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Function of the C-terminus of ϕ29 DNA polymerase in DNA and terminal protein binding

Verónica Truniger, José M. Lázaro and Margarita Salas*

Instituto de Biología Molecular 'Eladio Viñuela' (CSIC), Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

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ABSTRACT

The thumb subdomain, located in various family B DNA polymerases in the C-terminal region, has been shown in their crystal structures to move upon binding of DNA, changing its conformation to nearly completely wrap around the DNA. It has therefore been involved in DNA binding. In agreement with this, partial proteolysis studies of ϕ29 DNA polymerase have shown that the accessibility of the cleavage sites located in their C-terminal region is reduced in the presence of DNA or terminal protein (TP), indicating that a conformational change occurs in this region upon substrate binding and suggesting that this region might be involved in DNA and TP binding. Therefore, we have studied the role of the C-terminus of ϕ29 DNA polymerase by deletion of the last 13 residues of this enzyme. This fragment includes a previously defined region conserved in family B DNA polymerases. The resulting DNA polymerase Δ13 was strongly affected in DNA binding, resulting in a distributive replication activity. Additionally, the capacity of the truncated polymerase to interact with TP was strongly reduced and its initiation activity was very low. On the other hand, its nucleotide binding affinity and its fidelity were not affected. We propose that the C-terminal 13 amino acids of ϕ29 DNA polymerase are involved in DNA binding and in a stable interaction with the initiator protein TP, playing an important role in the intrinsic processivity of this enzyme during polymerization.

INTRODUCTION

The three-dimensional crystal structure of several DNA polymerases belonging to different families has shown that their synthetic and degradative activities reside in structurally separated domains. Comparison of their polymerization domains has shown their structural similarity to a right hand and allowed definition of three distinct subdomains, named palm, fingers and thumb (1). The palm contains the polymerization active site, while thumb and fingers have been proposed to be important for DNA and dNTP binding. During replication by DNA polymerases the DNA is translocated with each cycle of polymerization. Conformational changes in the DNA polymerase upon binding of template/primer and nucleotide, which could facilitate this movement and favour a correct polymerization reaction, have been shown to occur (2–4). The fingers subdomain was shown to undergo substrate-induced conformational changes upon binding nucleotide in addition to DNA (ternary complex), rotating towards the palm to form a binding pocket for the nascent base pair (2–4). In fact, several of the fingers residues have been shown to be, together with some of the palm subdomain, important for nucleotide insertion fidelity (5–12). On the other hand, comparison of the crystal structures with and without DNA of several family A DNA polymerases, such as *Taq* Pol I (13,14), *Bst* Pol I (15), *Escherichia coli* Pol I (16) and T7 DNA polymerase (17), has shown that the thumb subdomain changes its conformation to nearly completely wrap around the DNA. In the crystal structures of *E.coli* Pol I, *Taq* and T7 DNA polymerases with DNA, several residues of their thumb subdomains have been shown to contact nucleotides of the template or primer strands (14,16,17). The thumb subdomain has therefore been involved in DNA binding stability and processivity. The only family B DNA polymerase crystallized to date as a closed ternary complex, RB69 DNA polymerase (18), when compared with its open complex structure (19), also shows rotation of the thumb as a whole towards the palm by ~8°, wrapping around the template/primer DNA on its minor groove side. Two residues lying at the C-terminal end of this DNA polymerase, Lys874 and Lys878, have been shown to interact with the phosphate groups between the 6th, 7th and 8th nucleotides of the template strand (counting from the templating nucleotide) (20). Therefore, the C-terminal region of the thumb of RB69 DNA polymerase seems also to be involved in DNA binding. In the DNA polymerases belonging to family B, whose crystal structure has been solved, the thumb is localized in their C-terminal region [bacteriophage RB69 (19), *Thermococcus gorgonarius* (20), *Desulfurococcus* strain Tok (21), *Thermococcus* sp. 9^0N-7 (22) and *Pyrococcus kodakaraensis* KOD1 (23) DNA polymerases], which has been poorly studied.

*To whom correspondence should be addressed. Tel: +34 91 3978435; Fax: +34 91 3978490; Email: msalas@cbm.uam.es
Present address:
Verónica Truniger, CEBAS, Campus Universidad de Murcia, 30100 Espinardo, Murcia, Spain

In the case of ϕ29 DNA polymerase, a family B DNA polymerase, the extensive structure/function studies of highly conserved residues and the biochemical characterization provided an appropriate basis to understand the dynamics of DNA replication (24–26). The results obtained from mutational analysis of ϕ29 DNA polymerase (27–37) are in agreement with the data of the existing crystal structures of family B DNA polymerases (18–23). The high processivity required for DNA replication by DNA polymerases is generally achieved by stable binding of the DNA and association of their catalytic subunit with accessory proteins that reduce the rate of dissociation of the enzyme from the DNA, relative to translocation and further nucleotide addition. Due to its high intrinsic processivity (>70 kb) and strand-displacement capacity, ϕ29 DNA polymerase does not need the help of any helicase or processivity factor to completely replicate its 19 kb linear natural template (38). This suggests that this enzyme must have specific DNA binding subdomains involved in processivity. The thumb subdomain of ϕ29 DNA polymerase is supposed to be located in its C-terminal region distal to the 'KxY' motif (like in other family B DNA polymerases). By sequence alignment of family B DNA polymerases, Blanco and co-workers defined a conserved region (region 5) located in ϕ29 DNA polymerase at its C-terminal end, which comprised its last 12 amino acids (39). We have studied here the role of the C-terminus of ϕ29 DNA polymerase by deletion of its last 13 amino acids. The resulting truncated DNA polymerase Δ13 was strongly affected in DNA and terminal protein (TP) binding, resulting in a distributive replication activity.

MATERIALS AND METHODS

Nucleotides and proteins

Unlabeled nucleotides were purchased from Pharmacia P-L Biochemicals. [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham International plc. Restriction endonucleases were from New England BioLabs. T4 polynucleotide kinase was from Boehringer Mannheim.

