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Two Positively Charged Residues of ϕ29 DNA Polymerase, Conserved in Protein-primed DNA Polymerases, are Involved in Stabilisation of the Incoming Nucleotide

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In DNA polymerases from families A and B in the closed conformation, several positively charged residues, located in pre-motif B and motif B, have been shown to interact with the phosphate groups of the incoming nucleotide at the polymerisation active site: the invariant Lys of motif B and the nearly invariant Lys of pre-motif B (family B) correspond to a His in family A DNA polymerases. In ϕ29 DNA polymerase, belonging to the family B DNA polymerases able to start replication by protein-priming, the corresponding residues, Lys383 and Lys371, have been shown to be dNTP-ligands. Since in several DNA polymerases a third residue has been involved in dNTP binding, we have addressed here the question if in the DNA polymerases of the protein-primed subfamily, and especially in ϕ29 DNA polymerase, there are more than these two residues involved in nucleotide binding. By site-directed mutagenesis in ϕ29 DNA polymerase the functional role of the remaining two conserved positively charged amino acid residues of pre-motif B and motif B (besides Lys371 and Lys383) has been studied. The results indicate that residue Lys379 of motif B is also involved in dNTP binding, possibly through interaction with the triphosphate moiety of the incoming nucleotide, since the affinity for nucleotides of mutant DNA polymerase K379T was reduced in DNA and TP-primed reactions. On the other hand, we propose that, when the terminal protein (TP) is present at the polymerisation active site, residue Lys366 of pre-motif B is involved in stabilising the incoming nucleotide in an appropriate position for efficient TP-deoxynucleotidylation. Although mutant DNA polymerase K366T showed a wild-type like phenotype in DNA-primed polymerisation in the presence of DNA as template, in TP-primed reactions as initiation and transition it was impaired, especially in the presence of the ϕ29 DBP, protein p6.

Keywords: ϕ29 DNA polymerase; pre-motif B; motif B; dNTP binding; protein-priming

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Introduction

In addition to the DNA-primed polymerisation activities, common to most replicative DNA polymerases, the protein-primed DNA polymerases, like ϕ29 DNA polymerase, are able to recognize and use the terminal protein (TP) as primer to initiate DNA replication.[1,2] Thus, these polymerases must be structurally adapted to use as primers both protein and DNA. Initiation of replication by ϕ29 DNA polymerase occurs at both TP-DNA ends with the formation of the TP-dAMP initiation complex, and replication proceeds continuously without further priming events. Following initiation, transition includes the deoxynucleotidylation steps until the DNA primer is

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Abbreviations used: pol, DNA polymerase; exo, 3′–5′ exonuclease; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; DBP, double-stranded, DNA-binding protein; TP, terminal protein; TP-DNA, terminal protein-containing ϕ29 DNA.

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long enough for replication (10 nt). Dissociation of TP and DNA polymerase occurs after synthesis of this DNA primer.[3] Therefore, the same DNA polymerase is able to catalyse TP-primed initiation, the subsequent transition steps and, finally, DNA-primed elongation.

The movement of the fingers subdomain, shown to occur in several DNA polymerases in the transition between the open and closed complex during DNA-templated reactions,[4–8] brings the residues of pre-motif B and motif B closer to the active site, in order to form a binding pocket for the nascent base-pair. The residues forming the nucleotide-binding pocket are highly conserved among DNA polymerases belonging to families A and B. Most of them belong to motif B ("KLx$_2$NSxYG", fingers subdomain),[9] which has been shown to be important for primer/template binding and dNTP selection: in the crystal structure of the only family B DNA polymerase crystallised as closed ternary complex, RB69 DNA polymerase,[8] the Leu, Tyr and Gly residues of this motif have been shown to enclose the templating nucleotide. Consequently, these residues are important for DNA-dependent dNTP binding[10,11] and have been proposed to be directly involved in checking base-pairing correctness;[12–17] the invariant Lys residue of motif B together with the Leu and Tyr residues of motif A, Dx$_2$SLYP, have been shown to enclose the incoming nucleotide and to be important in nucleotide selection.[18–20] In the crystal structures of several DNA polymerases belonging to families A and B, three positively charged residues have been shown to be involved in binding the incoming nucleotide through interaction with its phosphate groups: one is the invariant Lys residue of motif B[8,21,22] (Lys663 of *Taq* and Lys560 of RB69 DNA polymerases, see Figure 1), another the nearly invariant His residue of pre-motif B in family A DNA polymerases,[21,22] corresponding to

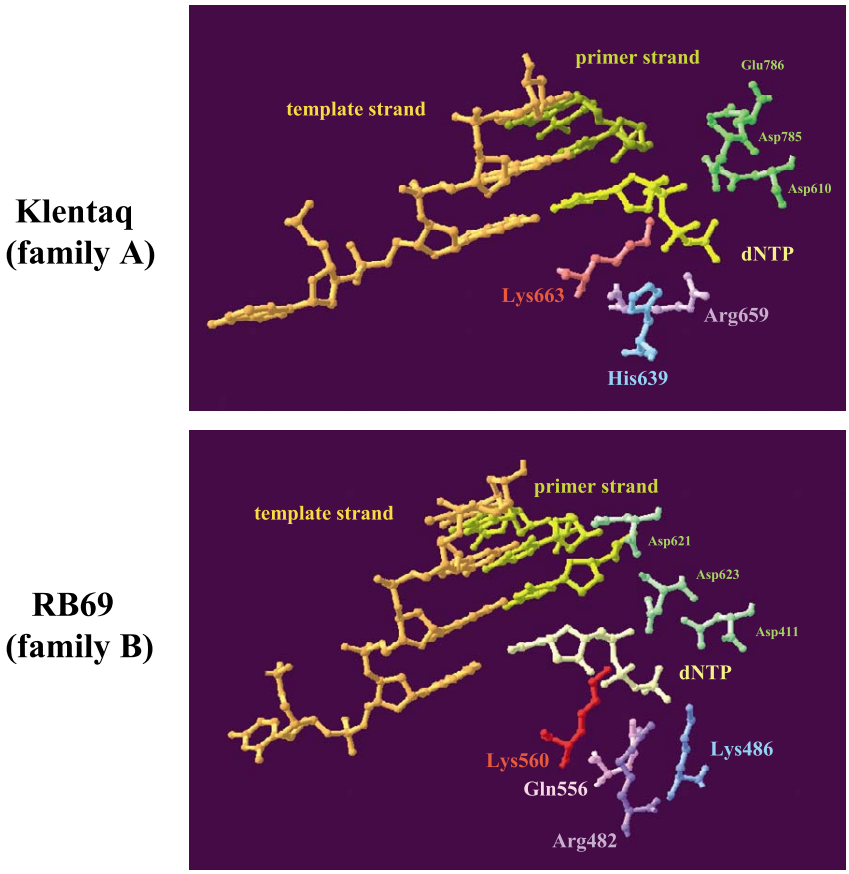

Figure 1. Location of the residues contacting the triphosphate moiety of the incoming nucleotide at the polymerisation active site. Crystal structure of the active site of the replicating complex of *Taq* DNA polymerase[21] and RB69 DNA polymerase.[8] The incoming nucleotide is coloured in yellow, the primer strand in dark yellow and the template strand in orange. The three catalytic aspartate residues are shown in green. The residues contacting the phosphate groups of the incoming nucleotide are coloured as follows: Asp610 and Asp785 of *Taq* and Asp623 of RB69 DNA polymerase, as mentioned, in green; the invariant Lys of motif B in red; the nearly invariant His of family A and Lys of family B DNA polymerases of pre-motif B in blue; the nearly invariant Arg of motif B of family A DNA polymerases in light purple; residue Arg482 of pre-motif B of RB69 DNA polymerase in dark purple. Residue Gln556 of RB69 DNA polymerase, corresponding to Lys379 of ϕ29 DNA polymerase, is coloured light pink. Crystallographic data are from PDB1 IG9 (RB69 DNA polymerase) and PDB1 3KTQ (*Taq* DNA polymerase).

