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COMMUNICATION

Functional importance of bacteriophage ϕ29 DNA polymerase residue Tyr148 in primer-terminus stabilisation at the 3´-5´ exonuclease active site

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Running Title: 3´-5´ exonuclease active site of ϕ29 DNA polymerase

Summary

Recent crystallographic resolution of ϕ29 DNA polymerase complexes with ssDNA at its 3′-5′ exonuclease active site has allowed the identification of residues Pro129 and Tyr148 as putative ssDNA ligands, the latter being conserved in the Kx_2h motif of proofreading family B DNA polymerases. Single substitution of ϕ29 DNA polymerase residue Tyr148 to Ala rendered an enzyme with a reduced capacity to stabilize the binding of the primer-terminus at the 3′-5′ exonuclease active site, not playing a direct role in the catalysis of the reaction. Analysis of the 3′-5′ exonuclease on primer/template structures showed a critical role for residue Tyr148 in the proofreading of DNA polymerisation errors. In addition, Tyr148 is not involved in coupling polymerisation to strand displacement in contrast to the catalytic residues responsible for the exonuclease reaction, its role being restricted to stabilisation of the frayed 3′ terminus at the exonuclease active site. Altogether, the results lead us to extend the consensus sequence of the above motif of proofreading family B DNA polymerases into Kx_2hxA**.** The different solutions adopted by proofreading DNA polymerases to stack the 3′ terminus at the exonuclease site are discussed. In addition, the results obtained with mutants at ϕ29 DNA polymerase residue Pro129 allow us to rule out a functional role as ssDNA ligand for this residue.

Keywords: ϕ29 DNA polymerase/3′-5′ exonuclease/site-directed mutagenesis/ssDNA binding/strand displacement

Abbreviations used: ssDNA, single-stranded DNA; (*p*NP-TMP), 5′-*p*-nitrophenyl ester of thymidine 5′-monophosphate; Pol Ik, Klenow fragment of DNA polymerase I; DTT, dithiothreitol; BSA, bovine serum albumin.

Most replicative DNA polymerases combine two processes to guarantee faithful synthesis of DNA, nucleotide selectivity and editing of the misinserted nucleotides. DNA polymerases show a high intrinsic nucleotide selectivity that provides the greatest contribution to base substitution fidelity since they bind correct dNTPs with about 10- to >1000-fold higher affinity than incorrect dNTPs as the geometry of the nascent base pair pocket accommodates exclusively canonical Watson and Crick base pairs (reviewed in[1] and references therein). Moreover, the rate of the phosphodiester bond formation with erroneous nucleotides decreases dramatically. Altogether, these functional features lead to an error rate for single base substitutions ranging from 10^{-3} to 10^{-6} [1]. In addition, if a nucleotide is misinserted onto the growing strand, the resulting mismatch will stall further DNA synthesis by the polymerase. Such a decrease in the elongation rate of the mismatch favours dissociation of the frayed primer-terminus from the polymerisation site, allowing it to be shuttled to the 3′-5′ exonuclease active site for further hydrolysis of the last phosphodiester bond. The 3′-5′ exonuclease active site of DNA polymerases is evolutionarily conserved across the A, B, and C families[2] and share a common mechanism catalyzed by two metal ions bound to the enzyme through four carboxylate groups[3,4] which belong to motifs Exo I, Exo II and Exo III, found in most prokaryotic and eukaryotic members endowed with a proofreading activity[2]. In addition, biochemical and structural analyses have demonstrated the contribution to the catalysis of the exonucleolytic reaction of two other conserved amino acid residues; a Tyr belonging to the Exo III motif, which interacts with the water nucleophile[2,3,5,6] and a Lys from the Kx$_2$*h* motif, specifically present in the exonuclease domain of family B DNA polymerases[7] and whose ε-amine group interacts with the hydroxyl of the above mentioned Tyr, with the catalytic Asp of the Exo III motif and with the scissile phosphate through a water-mediated hydrogen bond[8-10]. The 3′-5′ exonuclease activity has been estimated to

enhance DNA synthesis fidelity by factors ranging from a few-fold to more than 100-fold[1,11-19].