ϕ29 TP-DNA was isolated according to Peñalva and Salas (40). The TP was purified as described by Zaballos and Salas (41). The ϕ29 single-stranded DNA (ssDNA)-binding protein and the double-stranded DNA (dsDNA)-binding protein p6, obtained from *Bacillus subtilis* cells infected with phage ϕ29, were purified as described previously (42,43).

DNA templates and substrates

Oligonucleotides sp1 (5′-GATCACAGTGAGTAC) and sp1c+6 (5′-TCTATTGTACTCACTGTGATC), sp1c+13 (5′-ACTGGCCGTCGTTGTACTCACTGTGATC) and sp1c+18 (5′-ACTGGCCGTCGTTCTATTGTACTCACTGTGATC), which have a 5′-extension of 6, 13 and 18 nucleotides, respectively, in addition to the sequence complementary to sp1, were obtained from Isogen. The oligonucleotide sp1 was first purified by electrophoresis on 8 M urea–20% polyacrylamide gels and then 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. To analyze the polymerization activity on a primer/template structure, 5′-labeled sp1 (matched 3′-terminus) was hybridized to sp1c+6, sp1c+13 or sp1c+18 in the presence of 0.2 M NaCl and 60 mM Tris–HCl (pH 7.5). The molecules sp1/sp1c+6 (matched 15mer/21mer) and sp1/sp1c+18 (matched 15mer/33mer) were used as substrates for gel retardation and polymerization experiments of template/primer DNA. To analyze processive DNA polymerization coupled to strand displacement by ϕ29 DNA polymerase, the universal primer (Boehringer Mannheim) was hybridized to M13mp8 ssDNA as described above, and the resulting molecule was used as a primer/template suitable for a rolling-circle type of DNA replication.

Site-directed mutagenesis and expression of ϕ29 DNA polymerase mutants

The wild-type ϕ29 DNA polymerase gene, cloned into plasmid pJLPM [a derivative of pT7-4w2 (26)], which expresses ϕ29 DNA polymerase under the control of the T7 RNA polymerase-specific ϕ10 promoter (44), was used for construction of the truncated DNA polymerase. After one-step PCR to introduce a stop codon after amino acid residue 562 in the ϕ29 DNA polymerase sequence, the fragment containing the C-terminal half of the ϕ29 DNA polymerase gene missing the last 42 base pairs and including a unique AccI restriction site was subcloned into pJLPM digested with AccI and SmaI. The stop codon introduced resulted in a truncated ϕ29 DNA polymerase missing the last 13 amino acids of its C-terminus. The absence of any other changes was confirmed by sequencing. Expression of the mutant protein was carried out in the *E.coli* strain BL21(DE3), which contains the T7 RNA polymerase gene under the control of the IPTG-inducible lacUV5 promoter (45). The purification of wild-type and mutant ϕ29 DNA polymerases was performed as described previously by Lázaro *et al.* (26). MALDI-TOF-MS analysis of the tryptic peptides of both wild-type and Δ13 mutant DNA polymerases, and MS/MS fragmentation and analysis of the sequence of the corresponding peptides by LC-ESI-IT, SIM mode, was carried out to determine the C-terminal sequence of the Δ13 mutant DNA polymerase.

Buffers

The 10× reaction buffer used in all assays contained 500 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 40% glycerol and 1 mg/ml bovine serum albumin (BSA). The buffer used for dilution of the polymerase contained 25 mM Tris–HCl (pH 7.5), 120 mM NaCl, 1 mg/ml BSA and 50% glycerol.

DNA gel retardation assays

The interaction of either wild-type or mutant ϕ29 DNA polymerase with a primer/template structure was assayed using the 5′-labeled sp1/sp1c+6 (15mer/21mer) and sp1/sp1c+18 (15mer/33mer) DNAs. The incubation mixture, in a final volume of 20 μl, contained 12 mM Tris–HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulfate, 0.1 mg/ml BSA, 1.2 nM of 5′-labeled DNA and, as indicated, between 2.1 and 33.6 nM of either wild-type or mutant ϕ29 DNA polymerase (36). After incubation for 5 min at 4°C, the samples were subjected to electrophoresis in 4% (w/v) polyacrylamide gels (80:1, monomer:bis) containing 12 mM Tris–acetate (pH 7.5) and 1 mM EDTA, and run at 4°C in the same buffer at 8 V/cm, essentially as described previously (46). After autoradiography, enzyme/DNA stable interaction was detected as a shift in the position of the free labeled DNA, and quantitated by

densitometry of the autoradiograms corresponding to different experiments.

Polymerase/3′–5′ exonuclease (pol/exo) coupled assay

The DNA molecules sp1/sp1c+6 (15mer/21mer) and sp1/sp1c+18 (15mer/33mer) contain a six- and 18-nt 5′-protruding end, respectively, and can therefore be used as substrate for the 3′–5′ exonuclease activity (dsDNA) and also for DNA-dependent DNA polymerization. The reaction mixture, in a final volume of 12.5 µl, contained 1.25 µl reaction buffer, 10 mM $MgCl_2$, 1.2 nM of the 5′-labeled DNA, 16.8 nM of either wild-type or mutant ϕ29 DNA polymerase, and the indicated increasing concentrations of the four dNTPs. If indicated, a 100× molar excess of unlabeled DNA as trap was added at the same time as the reaction was started with the metal ion, after preincubation (10 min on ice) of the DNA polymerase with the same, but labeled, DNA substrate in the presence of 0.5 µM dNTPs. After incubation for 5 min at 25°C, the reaction was stopped by the addition of 3 µl of sequencing loading buffer. Samples were analyzed by 8 M urea–20% polyacrylamide gel electrophoresis and autoradiography. Polymerization or 3′–5′ exonuclease is detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labeled sp1 primer. Different dNTP concentrations are required for the wild-type ϕ29 DNA polymerase to polymerize the first nucleotide (from position 15 to 16), to polymerize effectively until the last nucleotide (from position 16 to 20, or from 16 to 32) and to replicate the last nucleotide of the template (from position 20 to 21 or from 32 to 33).