a nearly invariant Lys in family B DNA polymerases[8,23] (His639 of *Taq* and Lys486 of RB69 DNA polymerases, see Figure 1). Mutational analysis of these two Lys residues of RB69 DNA polymerase located in motif B and pre-motif B has supported their importance in dNTP binding.[15] In family A DNA polymerases, the Arg of motif B[21,22] (Arg659 of *Taq* DNA polymerase, see Figure 1) has been involved as third residue in contacting the phosphate groups of the incoming nucleotide. On the other hand, in RB69 DNA polymerase, belonging to the bacterial/viral family B DNA polymerases, an Arg located in pre-motif B has been shown to be the third positively charged residue involved in interaction with the phosphate groups of the dNTP (Arg482 of RB69 DNA polymerase, see Figure 1).[8] Taking into account the similarity of the open structure of RB69 DNA polymerase[24] with other crystal structures of family B DNA polymerases reported more recently (*Thermococcus gorgonarius*,[25] *Desulfurococcus* strain Tok,[26] *Thermococcus* sp. 9°N-7[27] and *Pyrococcus kodakaraensis* KOD1[28] DNA polymerases), the RB69 enzyme is a good structural model for the family B DNA polymerases. In agreement with this, site-directed mutagenesis performed with ϕ29 DNA polymerase, belonging to the family B DNA polymerases able to start replication by protein-priming, has supported experimentally the roles in dNTP binding of the Lys of motif B (Lys383)[18] and the Lys of pre-motif B (Lys371).[23] Since in the protein sequences of the DNA polymerases of this subgroup no positively charged amino acid residue can be found at the position corresponding to the Arg of pre-motif B, but positively charged amino acid residues can be found at the position corresponding to the Arg of motif B, we have addressed here the question if ϕ29 DNA polymerase has more amino acid residues involved in nucleotide binding besides Lys371 and Lys383. We can conclude that residue Lys379 of motif B, conserved in protein-primed DNA polymerases and located at the position corresponding to the invariant Arg involved in dNTP binding in family A DNA polymerases, is also involved in binding the incoming nucleotide in ϕ29 DNA polymerase. Additionally we propose that, when the TP is present at the polymerisation active site, residue Lys366 of pre-motif B is involved in stabilising the incoming nucleotide in an appropriate position for efficient TP-deoxynucleotidylation.

Results

Conserved positively charged residues in DNA-dependent DNA polymerases located in pre-motif B and motif B

In DNA polymerases from families A and B several positively charged residues, located in pre-motif B and motif B, have been involved in nucleotide binding: one is the invariant Lys residue of motif B (see Figure 2, marked with grey arrow), and a second one is the nearly invariant His (family A) or Lys (family B) residue of pre-motif B (Figure 2 (grey arrow);[8,18,21–23] In family A DNA polymerases a third residue, the invariant Arg (Figure 2, marked with a black arrow) located at a distance of four amino acid residues downstream to the invariant Lys of motif B, has been shown to be involved in binding the phosphate groups of the dNTP.[21,22] At the corresponding position, family B DNA polymerases of the bacterial/viral and cellular subfamily contain a nearly invariant Gln residue with unknown function, while DNA polymerases able to start replication by protein-priming have in ten out of 15 aligned sequences a positively charged amino acid residue (Arg or Lys) at this position (Figure 2, black arrow). To study the role of this residue, the corresponding Lys379 of ϕ29 DNA polymerase was subjected to site-directed mutagenesis. On the other hand, in RB69 DNA polymerase, belonging to the bacterial/viral subfamily of family B DNA polymerases, an Arg residue at pre-motif B (Figure 2, grey arrow) has been shown to contact, together with the two Lys residues of pre-motif and motif B, the phosphate groups of the nucleotide.[8] Interestingly, this Arg residue lies at a distance of four amino acid residues from the Lys residue of pre-motif B; similarly, in family A DNA polymerases, the Arg residue of motif B lies four residues from the Lys residue of the same motif. This Arg of pre-motif B is nearly invariant in the bacterial/viral and cellular family B DNA polymerases. No positively charged amino acid can be found at this position in the sequences of DNA polymerases from the protein-primed subfamily, but in nine cases (out of 15) a positively charged amino acid can be found at the preceding position (Figure 2, marked with black arrow). To study the role of this residue, the corresponding Lys366 of ϕ29 DNA polymerase was subjected to site-directed mutagenesis. Lys366 and Lys379 are the only two positively charged residues, besides the two Lys residues of motif B and pre-motif B, found in this region to be conserved in the sequences of DNA polymerases able to start replication by protein-priming (Figure 2). Both residues were changed into the non-conservative polar Thr. These mutations in the ϕ29 DNA polymerase were designed according to general suggestions for conservative substitutions[29] and secondary structure predictions.[30,31]

The DNA-binding capacity of the ϕ29 DNA polymerase mutants is not affected

The interaction of the two mutant ϕ29 DNA polymerases with a primer/template structure was studied by gel retardation (described in Materials and Methods). In this assay the wild-type enzyme gives rise to a single retardation band, which most likely corresponds to a protein–DNA interaction in which the primer terminus is stabilized at the polymerisation active site.[32]

