "Experimental Procedures," was detected in the ϕ 29 DNA polymerase preparation (not shown), indicating that the strand displacement ability is dependent on the polymerization reaction. This process does not require hydrolysis of rNTPs. In fact, addition of 0.5 mM rNTPs to these two DNA replication systems produced about 65% inhibition (Tables I and II). In addition, dNTPs are not directly hydrolyzed, being a low level of dNMPs detected due to the 3' → 5' exonuclease activity of the ϕ 29 DNA polymerase (not shown).

Binding of the ϕ 29 DNA Polymerase to Double- and Single-stranded DNA—When the binding of protein p2 to the double-stranded HindIII L fragment, 273 bp long, from the right ϕ 29 DNA end was studied by a gel retardation assay, some band shift was seen with 3 ng of protein p2, an amount of 48 ng of p2 being needed to get retardation of all of the band (Fig. 5A). The ordered binding pattern shown in Fig. 5A was not clearly seen when 20 mM $(\text{NH}_4)_2\text{SO}_4$ was omitted from the incubation mixture (not shown). With heat-denatured HindIII L fragment, the amount of protein p2 required to get DNA retardation was also similar (Fig. 5B). Similar results were obtained with DNA fragments lacking specific ϕ 29 terminal sequences (not shown). The addition of 20 mM $(\text{NH}_4)_2\text{SO}_4$ to the incubation mixture did not affect the binding pattern of the ϕ 29 DNA polymerase to single-stranded DNA (not shown). In addition, the binding of protein p2 to

single-stranded DNA was more resistant to salt than binding to double-stranded DNA. Up to 75 mM NaCl can be used without essentially affecting the binding of protein p2 to single-stranded DNA, but at this salt concentration the binding of p2 to double-stranded DNA is completely abolished (results not shown). Furthermore, the addition of 10 mM MgCl₂, needed for protein p2 catalysis, to the incubation mixture produced the exonucleolytic degradation of the single-stranded DNA fragment, whereas in these conditions the double-stranded DNA was neither digested nor retarded by identical amounts of the enzyme (results not shown). The above results suggest that binding to single-stranded DNA is more relevant for ϕ 29 DNA polymerase activity than that to double-stranded DNA.

DISCUSSION

A highly efficient ϕ 29 DNA replication system, able to carry out the complete replication of both DNA strands, only requires two proteins: the ϕ 29 terminal protein p3, that acts as a primer for initiation, and the viral DNA polymerase p2. The 66-kD ϕ 29 DNA polymerase owes its high catalytic efficiency to a remarkable processivity and to the ability to produce strand displacement during the polymerization process.

In other systems, accessory proteins confer processivity to the DNA polymerase. Thus, *E. coli* thioredoxin is an accessory protein that stabilizes the binding of T7 DNA polymerase to a primer template, stimulating the processivity of the polymerization reaction (Tabor *et al.*, 1987); the three accessory proteins encoded by genes 44, 45, and 62 of phage T4 increase the processivity and the rate of T4 DNA polymerase (Mace and Alberts, 1984); the DNA polymerase III holoenzyme of *E. coli* includes several auxiliary proteins that increase the processivity of the core polymerase (Maki and Kornberg, 1988). The eukaryotic DNA polymerase δ is processive in the presence of proliferating cell nuclear antigen, although it is essentially distributive in its absence (Downey *et al.*, 1988). In all these systems, the role of the accessory protein(s) is mainly to clamp the polymerase to the primer template, enabling processive DNA synthesis to occur. On the other hand, the ϕ 29 protein p2 is in itself a DNA polymerase with high processivity, and accessory proteins do not seem to be required. The strong binding of the ϕ 29 DNA polymerase to single-stranded DNA probably accounts for its inherent processivity.

Another important requirement for efficient replication of duplex DNA is the unwinding of the parental DNA helix. In this paper, we show that the ϕ 29 DNA polymerase also has unwinding properties, being able to use double helical DNA templates without any unwinding protein and even in the absence of rNTP breakdown. This probably means that enough energy for helix unwinding can also come from the deoxyribonucleoside triphosphate cleavage which accompanies polymerization, as proposed by Alberts and Sternglanz (1977).

The discovery of discontinuous DNA synthesis and the finding of RNA primers were the reasons to generally consider DNA replication as an inherently asymmetric process, with one strand being synthesized continuously and the other strand discontinuously. To account for the replication of both strands, two asymmetric DNA polymerases must be involved: one of them must be moderately processive since discontinuous synthesis requires that the polymerase molecule must recycle rapidly at the replication fork from a nascent (Okazaki) fragment of the lagging strand to a new primer terminus. The other, highly processive, may work on the leading strand.