Exonuclease activity was assayed in linear conditions, both in terms of time and enzyme quantity, using either the ssDNA molecule sp1 (15mer) or the dsDNA molecule sp1/sp1c+6. Total exonucleolytic activity was calculated from the intensity of each degradation product (produced in the absence of dNTPs), obtained by densitometry of the autoradiograms, corrected (multiplied) by the number of catalytic events needed to give rise to each of these degradation products. From these data, the catalytic efficiency (indicated in Table 1) of the mutant derivative was calculated relative to wild-type ϕ29 DNA polymerase.

Processive DNA polymerization assay (replication of primed M13-DNA)

The reaction mixture, in a final volume of 25 µl, contained 2.5 µl reaction buffer, 10 mM $MgCl_2$, 20 µM of each of the four dNTPs, 13 nM [α-^{32}P]dATP (2 µCi), 4.2 nM of oligonucleotide-primed M13 ssDNA and 16.8 nM of either wild-type or truncated ϕ29 DNA polymerase. If indicated, 36 µM of ϕ29 ssDNA binding protein (SSB) was added to the reaction. After incubation for the indicated times (between 10 and 60 min) at 30°C, the reaction was stopped by the addition of 10 mM EDTA and 0.1% SDS. After filtration through Sephadex G-50 spin columns, the Cerenkov radiation of the excluded volume was determined to calculate the amount of incorporated dNMPs. Elongation was analyzed by alkaline 0.7% agarose gel electrophoresis (47). Short replication products are difficult to visualize quantitatively in this alkaline gel. The position of unit length ϕ29 DNA in the agarose gels was detected by ethidium bromide staining. Autoradiography of the dried gels revealed the elongation efficiency.

Protein-primed initiation assay

The formation of TP–dAMP was performed either with or without TP-DNA as template. In both cases the reaction mixture, in a final volume of 25 µl, contained 2.5 µl reaction buffer, 20 mM ammonium sulfate, 0.03 µM [α-^{32}P]dATP (2 µCi), 83.2 nM of purified TP, and 16.8 nM of either wild-type or truncated ϕ29 DNA polymerase. When indicated, 34 µM of p6 was added to the reaction. The template-dependent assay (with 1.6 nM of TP-DNA) was performed with 10 mM $MgCl_2$ as metal activator and the incubation time was 5 min at 30°C. The assay without template was performed with 1 mM $MnCl_2$ as metal activator and the incubation was for 4 h at 5°C. Reactions were stopped by the addition of 10 mM EDTA, 0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns (in the presence of 0.1% SDS) and analyzed further by SDS–PAGE as described previously (40). The TP–dAMP complex was detected by autoradiography and quantitated by densitometric analysis. To calculate the Michaelis–Menten constant for dATP binding (K_m and V_{max}) in the TP-DNA initiation reaction (performed with 1 mM $MnCl_2$), different dATP concentrations and incubation times were assayed.

Misincorporation frequency during initiation was studied in the presence of TP-DNA and 1 mM $MnCl_2$, and 10 µM of either [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{32}P]dGTP or [α-^{32}P]dTTP (5 µCi). The assay was performed as described above.

TP-DNA replication (protein-primed initiation, transition and elongation)

The incubation mixture for the truncated replication assay (no dCTP supplied), in a final volume of 25 µl, contained 2.5 µl reaction buffer, 20 mM ammonium sulfate, 1.6 nM TP-DNA, 83.2 nM of purified TP, 0.1 or 1 µM of [α-^{32}P]dATP (2 µCi) or either 1 or 10 µM of each dGTP, dTTP and [α-^{32}P]dATP (2 µCi), 1 mM $MnCl_2$ and 16.8 nM of the corresponding DNA polymerase. When indicated, 34 µM of p6 was added. After incubation at 30°C for 1 min (wild type) or 10 min (Δ13 mutant DNA polymerase), the reactions were stopped by the addition of 10 mM EDTA, 0.1% SDS, and the samples were filtered through Sephadex G50 spin columns in the presence of 0.1% SDS. Separation of TP–dAMP (initiation complex) from TP–$(dAMP)_2$ and further truncated transition products was carried out in SDS-containing 12% polyacrylamide gels (360 × 280 × 0.5 mm) as described previously (48), and detected by autoradiography. Quantitation was performed by densitometric scanning of the autoradiograms.

The complete TP-DNA replication reaction was performed as described above, but with 20 µM of the four dNTPs and incubation times of 5, 15 and 30 min at 30°C. The Cerenkov radiation in the excluded volume of the Sephadex G50 spin columns was measured and used for quantitation.

Analysis of the interaction between DNA polymerase and TP

The interaction assay by glycerol gradient centrifugation was performed as described previously (37), but with 1 µg of TP and 2 µg of wild-type or truncated ϕ29 DNA polymerase.