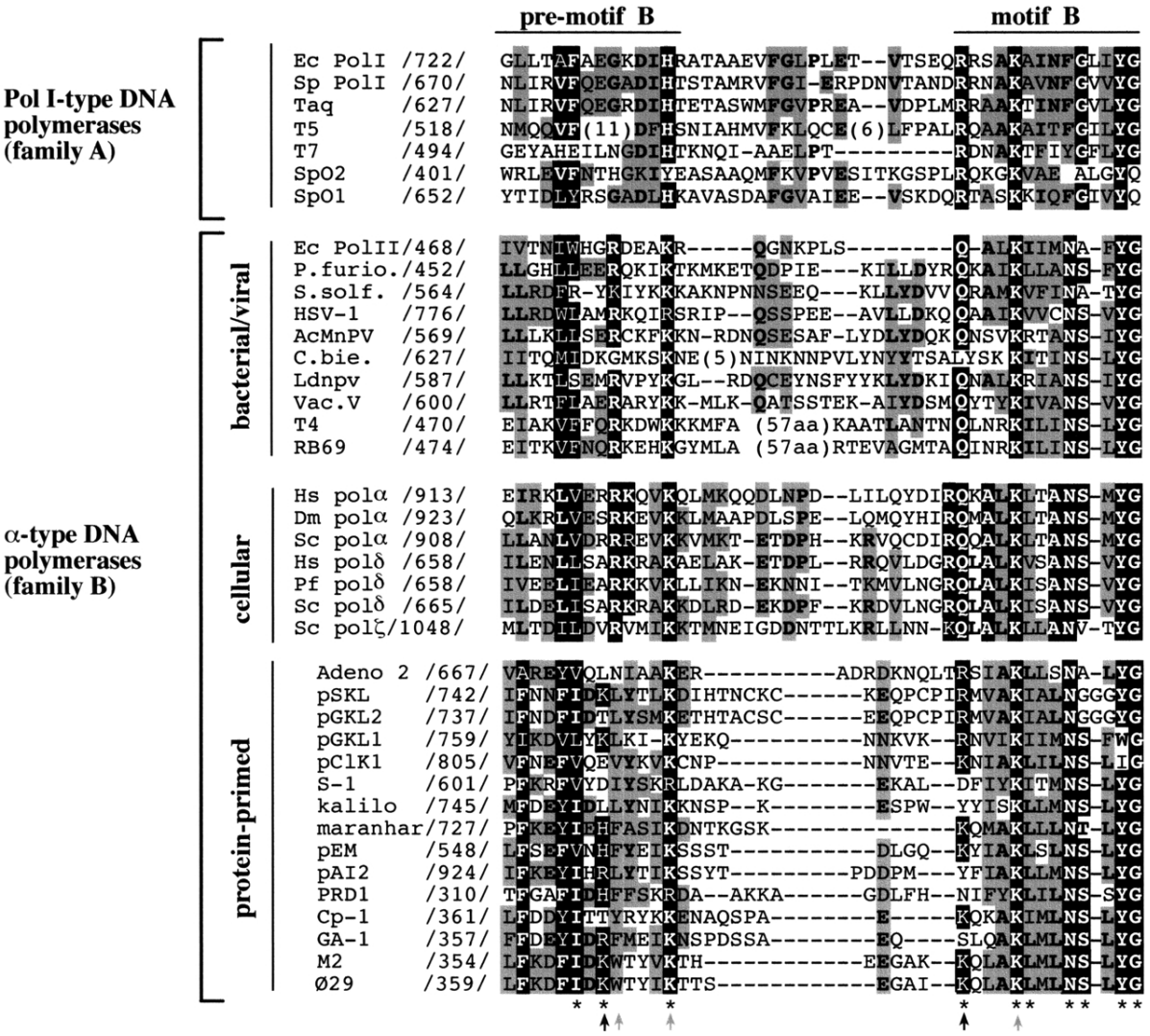

Figure 2. Location in pre-motif B and motif B of the positively charged residues involved in dNTP binding. Multiple alignment of the amino acid sequences of DNA-dependent DNA polymerases from pre-motif B to motif B. DNA polymerase nomenclature and sequence references were compiled by Braithwaite & Ito,[56] with the exception of pAL2 DNA polymerase codified by the linear plasmid pAL2 from *Podospora anserina*,[57] *Streptococcus pneumoniae* phage Cp1 DNA polymerase (GenBank data base/a.n. Z47794) and phage GA-1 DNA polymerase.[58] Numbers between slashes indicate the amino acid position relative to the N terminus of each DNA polymerase sequence. Invariant or highly conserved residues are indicated in white letters on a black background. Significant similarities are indicated by grey boxes. Identical residues aligned in more than half of the sequences are in bold type. Previously studied residues of ϕ29 DNA polymerase in this region are indicated with an asterisk.[17,18,23,59] The residues studied here, Lys366 and Lys379, and the other residues known to be involved in nucleotide binding through the phosphate group[23] are additionally indicated with black and grey arrows, respectively. The following conserved amino acid residues were considered: S and T; A and G; D and E; K, R and H; A, I, L, M, C and V; Y and F.

A similar result was obtained with both mutant DNA polymerases, suggesting that the mutations were essentially not affecting their DNA-binding capacity: mutant DNA polymerase K366T was slightly reduced in its DNA-binding capacity (60% of the wild-type activity), while mutant DNA polymerase K379T showed a slightly increased binding to DNA (160% of the wild-type activity, see Table 1). Also the capacity of both mutant polymerases to retard a single-stranded oligonucleotide (15mer) was similar to that of the wild-type polymerase (not shown).

Pol/exo balance on a primer/template structure

A change in the polymerase/exonuclease (pol/exo) balance from exonucleolysis to polymerisation on a primer/template structure is obtained by increasing the dNTP concentration (see Materials and Methods). This balance not only depends on the presence of the two activities, but particularly on the affinity of the polymerisation active site for the incoming nucleotide and on the partition of the primer strand between both active sites. As shown in Figure 3, different dNTP

Table 1. Enzymatic activities of the ϕ29 DNA polymerase mutants

		ϕ29 DNA polymerase		
Activity assay	Substrate	wt	K366T	K379T
DNA binding	15/21mer	100	60	160
Exonuclease	15/21mer	100	90	110
Pol/exo balance (nM)	15/21mer	30	30	100
K_m^{dNTP} (μM)	15/21mer	0.1	0.1	0.4
Processive polymerisation	Primed M13-DNA	100	72	85
TP-dAMP formation	TP-DNA (+p6)	100(4)	31(0.4)	34(4.4)
	No template	100	48	68
K_m^{dNTP} (μM) (V_{max},fmol/min)	TP-DNA	7.5(1.7)	8.9(0.7)	21.2(1.5)
	TP-DNA, + p6	1.2(3.0)	15.6(0.5)	n.d.
	No template	69(58)	63(18)	166(55)
TP-DNA replication	TP-DNA (+p6)	100(5)	20 (0.3)	15(15)

The different activity assays were carried out with the indicated substrates, as described in Materials and Methods. With the exception of the pol/exo assay and the K_m/V_{max} data, the numbers indicate percentage of activity obtained with the wild-type polymerase and are an average of several experiments. In the pol/exo-coupled assay, the dNTP concentration required (nM) for effective polymerisation (position 20) is indicated. The factor of stimulation by p6 is given in parentheses. n.d., not determined.

concentrations are required for the wild-type ϕ29 DNA polymerase to: (1) start replication and polymerise the first nucleotide (10 nM, from position 15 to 16); (2) to compete effectively the 3′-5′-exonuclease activity replicating until the penultimate nucleotide (30 nM, from position 16 to 20); (3) to replicate the last nucleotide of the primer/template substrate (10 μM, from position 20 to 21). A similar phenotype for replication of this primer/template structure was observed for mutant DNA polymerase K366T (Figure 3). On the other hand, mutant DNA polymerase K379T needed over three times higher nucleotide concentration to replicate from position 15 to 20 (Figure 3). Since the exonuclease activity of both mutant DNA polymerases, K366T and K379T, was wild-type like (see Figure 3 (lanes 0) and Table 1) and the retardation experiments had also shown no great effect in DNA binding, the affinity of the exonuclease and polymerisation active sites of these mutant DNA polymerases for the primer strand did not seem to be affected by the mutations. Determination of the K_m for nucleotides during polymerisation of the same primer/template substrate (see Materials and Methods) revealed a K_m similar to that of the wild-type enzyme for mutant DNA polymerase K366T and a four times higher K_m for mutant DNA polymerase K379T (Table 1). These results suggested that mutant DNA polymerase K379T had a reduced affinity for the incoming nucleotide.