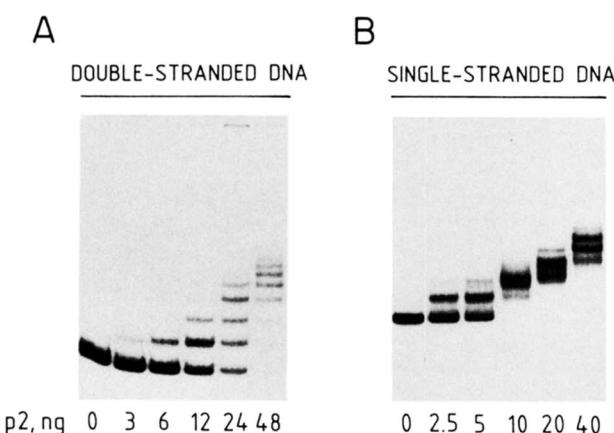
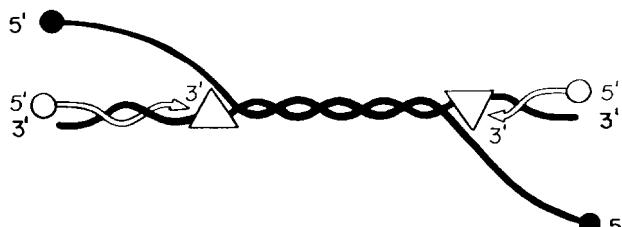


FIG. 5. Binding of protein p2 to double- and single-stranded DNA. ^{32}P -Labeled ϕ 29 DNA HindIII L fragment, either native (A) or heat-denatured (B), was incubated with the indicated amounts of protein p2, in the presence or absence of 20 mM $(\text{NH}_4)_2\text{SO}_4$, respectively, and the protein-DNA complexes were separated by polyacrylamide gel electrophoresis as indicated under "Experimental Procedures." After electrophoresis, the gels were dried and autoradiographed.

This asymmetry can be obtained by the following. 1) Association of the same core polymerase with different auxiliary subunits in order to be functionally different both in binding and in using the DNA template (Fig. 6B). This asymmetric dimeric polymerases and a super assembly of these (replisome) have been proposed to couple lagging and leading strand synthesis in the *E. coli* chromosome (McHenry and Johanson, 1984; Maki *et al.*, 1988) and in that of phage T4 (Alberts, 1984). 2) Involvement of two distinct DNA polymerases, as it has been proposed for the replication of the mammalian genome. DNA polymerase δ , highly processive in the presence of proliferating cell nuclear antigen and able to carry out strand displacement synthesis would be the leading strand replicase, whereas DNA polymerase α , moderately processive and tightly associated with a DNA primase, would be the lagging strand replicase (Downey *et al.*, 1988). A similar asymmetry has been proposed for yeast DNA replication, where DNA polymerases I and II are the counterparts of DNA polymerase α and δ , respectively (Campbell, 1988).

However, this asymmetry is not necessary in the case of the linear double-stranded DNA of bacteriophage ϕ 29. In this case, the existence of two replication origins placed at both ends of the duplex DNA chromosome allows both strands to be replicated continuously (Fig. 6A), and, therefore, this mechanism of symmetric replication would not require asymmetric complexes of DNA polymerase with other accessory proteins to control the different mechanics of continuous and discontinuous synthesis. In this sense, the catalytic properties

A. SYMMETRIC MODEL



B. ASYMMETRIC MODEL

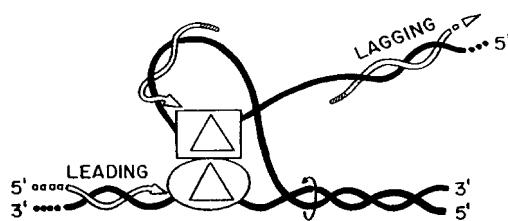


FIG. 6. Symmetrical and asymmetrical modes of DNA replication. *A*, proposed model for ϕ 29 DNA replication. This model involves two replication origins, protein-primed initiation, and continuous synthesis of both DNA strands carried out by 2 units of an efficient (highly processive) monomeric DNA polymerase. *B*, proposed model for *E. coli* and T4 DNA replication (McHenry and Johanson, 1984; Maki *et al.*, 1988; Alberts, 1984). This model involves one replication origin, RNA-primed initiation, and continuous (leading strand) and discontinuous (lagging strand) DNA synthesis carried out by two efficient and asymmetrical units of multimeric DNA polymerases.

of the ϕ 29 DNA polymerase, high processivity and strand displacement ability, seem to be well designed to support this symmetric mode of replication.

As the two replication forks move, long stretches of single-stranded ϕ 29 DNA are displaced as replicative intermediates. This transient situation, probably disadvantageous for a large DNA, may require the function of single-stranded DNA binding protein(s) to protect the displaced DNA against nuclease action. In addition to the terminal protein and the DNA polymerase, ϕ 29 DNA replication *in vivo* requires the products of the viral genes 1, 5, 6, and 17 (Talavera *et al.*, 1972; Carrascosa *et al.*, 1976). Although the *in vivo* role of these proteins remains to be determined, *in vitro* results (Prieto *et al.*, 1988)² suggest that several functions such as DNA protection and maintenance of a proper DNA topology could be carried out by the ϕ 29 proteins p5 and p6, respectively.

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