The assay to study the competition between truncated and wild-type ϕ29 DNA polymerases for binding the TP was

Table 1. Enzymatic activities of the truncated ϕ29 mutant DNA polymerase

Activity assay	Substrate, DNA binding proteins	ϕ29 DNA polymerase	
		Wild type	Δ13
Stable DNA binding	15mer/21mer (15mer)	100 (100)	ND (ND)
	15mer/33mer	100	2
Pol/Exo balance, [dNTP] nM[a]	15mer/21mer	10/30	10/100
	15mer/33mer	<10/<10	$<10/10^4$
Exonuclease	15mer/21mer (15mer)	100 (100)	1 (<1)
M13 replication	Primed M13-DNA, ±SSB	100	6/28
TP–dAMP formation	No template	100	1
	TP-DNA, +/–p6	100/100	4/3
K_m (μM)/V_{max} (fmol/min)	TP-DNA	1.7/20	1.8/0.9
TP-DNA replication	TP-DNA	100	<1

ND, not detectable.
[a]In the pol/exo coupled assay, the dNTP concentration required (nM) for insert of the first nucleotide and for effective polymerization (position 16/position 20) is indicated.
The different activity assays were carried out with the indicated substrates, as described in Materials and Methods. Numbers (with the exception of the pol/exo data and TP–dAMP constants) indicate percentage of activity obtained with the wild-type polymerase (100%) and are an average of several experiments.

performed as the initiation reaction without TP-DNA described above, but with 20 ng of wild-type DNA polymerase and 8 ng of TP (pol:TP = 1:1 molar ratio). Increasing amounts of the truncated polymerase Δ13, nearly inactive in this reaction, were added (10, 20, 50, 100 and 200 ng). ϕ29 DNA polymerase mutant D249E [catalytically inactive but displaying a normal interaction with TP (28)] was used as a positive control in this interference assay. Incubation and further treatment of the samples was performed as described above for the template-independent, protein-primed initiation assays.

RESULTS

Construction of a truncated ϕ29 DNA polymerase at the C-terminus

Blanco *et al.* defined a C-terminal region (named region 5) conserved in DNA polymerases belonging to family B (39). To study its possible function, we constructed a truncated ϕ29 DNA polymerase lacking the C-terminal 13 amino acids containing this region (see Materials and Methods). A stop codon was inserted by PCR mutagenesis after amino acid 562 of the ϕ29 DNA polymerase sequence (total length 575 amino acids). The wild-type and truncated ϕ29 DNA polymerases were purified to homogeneity as described by Lázaro *et al.* (26). MALDI-TOF-MS analysis of the tryptic peptides of both the wild-type and the Δ13 mutant DNA polymerase indicated that the two proteins contained the same peptides, except two with the following masses: in the case of the wild-type polymerase, 1884.05 and 2384.1, which corresponds with the theoretical mass of two C-terminal peptides with sequences PVQVPGGVVLVDDTFTIK and MoxKPVKPVQVPGGV-VLVDDTFTIK, respectively; and in the case of the Δ13 mutant the masses obtained were 539.32 and 1039.6, which correspond to the theoretical masses of two C-terminal peptides with sequences PVQVP and MoxKPKPVQVP, respectively. To confirm that the two peptides of the Δ13 mutant are the ones at the C-terminal end we carried out a fragmentation MS/MS and analysis of the sequence of each peptide by LC-ESI-IT, SIM mode. The results obtained confirmed that the sequences of the two peptides correspond to the C-terminal ones with sequences PVQVP and MoxKPKPVQVP, respectively. Therefore, the mutant Δ13 DNA polymerase lacks the last 13 amino acids.

The C-terminal truncated ϕ29 DNA polymerase is strongly affected in DNA binding

Mutant DNA polymerase Δ13 was assayed for its DNA binding capacity using primer/template structures of different template length (21 and 33 base pairs, as described in Materials and Methods). In a native gel, the wild-type enzyme bound to the primer/template structure results in a single retardation band (see Fig. 1A and B), which most likely corresponds to a protein–DNA interaction in which the primer terminus is stabilized at the polymerization active site (34). Comparison of Figure 1A and B shows that the wild-type ϕ29 DNA polymerase binds the DNA structure with the longer template strand more efficiently than the shorter one. Under the same conditions the truncated DNA polymerase was not able to bind the shorter DNA substrate (15mer/21mer; Fig. 1A). On the other hand, as shown in Figure 1B, DNA polymerase Δ13 was able to bind nearly all of the longer DNA substrate (15mer/33mer), but most of the binding was not stable enough to reach the Pol–DNA complex position (Fig. 1B; Table 1). The ssDNA binding activity of the truncated mutant polymerase was also undetectable (Table 1). These results indicate that deletion of the C-terminal 13 amino acids strongly affects the DNA binding capacity of ϕ29 DNA polymerase.

Distributive polymerization activity of the truncated mutant DNA polymerase on a primer/template structure

The polymerization coupled to exonuclease activity of DNA polymerases can be studied on a primer/template molecule by increasing the dNTP concentration progressively (polymerase/3′–5′ exonuclease coupled assay; see Materials and Methods). As can be seen in Figure 2A and Table 1 (with a 15mer/21mer DNA), different dNTP concentrations were required for the wild-type ϕ29 DNA polymerase to start replication and polymerize the first nucleotide (10 nM, from position 15 to 16), to replicate effectively until the penultimate nucleotide

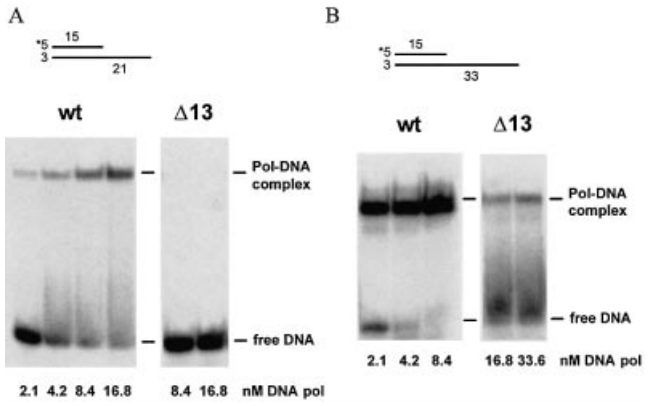

Figure 1. Strongly reduced DNA binding capacity of the truncated Δ13 ϕ29 DNA polymerase. The 5′-labeled hybrid molecules were incubated with increasing amounts (nM) of the wild-type or truncated Δ13 ϕ29 DNA polymerase, in the conditions described in Materials and Methods. After non-denaturating gel electrophoresis, the mobility of free DNA and that of the retarded polymerase–DNA complex (as indicated) was detected by autoradiography. (**A**) Gel retardation analysis performed with the indicated molecule sp1/sp1c+6 (15mer/21mer). (**B**) Gel retardation analysis performed with the indicated molecule sp1/sp1c+18 (15mer/33mer), with a 12-nt longer template strand.