Non-templated +1 addition activity on a blunt-ended DNA substrate

An apparently reduced affinity for the incoming nucleotide can result directly from a reduced

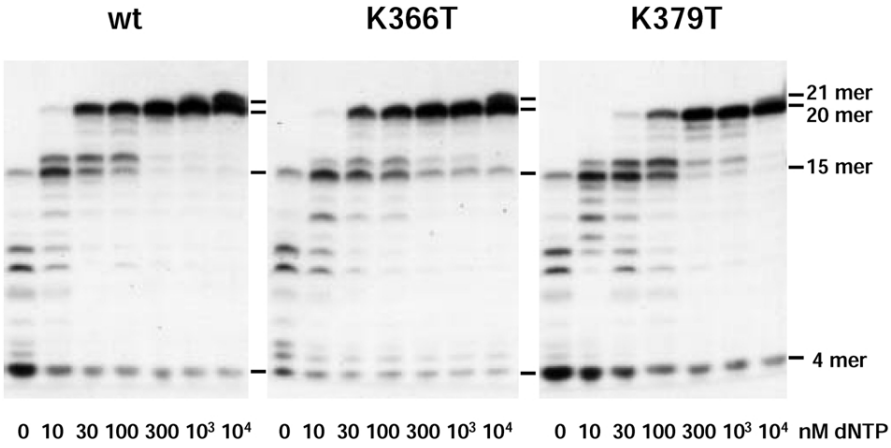

Figure 3. DNA polymerisation coupled to exonucleolysis. The pol/exo assays were carried out as described in Materials and Methods, using the ^{32}P-labelled molecule sp1/sp1c + 6 (15/21mer) as primer/template DNA. After autoradiography of the 8 M urea/20% polyacrylamide gel, polymerisation or 3′-5′ exonuclease are detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labelled sp1 primer. The positions of the non-elongated primer (15mer), elongated primer (16mer, 20mer and 21mer) and degraded primer (4mer) are shown. The dNTP concentration required to compete exonucleolysis, allowing efficient replication of the template, is shown in Table 1 for the wild-type and mutant ϕ29 DNA polymerases.

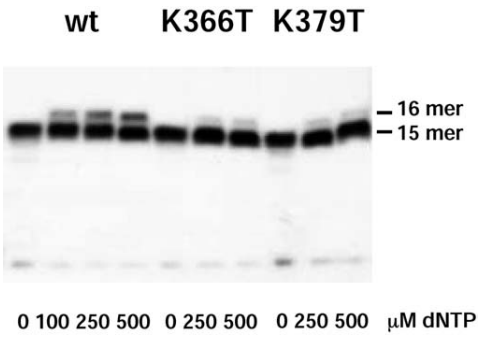

Figure 4. +1 addition on a blunt-ended DNA substrate by the mutant DNA polymerases. Template-independent addition of a nucleotide on the blunt-ended sp1/sp1c (15/15mer), performed as described in Materials and Methods. To avoid exonuclease activity on the substrate, the reaction was incubated on ice. The positions of the non-elongated primer (15mer) and elongated primer (16mer), observed on the autoradiogram of an 8 M urea/20% polyacrylamide gel, are indicated.

interaction of the DNA polymerase with the incoming nucleotide or indirectly from an incorrect positioning of the templating nucleotide. Therefore, a polymerisation reaction in the absence of DNA as template was performed. Like several other DNA polymerases,[33] ϕ29 DNA polymerase (J. A. Esteban *et al*. unpublished data) is able to add a nucleotide on a blunt-ended dsDNA molecule (non-templated +1 addition). As can be observed in Figure 4, mutant DNA polymerases K366T and K379T needed a more than five times higher dNTP concentration than the wild-type DNA polymerase to perform this reaction.

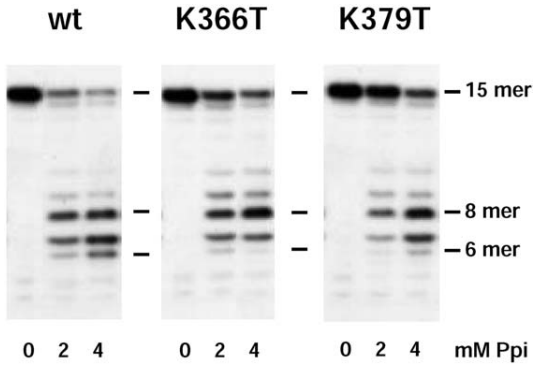

Figure 5. Pyrophosphorolytic activity of the mutant DNA polymerases. The reverse activity to the polymerisation reaction on the primer/template substrate sp1/sp1c + 6 (15/21mer), the pyrophosphorolytic degradation, can be observed upon addition of increasing concentrations of inorganic pyrophosphate (performed as described in Materials and Methods). To avoid exonuclease activity on the substrate the reaction was incubated on ice. In the autoradiogram of the 8 M urea/20% polyacrylamide gel, the positions of the non-elongated primer (15mer) and some degradation products are indicated.

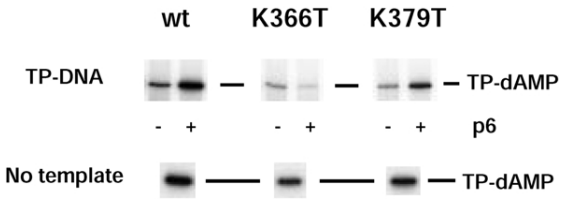

Figure 6. The mutant DNA polymerases are impaired in TP-primed initiation activity. Template-dependent (TP-DNA) and template-independent (no template) formation of TP-dAMP complex studied using the wild-type and mutant DNA polymerases. The reactions were carried out as indicated in Materials and Methods. Due to the different metal ions and incubation times used in these assays, the template-dependent and template-independent TP-dAMP formation is not directly comparable. When indicated, 34 μM ϕ29 DBP p6 (+) was added. The TP-dAMP complex is seen as a band in the autoradiogram of the SDS-PAGE gel. Mean activity values relative to the wild-type are given in Table 1.

Therefore, the reduced affinity of mutant DNA polymerase K379T for the incoming nucleotide seemed to be independent of the presence of a template. Interestingly, although mutant DNA polymerase K366T had shown wild-type like polymerisation activity on a primer/template structure, it had a reduced polymerisation activity in the absence of a DNA template.

Pyrophosphorolytic activity of the ϕ29 DNA polymerase mutants

ϕ29 DNA polymerase has two 3′-5′ degradative activities: the exonucleolysis and the pyrophosphorolysis, an inorganic pyrophosphate-dependent degradative activity regarded as the reversal of the polymerisation reaction.[34] As can be seen in Figure 5, addition of increasing pyrophosphate concentrations resulted in a degradative activity on the primer/template molecule (15/21mer), distinguishable from the exonuclease activity (the latter was reduced by lowering the incubation temperature and can be detected in the absence of pyrophosphate). It can be observed that mutant DNA polymerases K366T and K379T required higher pyrophosphate concentrations than the wild-type enzyme for similar degradation activities.

The processive polymerisation activity of the ϕ29 DNA polymerase mutants is not affected

For replication of primed M13-ssDNA, the intrinsic processivity of ϕ29 DNA polymerase is required.[35] 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 ("rolling circle" replication, see Materials and Methods). Both mutant DNA polymerases showed a similar replication activity as

the wild-type enzyme on this substrate (Table 1). The intrinsic processivity and strand displacement capacity of ϕ29 DNA polymerase was not affected by the mutations, since both mutant polymerases replicated ϕ29 DNA to full length at similar incubation times as the wild-type polymerase.

Reduced protein-primed initiation activity of the ϕ29 DNA polymerase mutants

Initiation of protein-primed replication is templated by the second nucleotide at the 3′ end of the TP-DNA[36] (with the parental primer protein TP covalently linked to its 5′ end) and consists in the formation of a covalent phosphoester linkage between the hydroxyl group of Ser^{232} of the free primer protein TP and 5′-dAMP[37] catalyzed by ϕ29 DNA polymerase.[38] As shown in Figure 6, the wild-type DNA polymerase forms a band corresponding to TP-dAMP, the product of the initiation reaction (performed in the presence of Mg^{2+}, see Materials and Methods). It can be observed that the initiation activity of both mutant DNA polymerases was reduced threefold (Figure 6 and Table 1). For an efficient initiation reaction, ϕ29 DNA polymerase requires stable interactions with the three substrates TP-DNA, TP and initiating nucleotide. The interaction of both mutant DNA polymerases with DNA had been shown not to be affected in the gel retardation assay. Since ϕ29 DNA polymerase is able to covalently link any of the four dNMP residues to the TP,[39] the TP-deoxynucleotidylation activity can be measured in the absence of template DNA. Under these conditions (performed in the presence of Mn^{2+}, see Materials and Methods) the mutant DNA polymerases K366T and K379T showed a similar TP-deoxynucleotidylation activity in the absence (Figure 6 and Table 1) as in the presence of TP-DNA (not shown), indicating that the reduction in their initiation activity was independent of the presence of template and indirectly supporting the finding that their DNA-binding capacity was not affected. By glycerol gradient centrifugation, the interaction of both mutant DNA polymerases with TP was also shown to be normal, since the mutant DNA polymerases (66 kDa), like the wild-type enzyme, and TP (31 kDa) cosedimented, at a position corresponding to approximately 95 kDa, thus forming a stable TP/DNApol heterodimer (not shown). Therefore, a defective interaction with the initiating nucleotide dATP or the catalysis itself could be the reason for the decreased initiation activity of the mutant polymerases. Determination of the catalytic constants for the initiation reaction in the presence of TP-DNA of the mutant DNA polymerases (see Materials and Methods) revealed, in agreement with its reduced affinity for dNTPs during polymerisation, mutant DNA polymerase K379T had a three times higher K_m for the initiating nucleotide and a similar V_{max} when compared to that of the wild-type DNA polymerase (Table 1); on the other hand, mutant DNA polymerase K366T had a similar K_m as the wild-type enzyme, but a 2.5 times reduced V_{max} (Table 1). In non-templated initiation, the K_m of mutant DNA polymerase K379T was 2.4 times higher and the V_{max} similar as that of the wild-type polymerase (Table 1), indicating that the reduced affinity for the initiating nucleotide was independent of the presence of template. A similar conclusion could be drawn for mutant DNA polymerase K366T, which showed a similar K_m as the wild-type enzyme but a reduced V_{max} during initiation in the presence and in the absence of template (Table 1).

A surprising result was obtained by the addition of the ϕ29 DBP, protein p6, to the initiation reaction. Protein p6 is known to form a nucleoprotein complex at both ϕ29 DNA ends, producing a conformational change in the DNA that probably leads to local opening of the DNA duplex,[40] favouring the initiation reaction due to a decrease in the K_m for the initiating nucleotide, dATP.[41] In agreement with this, addition of p6 caused an increase in the initiation activity of the wild-type DNA polymerase (Figure 6). A similar increase in its initiation activity was observed for mutant DNA polymerase K379T (Figure 6 and Table 1). Surprisingly, in the presence of p6 a threefold reduction of the initiation activity of mutant DNA polymerase K366T was observed (Figure 6 and Table 1). A similar result upon addition of p6 was obtained for its initiation activity on a double-stranded, but not on a single-stranded oligonucleotide containing the first 29 bp of the sequence of the right end of ϕ29 DNA (oriR29), indicating that the negative effect of p6 was dependent on its binding at the ϕ29 origin of replication (not shown). The decrease in the activity of mutant DNA polymerase K366T upon addition of p6 was independent of the TP-DNA end (oriL or oriR) used as template (not shown). Since mutant DNA polymerase K379T had shown the same increase upon addition of p6 as the wild-type polymerase, determination of the catalytic constants for the initiation reaction in the presence of p6 was

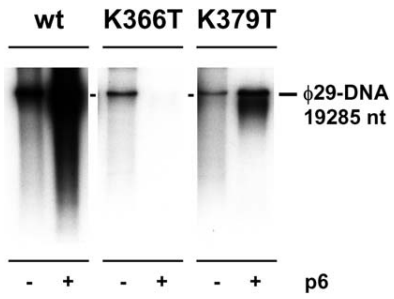

Figure 7. The mutant DNA polymerases are impaired in replication of TP-DNA. The assays were carried out as described under Materials and Methods, in the presence of 34 μM ϕ29 DBP p6 when indicated (+). The position of the completely replicated ϕ29 DNA is indicated. Mean activity values relative to the wild-type are given in Table 1.

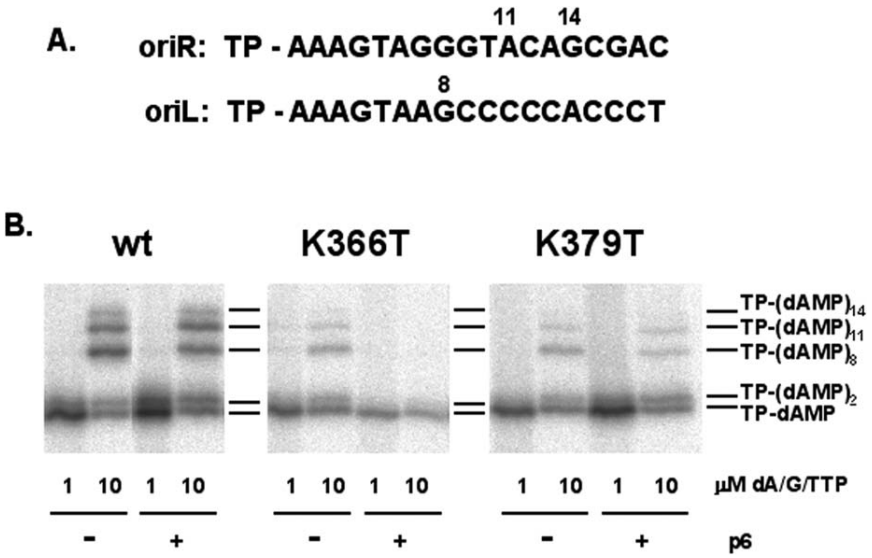

Figure 8. The mutant DNA polymerases are impaired in TP-$(dAMP)_2$ formation and transition. The early steps of TP-DNA replication, including initiation, TP-$(dAMP)_2$ formation and transition, were studied in a truncated replication assay. A, The truncated products appearing due to the absence of dCTP 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 Mg^{2+} as metal activator and 1 µM or 10 µM dATP/dGTP/dTTP. When indicated, 34 µM ϕ29 p6 was added (+). The TP-dAMP (initiation), TP-$(dAMP)_2$ and further truncated transition products synthesized, are indicated.

performed only for mutant DNA polymerase K366T. It had a 13 times increased K_m for the initiating nucleotide and a six times reduced V_{max} in comparison to the wild-type enzyme (Table 1).