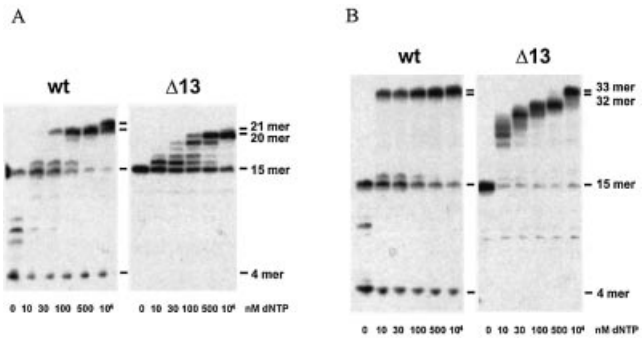

Figure 2. DNA polymerase/exonuclease coupled assay with the truncated Δ13 ϕ29 DNA polymerase. The assays were carried out as described in Materials and Methods, using 5′-labeled hybrid molecules as primer/template DNA. After autoradiography of the 8 M urea–20% polyacrylamide gel, polymerization or 3′–5′ exonuclease activity of the wild-type and truncated Δ13 ϕ29 DNA polymerases are detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labeled sp1 primer. The positions of the non-elongated primer (15mer), elongated primer (20-, 21- and 32-, 33mer) and degraded primer (4mer) are shown. The dNTP concentration (nM) required for replication of the first nucleotide (position 16) and efficient replication of the template (position 20) is shown in Table 1. (**A**) Polymerase/exonuclease (pol/exo) analysis performed with the molecule sp1/sp1c+6 (15mer/21mer). (**B**) Pol/exo analysis performed with the longer molecule, sp1/sp1c+18 (15mer/33mer).

(30 nM, from position 16 to 20), and to replicate the last nucleotide of the template (10 μM, from position 20 to 21). In agreement with the fact that the binding affinity of the wild-type DNA polymerase for the longer 15mer/33mer substrate was higher than that for the 15mer/21mer (Fig. 1), 10 nM of dNTPs were enough for effective polymerization on this substrate (from position 15 to 32; see Fig. 2B and Table 1). On the other hand, the truncated DNA polymerase Δ13 required the same nucleotide concentration as the wild-type DNA polymerase for insertion of the first nucleotide in the 15mer/21mer DNA (therefore being similarly active in this polymerization step), but it needed a three times higher dNTP concentration to reach position 20 (Fig. 2A; Table 1). The replication pattern of the truncated DNA polymerase resembled a ladder, suggesting that its polymerization activity was distributive. This possibility was tested with this primer/template structure in a further experiment: after preincubation of the DNA polymerase with the labeled DNA, a 100× molar excess of the same, but unlabeled, DNA was added as competitor at the same time as the metal ion. As can be observed in Figure 3, the mutant DNA polymerase Δ13 fell off the DNA even before replication could start, indicating that its stability on the DNA was very poor, resulting in distributive replication activity. As a control, the wild-type DNA polymerase did not fall off the DNA in the presence of the DNA trap (Fig. 3). The polymerization activity of the truncated polymerase was also impaired in longer replication reactions, requiring a thousand times higher nucleotide concentration than the wild-type DNA polymerase to fully replicate an 18-nt long template (Fig. 2B; Table 1). As in the case of the wild-type DNA polymerase, the better binding affinity of mutant DNA polymerase Δ13 for this longer 15mer/33mer DNA resulted in a higher replication efficiency than with the shorter 15mer/21mer. It can be also observed in Figure 2A and B [in the lane with no (0 nM) dNTPs added] that nearly no dsDNA exonuclease activity was detectable

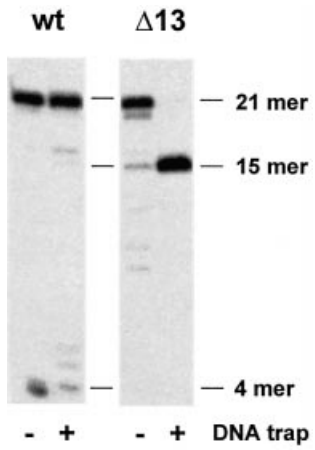

Figure 3. Distributive replication activity of mutant DNA polymerase Δ13. Replication activity on the 15mer/21mer primer/template structure with the truncated polymerase in the presence (+) of a 100-fold molar excess of the same but unlabeled DNA as trap. This DNA trap was added after preincubation of the DNA polymerases with the labeled DNA, at the same time as the reaction was started with the metal ion and 0.5 μM dNTPs. The positions of the non-elongated primer (15mer), elongated primer (21mer) and degraded primer (4mer) on the autoradiography of the 8 M urea–20% polyacrylamide gel are shown.

with mutant DNA polymerase Δ13 (Table 1; determined as described in Materials and Methods), most probably because of its strongly reduced DNA binding capacity. The appearance of exonucleolytic degradation by the wild-type DNA polymerase (4mer), even at the highest dNTP concentration, indicated that the substrate contained a small portion of labeled, but not hybridized, ssDNA. Since the truncated mutant DNA polymerase showed no degradation at all, it could be speculated that it had also no exonucleolytic activity