Reduced TP-DNA replication activity of the ϕ29 DNA polymerase mutants

Efficient initiation and TP-$(dAMP)_2$ formation ("sliding back")[34] followed by correct transition are required for TP-DNA replication. As can be seen in Figure 7 and Table 1, the replication activity of both mutant DNA polymerases was slightly more reduced than their initiation activity. Addition of p6 caused, in line with the observed enhancement in initiation, a fivefold increase in the replication activity of the wild-type and K379T mutant DNA polymerases (Figure 7 and Table 1). On the other hand, a 16-fold reduction of the replication activity of mutant DNA polymerase K366T was observed upon addition of p6 (a stronger effect than that observed on the initiation activity of this mutant DNA polymerase).

A detailed study of the first replication steps, TP-$(dAMP)_2$ formation and transition, was performed in a truncated replication assay of TP-DNA (see Materials and Methods). As can be seen in Figure 8 (and in agreement with its reduced nucleotide-binding affinity), mutant DNA polymerase K379T needed a higher nucleotide concentration for the TP-$(dAMP)_2$ formation than the wild-type enzyme. Also the transition was less efficient than that of the wild-type DNA polymerase (less truncated products, TP-$(dAMP)_{8/11/14}$, formed). Addition of p6 had a slight stimulatory effect on the TP-dAMP and TP-$(dAMP)_2$ formation of the wild-type and K379T DNA polymerases, but nearly no effect on the transition activities of both DNA polymerases (Figure 8). On the other hand, addition of p6 caused, in the case of mutant DNA polymerase K366T, a reduction in the formation of TP-dAMP and TP-$(dAMP)_2$, and no truncated products were observed (Figure 8). The strongly affected transition activity of this mutant DNA polymerase in the presence of p6 explains why under these conditions its replication activity was even more strongly reduced than its initiation activity.

As a control, addition of p6 had no effect on the DNA-primed replication activity of mutant DNA polymerase K366T (primer/template structure and primed-M13 ssDNA) and on its template-independent TP-deoxynucleotidylation activity (not shown).

Discussion

Previous studies have shown that two residues of ϕ29 DNA polymerase, the invariant Lys of motif B (Lys383)[18] and the highly conserved Lys residue of pre-motif B (Lys371),[23] are involved in binding the incoming nucleotide. The results of the mutational analysis of ϕ29 DNA polymerase described here indicate that two additional positively charged residues, Lys366 and Lys379, located in pre-motif B and motif B, respectively, are involved in stabilisation of the incoming nucleotide in ϕ29 DNA polymerase.

Mutation of Lys379 (motif B) resulted in a mutant DNA polymerase (K379T) with

reduced affinity for the incoming nucleotide in DNA-primed polymerisation and TP-primed initiation and elongation reactions. The mutation did not affect either the DNA or the TP-binding capacity of the mutant DNA polymerase. Since the affinity of mutant DNA polymerase K379T for the initiating nucleotide was also affected in a non-templated TP-deoxynucleotidylation reaction and in the similar DNA-primed polymerisation reaction (addition of one nucleotide to a blunt-ended DNA substrate), it could be deduced that the reduced nucleotide-binding affinity of mutant DNA polymerase K379T was independent of the presence of template. This supports the involvement of Lys379 in dNTP binding, even when no templating nucleotide is positioned at the polymerisation active site. A similar result had been obtained for ϕ29 DNA polymerase residue Lys371,[23] indicating that during these non-templated reactions, the positively charged residues of motif B and pre-motif B are interacting with the triphosphate groups of the incoming nucleotide, as it occurs in the closed conformation. Although the exonuclease activity of mutant DNA polymerase K379T was similar to that of the wild-type enzyme, its pyrophosphorolytic activity was reduced, indicating that residue Lys379 could be involved in binding the β or γ phosphate groups of the incoming nucleotide and that during this reversal activity of polymerisation, pyrophosphate, DNA substrate and DNA polymerase are forming a closed "ternary" complex.

Lys379 of ϕ29 DNA polymerase aligns with the Arg of motif B, invariant in the protein sequences of DNA polymerases belonging to family A, which has been shown to contact the γ-phosphate group of the incoming nucleotide (Arg659 of *Taq*,[21] see Figures 1 and 2). Mutation of Arg659 of *Taq* DNA polymerase results in loss of activity and it has been proposed to be involved in dNTP binding.[42] Interestingly, the cellular and bacterial/viral subfamilies of the family B DNA polymerases have a nearly invariant Gln with unknown function at the corresponding position (Gln556 in RB69 DNA polymerase, Figures 1 and 2). Only family B DNA polymerases belonging to the protein-primed subfamily have also a positively charged amino acid at this position of motif B (like family A DNA polymerases), corresponding to Lys379 of ϕ29 DNA polymerase. The involvement of this residue of ϕ29 DNA polymerase in binding the incoming nucleotide proposed here agrees with the role of the corresponding invariant Arg of family A DNA polymerases and can possibly be extrapolated to the corresponding positively charged residues of the other protein-primed DNA polymerases. Since the residues corresponding to Lys379 of ϕ29 DNA polymerase are only partially conserved as positively charged amino acid residues in DNA polymerases belonging to the protein-primed subfamily, the role of this residue in dNTP-binding must be less critical than that of the residues corresponding to the invariant Lys383 and the highly conserved Lys371 of ϕ29 DNA polymerase. In agreement with this, the phenotype of mutant DNA polymerase K379T was less drastic than that of mutant DNA polymerase K371T.[23] Interestingly, in the crystal structure of RB69 DNA polymerase the positions of residues Arg482 (shown to be the third dNTP-ligand in RB69 DNA polymerase) and Gln556 (corresponding in the alignment to Lys379 of ϕ29 DNA polymerase) are very similar with respect to the incoming nucleotide (Figure 1), indicating that Lys379 could be a direct dNTP-ligand. Although in family A and B DNA polymerases some of the residues involved in dNTP binding are different, the structure of their polymerisation active sites including the nucleotide-binding pocket seems to be very similar (Figure 1).

The Arg located in pre-motif B, nearly invariant in the bacterial/viral subfamily of family B DNA polymerases was shown in the crystal structure of RB69 DNA polymerase[8] (Arg482, Figure 1), as well as by mutational analysis[15] in this DNA polymerase, to contact the γ-phosphate group of the incoming nucleotide. Most probably the corresponding invariant Arg residue of the cellular DNA polymerases (Figure 2) plays a similar role as dNTP ligand. The DNA polymerases able to start replication by protein-priming have no positively charged residue at this position. But interestingly, they have a partially conserved positively charged residue at the preceding position of their sequences, corresponding to Lys366 in ϕ29 DNA polymerase. Since the mechanism of initiation is proposed to be similar in all DNA polymerases starting replication with a protein as primer, amino acid residues playing a specific role in TP-primed initiation of replication should be conserved among the DNA polymerase sequences of this subfamily. The results obtained here with ϕ29 DNA polymerase mutant K366T indicate that this residue is especially important in TP-primed reactions: in DNA-binding and in templated DNA-primed polymerisation (primer/template and processive primed-M13 DNA replication) this mutant DNA polymerase was not affected; on the other hand, its velocity in TP-dAMP formation was reduced, affecting its TP-deoxynucleotidylation activity independently of the presence or absence of template. A drastic effect on the initiation (TP-DNA or dsoriR), transition and replication activities of mutant DNA polymerase K366T was observed when p6 was forming a nucleoprotein complex at the ϕ29 DNA ends: a strong increase in the K_m for the initiating nucleotide and reduction in the V_{max} of the initiation reaction, as well as a strong impairment of the transition, were observed. Protein p6 has been described to favour the initiation reaction due to a decrease in the K_m for the initiating nucleotide, dATP,[41] which possibly is the result of a favourable presentation of the templating nucleotide at the polymerisation active site.[43] For DNA-templated TP-dAMP formation, ϕ29 DNA polymerase has to bind the ϕ29 replication origin to use one strand as template

once the TP/DNA polymerase heterodimer has been formed. It is likely that this specific reaction requires very precise interactions to position TP, templating and incoming nucleotide at the polymerisation active site. Therefore, it can be expected that specific amino acid residues must control this positioning to be correct. We propose that Lys366 of ϕ29 DNA polymerase could play a role in stabilising the incoming nucleotide in an appropriate position for efficient TP-deoxynucleotidylation: mutation of Lys366 into Thr could affect the positioning of the initiating nucleotide causing a decrease in the V_{max} of the initiation reaction (in the presence and absence of template); on the other hand, when binding of p6 at the ϕ29 DNA ends results in a slight change in the presentation of the templating nucleotide (increasing the pairing efficiency of the nascent base-pair in the wild-type enzyme), the affected stabilisation of the initiating nucleotide in mutant DNA polymerase K366T could impair the base-pair formation between templating and incoming nucleotide, affecting both K_m and V_{max} during initiation. This would explain the reduced TP-DNA initiation, transition and replication activities of this mutant polymerase, as well as the strong reduction in these activities upon addition of p6. Interestingly, mutant DNA polymerase K366T was also affected in non-templated DNA-primed polymerisation and in pyrophosphorolysis. The proposed stabilisation of the incoming nucleotide by residue Lys366 could, therefore, be especially important in the absence of template and/or when TP is present, maybe occurring through the interaction with the β or γ phosphate groups of the incoming nucleotide.

We can conclude that Lys379 of ϕ29 DNA polymerase, conserved among DNA polymerases of family B able to start replication by protein priming, is involved in dNTP binding. On the other hand, residue Lys366 of ϕ29 DNA polymerase may play a role in stabilising the incoming nucleotide in an appropriate position for efficient TP-deoxynucleotidylation.

Materials and Methods

Nucleotides and proteins

Unlabelled 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. Phage T4 polynucleotide kinase was from Boehringer Mannheim.

ϕ29 TP-DNA was isolated as described by Peñalva & Salas.[44] The TP ($M_r = 30,918$) was purified as described.[45] The ϕ29 double-stranded DNA-binding protein p6 (DBP, $M_r = 11,873$), obtained from *Bacillus subtilis* cells infected with phage ϕ29, was purified as described.[46]

DNA templates and substrates

Oligonucleotides sp1 (5′GATCACAGTGAGTAC), its complementary oligonucleotide sp1c (5′GTACTCACTG TGATC) and sp1c + 6 (5′TCTATTGTACTCACTGT GATC), that has a 5′-extension of 6 nt in addition to the sequence complementary to sp1, were prepared with a DNA synthesizer from Applied Biosystems. The oligonucleotide sp1 was first purified by electrophoresis on 8 M urea/20% (w/v) polyacrylamide gels and then 5′-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. To analyze the DNA binding, polymerisation and exonuclease activities on a primer/template structure, 5′-labelled sp1 was hybridised to sp1c + 6 in the presence of 0.2 M NaCl and 60 mM Tris–HCl (pH 7.5). Polymerisation in the absence of template was performed with the blunt-ended hybrid molecule sp1/sp1c (matched 15/15mer). To analyze processive DNA polymerisation 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. The sequences of the oligonucleotides oriR29 (29mer) and its complementary strand were identical with those of the right origin of ϕ29 DNA.[36]

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),[47] which expresses ϕ29 DNA polymerase under the control of the T7 RNA polymerase-specific ϕ10 promoter,[48] was used for site-directed mutagenesis carried out by two-step PCR-amplification.[49] The amplified fragments contained the C-terminal half of the ϕ29 DNA polymerase gene, including unique *Acc*I and *Bam*HI restriction sites. These fragments were subcloned into the same unique restriction sites of pJLPM. The presence of the desired mutations and the absence of other changes were confirmed by sequencing with a set of synthetic oligonucleotides complementary to the ϕ29 DNA polymerase gene as sequencing primers. Expression of the mutant protein was carried out in the *Escherichia coli* strain BL21 (DE3), which contains the T7 RNA polymerase gene under the control of the IPTG-inducible lacUV5 promoter.[50] The purification of wild-type and mutant ϕ29 DNA polymerases was done as described.[47]

Buffers

The 10 × reaction buffer used in all assays contained 500 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 40% (v/v) glycerol and 1 mg/ml of BSA. The buffer used for dilution of the polymerase contained 25 mM Tris–HCl (pH7.5), 120 mM NaCl, 1 mg/ml of BSA and 50% (v/v) glycerol.

DNA gel retardation assays

The interaction of either wild-type or mutant ϕ29 DNA polymerases with a primer/template structure was assayed using the 5′-labelled sp1/sp1c + 6 (15/21mer) DNA.[32] The incubation mixture, in a final volume of 20 μl, contained 12 mM Tris–HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulphate, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 0.1 mg/ml of BSA, 1.2 nM 5′-labelled sp1/sp1c + 6, and as indicated 2.1, 4.2 or 8.4 nM either wild-type or mutant ϕ29 DNA polymerase. After incubation for five minutes on ice, 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.[51] In the autoradiogram of the gel, enzyme/DNA stable interaction was detected as a shift in the position of the free labelled DNA, and quantified by densitometry corresponding to different experiments.