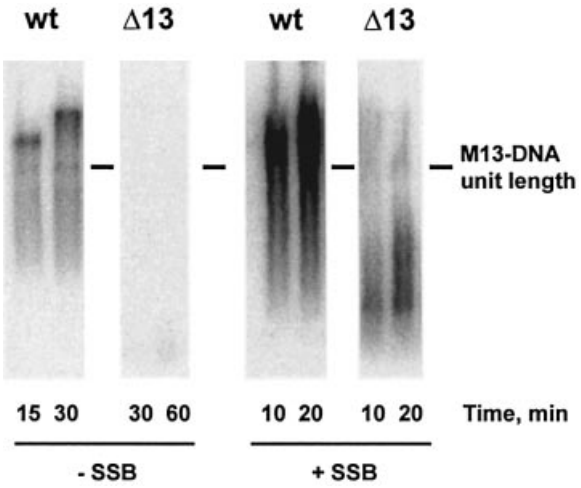

Figure 4. Strongly reduced replication activity on primed M13-DNA of the truncated Δ13 ϕ29 DNA polymerase. The assays were carried out as described in Materials and Methods with the indicated incubation times, in the absence (–SSB) or presence (+SSB) of 36 μM of ϕ29 SSB. The position of unit-length M13-DNA (first round of replication) is indicated in the autoradiography of the alkaline agarose gel shown. Mean activity values relative to the wild type are given in Table 1.

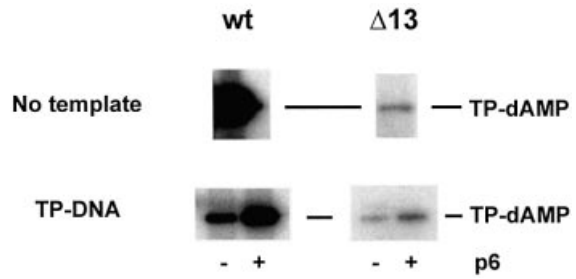

Figure 5. Reduced TP-primed initiation activity of the truncated Δ13 ϕ29 DNA polymerase. Template-dependent (TP-DNA) and template-independent (no template) formation of TP–dAMP (initiation) was studied using the wild-type or truncated Δ13 DNA polymerase. The reactions were carried out as indicated in Materials and Methods, using either 1 mM $MnCl_2$ or 10 mM $MgCl_2$ as metal activator, and 34 μM of ϕ29 DSB p6 when indicated (+). The TP–dAMP complex is seen as a band after autoradiography of the SDS–PAGE gel. Mean activity values relative to wild type are given in Table 1.

on ssDNA. This was confirmed in separate experiments, and was probably the result of its affected ssDNA binding activity (Table 1).

Strongly reduced replication activity of mutant DNA polymerase Δ13 on a long DNA substrate

Processive replication was tested on a long DNA substrate, primed M13-ssDNA. For replication of this substrate, the intrinsic processivity of ϕ29 DNA polymerase is required (40). The wild-type DNA polymerase is able to replicate M13-DNA processively and to produce, because of its intrinsic strand displacement capacity, DNA molecules greater than unit-length M13-DNA (Fig. 4, 'rolling circle' replication; see Materials and Methods). On the other hand, and as expected because of its distributive replication activity, mutant DNA polymerase Δ13 was only able to replicate short pieces of this substrate, even with long incubation times, incorporating only 6% of the amount of dNMPs incorporated by the wild-type DNA polymerase (Fig. 4; Table 1). ϕ29 SSB covers all the M13-ssDNA and concentrates binding of the ϕ29 DNA polymerase at the dsDNA portion; on the other hand, due to its helix-destabilizing activity, it reduces the formation of secondary structures in the DNA, favoring processivity (49). Thus, in the presence of ϕ29 SSB, the replication efficiencies of wild-type and truncated DNA polymerases were increased (Fig. 4). Mutant polymerase Δ13 produced more and longer replication products, and incorporated 28% of the amount of dNMPs incorporated by the wild-type DNA polymerase (Fig. 4; Table 1).

The initiation activity of mutant DNA polymerase Δ13 is strongly reduced

ϕ29 DNA polymerase is able to covalently link any of the four dNMP residues to the TP in the absence of template DNA (50). For this reaction the only interactions of the DNA polymerase required are those occurring with the TP and the initiating nucleotide. Since the C-terminal deletion strongly reduced the DNA binding capacity of ϕ29 DNA polymerase, it was important, when studying the effect of this deletion on protein-primed initiation, to perform this assay in the absence of template (see Materials and Methods). As shown in Figure 5, in the absence of TP-DNA the wild-type DNA polymerase forms a band corresponding to TP–dAMP (dATP added as initiating nucleotide; overexposed to see some activity with mutant polymerase Δ13). On the other hand, the truncated DNA polymerase Δ13 showed a strongly reduced TP-deoxynucleotidylation activity (Fig. 5; Table 1), indicating that besides its reduced DNA binding capacity this mutant DNA polymerase was also affected in either TP-binding or recognition of the initiating nucleotide. In the presence of TP-DNA as template, its activity was also very low, reaching 4% of the wild-type activity (Fig. 5; Table 1). The same affinity for the initiating nucleotide was found for both the mutant DNA polymerase and that of the wild-type DNA polymerase, indicating that its interaction with the initiating nucleotide was not affected (Table 1). On the other hand, its velocity in the initiation reaction compared with that of the wild-type DNA polymerase was ~20 times lower (Table 1).

During viral DNA replication, ϕ29 double-stranded DNA binding protein (DSB) p6 leads to local opening of the DNA duplex (51) and favors the initiation reaction due to a decrease in the K_m for the initiating nucleotide, dATP (52). As can be seen in Figure 5, the initiation activity of both DNA polymerases, wild-type and truncated, increased ~4.5 times upon addition of p6 (see also Table 1).

As can be seen in Figure 6, during the initiation reaction with TP-DNA the truncated DNA polymerase Δ13 had similar error frequencies for the three incorrect nucleotides, dCTP, dGTP and dTTP, as the wild-type DNA polymerase, indicating that its ability to discriminate wrong nucleotides was not affected. It is important to note here that the exonuclease activity of the wild-type ϕ29 DNA polymerase is not able to act on the initiation product TP–dAMP to correct misincorporations (53).

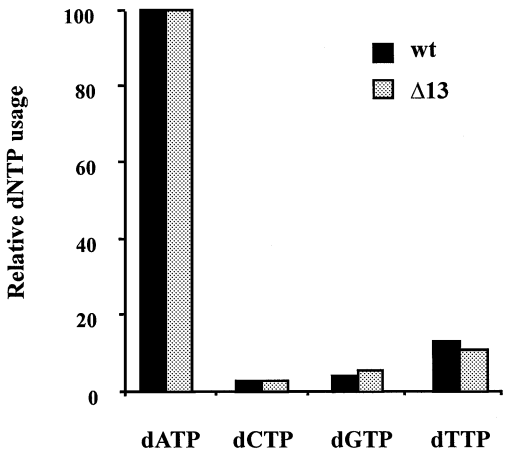

Figure 6. Misincorporation frequency in TP-primed initiation reactions. Error frequency of the wild-type and truncated Δ13 DNA polymerases during TP-DNA templated initiation (see Materials and Methods). The dNTP usage is given as percentage of the correct TP–dAMP formed.

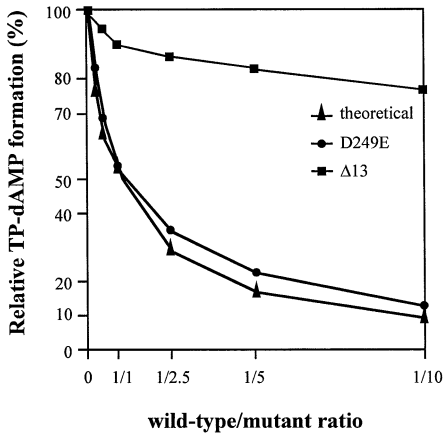

Figure 7. Competition of the truncated Δ13 with the wild-type ϕ29 DNA polymerase for binding the TP. Template-independent formation of TP–dAMP by the wild-type DNA polymerase performed in the presence of increasing amounts of the truncated mutant DNA polymerase Δ13 (see Materials and Methods), which is essentially inactive in this reaction (Table 1). A DNA polymerase, catalytically inactive in template-independent TP–dAMP formation but displaying a normal interaction with TP, would reduce the TP–dAMP formation by competition with the wild-type DNA polymerase following the theoretical curve. This was the case for mutant DNA polymerase D249E (30), used as a control of complete competition. The TP–dAMP formed in the different competition conditions relative to that formed in the absence of competition (100%) is indicated.

Weak interaction of mutant DNA polymerase Δ13 with TP

Since mutant DNA polymerase Δ13 showed nearly no template-independent TP-deoxynucleotidylation activity (Table 1) and its affinity for the initiating nucleotide did not seem to be reduced, its interaction with the primer TP was studied. Glycerol gradient sedimentation allows direct analysis of the interaction between DNA polymerase and TP (see Materials and Methods). Incubation of the wild-type ϕ29 DNA polymerase (66 kDa) with TP (31 kDa) led to the formation of a 90 kDa heterodimer, resulting in cosedimentation of DNA polymerase and TP. However, under the same conditions, the mutant DNA polymerase Δ13 was not able to interact stably with the TP, with both sedimenting as independent peaks (not shown). A more sensitive but indirect assay for the DNA polymerase–TP interaction is the interference experiment, in which the wild-type and mutant DNA polymerases compete for limited amounts of TP in an initiation reaction in the absence of template (see Materials and Methods). Mutant DNA polymerase D249E is inactive in template-independent TP–dAMP formation, but interacts normally with TP (28) and was used as positive control. Figure 7 shows that the inhibition profile, resulting from the competition between the catalytically inactive mutant DNA polymerase D249E and the wild-type DNA polymerase, paralleled the theoretical one. On the other hand, mutant DNA polymerase Δ13 still retained some capacity to compete with wild-type DNA polymerase for the TP (Fig. 7).

Reduced transition activity during protein-primed TP-DNA replication by truncated DNA polymerase Δ13

ϕ29 TP-DNA replication requires efficient initiation, TP–$(dAMP)_2$ formation and transition, which includes the deoxynucleotidylation steps following TP–$(dAMP)_2$ formation until the DNA primer is long enough for DNA-primed elongation. Dissociation of TP and DNA polymerase occurs after the synthesis of a 10-nt long DNA primer (54). As described by Blanco *et al.* (55), because of the pressure of its exonuclease activity, the wild-type ϕ29 DNA polymerase requires a higher nucleotide concentration for the TP–$(dAMP)_2$ formation and transition than for the initiation reaction. While the exonuclease activity of ϕ29 DNA polymerase is unable to act on TP–dAMP, it does do so on TP–$(dAMP)_2$ and longer transition products (53). To analyze the transition steps, a truncated replication reaction was performed with either only dATP at higher concentration than for the initiation reaction, or dATP, dGTP and dTTP (in the absence of dCTP; see Materials and Methods). Figure 8 shows that the truncated mutant DNA polymerase Δ13 was able to produce TP–$(dAMP)_2$ at a lower dATP concentration than the wild-type DNA polymerase. Figure 8 also shows the truncated products obtained during transition with the wild-type DNA polymerase due to the lack of dCTP, resulting in the formation of TP–$(dAMP)_8$ (starting at oriL) and TP–$(dAMP)_{11}$ (starting at oriR), and TP–$(dAMP)_{14}$ resulting from a misincorporation at position 12 (starting at oriR). On the other hand, nearly no truncated transition products could be observed under the same conditions with mutant DNA polymerase Δ13. The addition of ϕ29 p6 increased its TP–dAMP and TP–$(dAMP)_2$ formation, but not its transition activity (not shown).

As expected from the low initiation and transition activities of mutant ϕ29 DNA polymerase Δ13, no replication of TP-DNA was detected (Table 1).