Polymerase/3′-5′ exonuclease coupled assay

The DNA molecule sp1/sp1c + 6 (15/21mer) contains a 6 nt long 5′-protruding end, and therefore, the primer strand can be used both as substrate for the 3′-5′ exonuclease activity and also for DNA-dependent DNA polymerisation. The reaction mixture, in a final volume of 12.5 μl, contained 1.25 μl of reaction buffer, 10 mM $MgCl_2$, 1.2 nM 5′-labelled DNA (sp1/sp1c + 6), 16.8 nM either wild-type or mutant ϕ29 DNA polymerase, and the indicated increasing concentrations of the four dNTPs. After incubation for five minutes at 30 °C, the reaction was stopped by addition of 3 μl of sequencing loading buffer. Samples were analysed by 8 M urea/20% polyacrylamide gel electrophoresis and autoradiography. Polymerisation or 3′-5′ exonuclease is detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labelled sp1 primer. Different dNTP concentrations are required for the wild-type ϕ29 DNA polymerase to polymerise the first nucleotide (from position 15 to 16), to compete effectively the 3′-5′-exonuclease activity replicating until the last nucleotide (from positions 16 to 20), and to replicate the last nucleotide of the template (from positions 20 to 21). The exonucleolytic activity was obtained by calculating the number of catalytic events giving rise to each degradation product. From these data (obtained by densitometry of the autoradiograms), the catalytic efficiency (indicated in Table 1) of each mutant derivative, assayed in linear conditions both in time and enzyme amount, was calculated relative to the wild-type ϕ29 DNA polymerase.

The blunt-ended DNA substrate for the +1 addition assay was the 5′-labelled molecule sp1/sp1c (15/15mer). The reactions were performed as described for the pol/exo asays, but using dNTP concentrations ranging between 100 μM and 500 μM and incubating on ice (to avoid exonuclease activity on the DNA substrate). The non-templated +1 addition is seen as a band appearing at position 16.

Measurement of nucleotide-binding affinity

The analysis of the Michaelis–Menten constant for nucleotide binding (K_m^{dNTP}) in DNA polymerisation (using the 15/21mer primer/template structure (sp1/sp1c + 6) and $MgCl_2$ as metal activator) was carried out as described,[24] but the assay was performed on ice, to avoid exonucleolytic degradation of the primer/template structure. Therefore, no extra dCTP was added to the assay to prevent degradation of the DNA.

Pyrophosphorolysis

The pyrophosphorolytic activity was measured on the primer/template substrate sp1/sp1c + 6 (15/21mer). The reaction mixture was the same as for the pol/exo assays, but without dNTPs and using $MgCl_2$ as metal activator. Increasing concentrations of tetrasodium pyrophosphate (Sigma) were added (1–4 mM), as indicated. To reduce the exonuclease activity on this substrate, the reactions were incubated for ten minutes on ice. The exonuclease activity of the DNA polymerases was seen when no pyrophosphate was added, while the degradative activity seen with increasing concentrations of pyrophosphate corresponds to the pyrophosphorolytic activity. Higher concentrations than 4 mM pyrophosphate inhibited the degradative activity of wild-type and mutant DNA polymerases.

Replication of primed M13-DNA

The reaction mixture, in a final volume of 25 μl, contained 2.5 μl of RB, 10 mM $MgCl_2$, 20 μM each of the four dNTPs, 13 nM [α-^{32}P]dATP (2 μCi), 4.2 nM oligonucleotide-primed M13 ssDNA and 16.8 nM either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 30 °C, the reaction was stopped by addition of 10 mM EDTA and 0.1% (w/v) SDS. After filtration through Sephadex G-50 spin columns, the Čerenkov radiation of the excluded volume was determined to calculate the amount of incorporated dNMPs during this processive reaction. Elongation was studied by alkaline 0.7% agarose gel electrophoresis.[52] The position of unit length ϕ29 DNA in the agarose gels was detected by ethidium bromide staining. The autoradiogram 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 of reaction buffer, 20 mM ammonium sulphate, 0.03 μM [α-^{32}P]dATP (2 μCi), 83 nM purified TP, and 16.8 nM either wild-type or mutant ϕ29 DNA polymerase. When indicated, 34 μM ϕ29 DBP p6 was added. The conditions in the template-dependent assay (with 1.6 nM of TP-DNA) were 10 mM $MgCl_2$ as metal activator and an incubation time of five minutes at 30 °C. The assay without template was performed with 1 mM $MnCl_2$ as metal activator and the incubation was for four hours at 30 °C. Under these conditions the reactions were proved to be linear. TP-primed initiation on an oligonucleotide (ss-oriR or ds-oriR, 29mer) was performed as described by Méndez *et al*.[36] using the reaction mixture described above with 72 nM the respective DNA polymerase, 154 nM TP, 0.85 μM oriR (ds or ss), 10 mM $MgCl_2$ as the metal activator and incubating the samples for ten minutes at 15 °C. Reactions were stopped by addition of 10 mM EDTA, 0.1% SDS, filtration through Sephadex G-50 spin columns (in the presence of 0.1% SDS) and further analysis by SDS-PAGE as described.[44] The TP-dAMP complex was detected on the autoradiogram and quantified by densitometric analysis. To calculate the Michaelis–Menten constant for dATP binding (K_m and V_{max}) in the TP-DNA initiation reaction, different dATP concentrations and incubation times were assayed. The analysis of the interaction between DNA polymerase and TP by glycerol gradient centrifugation was performed as described.[53]

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

Complete replication of TP-DNA was performed as described above for the initiation experiment in the presence of 10 mM $MgCl_2$, 50 mM NaCl,[54] and 20 μM

each dGTP, dCTP, dTTP and [α-^{32}P]dATP (2 μCi). ϕ29 DBP p6 (34 μM) was added when indicated. After incubation at 30 °C for 15 minutes the reactions were stopped by addition of 10 mM EDTA, 0.1% SDS. Subsequent to filtration through Sephadex G-50 spin columns, the Čerenkov radiation in the excluded volume was measured and used for quantification. Then, the TP was digested by proteinase K treatment (5 mg, one hour at 30 °C) to be able to analyse the samples on 0.7% TBE–agarose gels.[55] The position of unit length ϕ29 DNA in the agarose gels was detected by ethidium bromide staining after electrophoresis. The autoradiogram of the dried gels revealed the elongation efficiency.

Truncated replication experiments (in the absence of dCTP) were performed as described above (Mg^{2+} as metal ion, no NaCl added) but with 1 μM or 10 μM each dGTP, dTTP and [α-^{32}P]dATP (2 μCi). When indicated, 34 μM p6 was added. After incubation at 30 °C for five minutes the reactions were stopped by 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. Separation of TP-dAMP from TP-$(dAMP)_2$ and the truncated transition products was carried out in SDS-containing 12% polyacrylamide gels (360 mm × 280 mm × 0.5 mm) as described,[36] and detected by autoradiography. Quantification was performed by densitometric scanning of the autoradiograms.

Acknowledgements

This investigation has been aided by research grant 2R01 GM27242-23 from the National Institutes of Health, by grant PB98-0645 from the Dirección General de Investigación Científica y Técnica and by an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular "Severo Ochoa". V.T. was a postdoctoral fellow from the Comunidad Autónoma de Madrid.

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Edited by J. Karn

(Received 24 July 2003; received in revised form 8 October 2003; accepted 13 October 2003)