DISCUSSION

In this work the effect of deletion of the last 13 amino acids of ϕ29 DNA polymerase is studied: the resulting truncated DNA polymerase Δ13 had a strongly reduced DNA binding capacity. This defect in DNA binding strongly affected its processivity during polymerization. A DNA trap would capture the mutant polymerase from its bound DNA substrate,

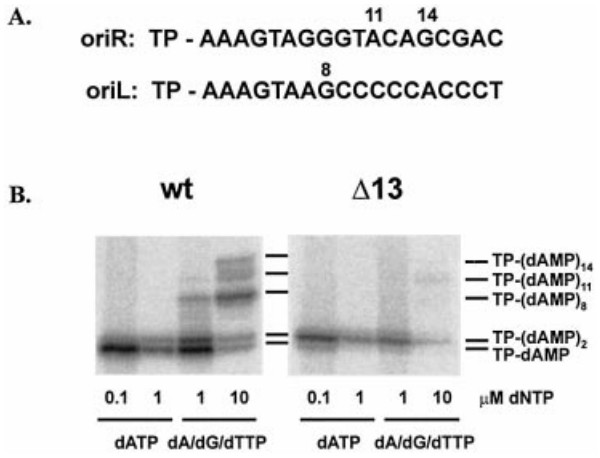

Figure 8. Reduced transition during truncated TP-DNA replication with the mutant Δ13 ϕ29 DNA polymerase. (**A**) The truncated products formed due to the absence of dCTP during the first steps of replication of ϕ29 TP-DNA, starting from the right [oriR, TP-$(dNMP)_8$] or left end [oriL, TP-$(dNMP)_{11}$ and TP-$(dNMP)_{14}$], are indicated. (**B**) High resolution SDS–PAGE (as indicated in Materials and Methods) of truncated replication reactions carried out with Mn^{2+} as metal activator and 0.1 or 1 μM of either dATP only, or 1 or 10 μM of dATP/dGTP/dTTP. The incubation time was 1 min for the wild-type DNA polymerase and 10 min for mutant polymerase Δ13. The TP–dAMP (initiation), TP–$(dAMP)_2$ and further truncated transition products synthesized are indicated.

even before replication could start. During primed M13-DNA replication, ϕ29 SSB, known to concentrate binding of ϕ29 DNA polymerase at the dsDNA portion and to reduce the formation of secondary structures in the DNA (49), was able to increase the polymerization efficiency of mutant DNA polymerase Δ13, resulting in more and longer replication products. The dNTP binding affinity of the truncated DNA polymerase was similar to that of the wild-type DNA polymerase. It was able to recognize the templating and incoming nucleotides correctly, since its misincorporation frequency during TP-DNA initiation reactions was not affected in comparison to the wild-type DNA polymerase. Since the truncated DNA polymerase showed similar efficiencies as the wild-type DNA polymerase in dNTP binding, nucleotide insertion fidelity and polymerization of one nucleotide (before movement along the DNA is required), its structure is supposed to be similar. In spite of its impaired processivity, mutant DNA polymerase Δ13 was able to produce TP–$(dAMP)_2$ at a lower dATP concentration than the wild-type DNA polymerase, probably due to its poor exonuclease activity. On the other hand, since nearly no truncated transition products could be observed with mutant DNA polymerase Δ13, it seems that transition requires a processive movement of ϕ29 DNA polymerase along the TP-DNA. We can conclude that the last 13 residues of ϕ29 DNA polymerase are important for stable DNA binding and processivity. This is in agreement with the results obtained with two other DNA polymerases: deletion of 24 amino acids of the tip of the thumb in Klenow polymerase resulted in a truncated DNA polymerase with strongly reduced DNA binding affinity and reduced processivity (56). Additionally, deletion of the C-terminal 17 amino acids of the T4 DNA polymerase, belonging to family B and homologous to RB69 DNA polymerase, affected its DNA binding affinity for short dsDNA substrates, for which no help of accessory proteins is needed (57). Region 5 is still present in this deletion (39). Therefore, just the C-terminal residues of different DNA polymerases seem to be important for DNA binding, although no especially conserved residues can be found in these regions. But, since ϕ29 DNA polymerase has an intrinsic ability to replicate processively, without the help of accessory proteins improving clamping of the DNA, the role of its C-terminus could also be specific for this DNA polymerase.

Using partial proteolysis it had been shown that in the C-terminal region of ϕ29 DNA polymerase (distal to motif 'KxY'), in its open conformation, several cleavage sites for endoproteinase LysC were accessible, but that this accessibility disappeared upon DNA or TP binding (58). These results indicated that this region of ϕ29 DNA polymerase, possibly containing its thumb subdomain, changed its conformation upon DNA and/or TP binding, resulting in reduction of the accessibility of these cleavage sites to the protease. It could therefore be involved in DNA and TP binding. Both primers, TP and DNA, are supposed to have a common binding site, the dsDNA binding channel (58). In agreement with this we have shown, besides the importance of the C-terminus of ϕ29 DNA polymerase for DNA binding, its importance for TP binding. The mutant DNA polymerase Δ13 was strongly affected in its interaction with TP, therefore showing only weak initiation activity. This is also in line with the finding that the same mutations affect both DNA and TP binding: several residues of ϕ29 DNA polymerase have been shown to be involved in interactions with DNA and TP, such as Thr434 and Arg438 from the 'Tx_2GR' motif (34), Lys498 and Tyr500 from the 'KxY' motif (35) and the residues of the 'YxGG/A' motif (36). Additionally, it has been shown that residues from the N-terminal domain-like Tyr59, His61 and Phe65 from the ExoII motif (59) and Ser122 and Phe128 from the S/TLx_2h motif (60,61) are involved in DNA and TP binding, and that the separately expressed C-terminal domain was unable to interact stably with any of the two primers (27). Our results suggest that the last 13 residues of ϕ29 DNA polymerase are important for stable binding of both TP and DNA. The conformational change in the C-terminal region of ϕ29 DNA polymerase, proposed to be occurring upon binding of both DNA and TP (58), possibly improves the substrate binding stability of the wild-type ϕ29 DNA polymerase.

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