Usually, polymerisation and exonuclease sites form part of the same polypeptide chain, being placed at two structurally independent domains. As these sites are spatially separated 30-40 Å[9,20-24], prevention of the accumulation of polymerisation errors onto the growing strand will depend on the proficient transfer of the 3′ primer-terminus between both active sites. Structural analysis of polymerisation *versus* editing complexes of several proofreading DNA polymerases have shown the thumb subdomain, which forms part of the polymerisation domain, as the main one involved in transferring the primer-terminus during proofreading. Thus, a rotation of the tip of the thumb conveys the primer strand from the polymerisation to the exonuclease active site as it maintains the contacts with the phosphates of the primer-terminus in both, polymerisation and editing modes[25-30]. In addition, other contacts established with the template strand also seem to modulate primer-terminus transference[31,32]. For bacteriophage ϕ29 DNA polymerase, the nearly static structure of its thumb subdomain has led to suggest an alternative transfer mode by which the shuttling would be accomplished by a passive diffusion of the frayed primer-terminus to the exonuclease active site[33].

In addition, for an efficient hydrolysis of the phosphodiester bond, the melted DNA has to be correctly stabilized and oriented at the 3′-5′ exonuclease active site by ssDNA binding residues. The resolution of the cocrystal structures of several proofreading DNA polymerase complexes with ssDNA at their exonuclease active site[3,9,10,20,23,30] allowed the identification of some of those ssDNA ligands. Thus, the Thr residue from the Exo I motif ("ExDT") is H-bonded to the 3′-OH of the ssDNA; the Asn residue from the Exo II motif ("Nx_{2-3}(F/Y)D") is H-bonded to the penultimate nucleotide, and the Phe residue from the Exo II motif forms extensive van der Waals contacts with the 3′-deoxyribosyl moiety and with the adjacent sugar[3]. All of the above mentioned residues were predicted to be evolutionarily conserved,

and involved in primer-terminus stabilisation at the 3′-5′ exonuclease active site of proofreading DNA polymerases[34,35].

Here, we describe the functional importance as ssDNA ligand of a Tyr residue belonging to the Kx$_2$*h* motif of family B (eukaryotic-type) DNA polymerases. This study was carried out by site-directed mutagenesis at the corresponding residue Tyr148 of ϕ29 DNA polymerase, a model multifunctional enzyme for eukaryotic DNA polymerases, which displays a very strong and processive 3′-5′ exonuclease activity[5,36,37].

Stabilisation of the ssDNA at the exonuclease domain of bacteriophage ϕ29 DNA polymerase

The structures of the ϕ29 DNA polymerase with a pentanucleotide bound to its exonuclease domain[23] supported sequence comparisons and mutational data that had shown the catalytic residues to be Asp12 and Glu14 (Exo I), Asp66 (Exo II), Asp169 and Tyr165 (Exo III), and Lys143 (Kx$_2$*h*) [2,5-7]. They also confirmed the previous identification of ssDNA ligands responsible for primer-terminus stabilisation at the 3′-5′ exonuclease active site, Thr15 (Exo I), Asn62 (Exo II) and Phe65 (Exo II) that contact the two 3′ terminal nucleotides of a ssDNA[34,35] (see Figure 1, right panel). These structures also permitted the identification of new residues that appeared to contact with the ssDNA, as Leu567 (from the thumb subdomain), placed between and interacting with the two 3′ terminal bases in the exonuclease active site[23] and whose importance as ssDNA ligand has been recently reported[38]; and Tyr148 from the Kx$_2$*h* motif[7] that stacks on the 3′ terminal base of the bound pentanucleotide (Figure 1, right panel). Multiple sequence alignment of the Kx$_2$*h* motif of family B DNA polymerases shows the conservation of an aromatic group in the equivalent position to ϕ29 DNA polymerase residue Tyr148, mainly Tyr, His or in some cases Trp[7] (see also Figure 1, left panel) underlining the putative physiological importance of this residue as

ssDNA ligand at the exonuclease site, leading us to expand the consensus sequence of the above motif into Kx$_2$*h*x*A*.

In addition, previous crystallographic structures of ϕ29 DNA polymerase pointed to Pro129, a non-conserved residue which belongs to the (S/T)Lx$_2$*h* motif of proofreading DNA polymerases[35,39], as a ssDNA ligand which would contact with the third 3′ terminal nucleotide[23] (see also Figure 1, right panel). However, more recent ϕ29 DNA polymerase structures seemed to preclude a role for this residue in primer-terminus stabilisation[8].

Site-directed mutagenesis of ϕ29 DNA polymerase residues Pro129 and Tyr148.

To study the physiological role of the ϕ29 DNA polymerase residues described above, Pro129 was changed into Ala (P129A) and Gly (P129G) to shorten the side chain, whereas Tyr148 was mutated to Ala (Y148A), to remove the aromatic group, and to Phe (Y148F) to conserve it. ϕ29 DNA polymerase mutants were obtained by using the QuickChange site-directed mutagenesis kit provided by Amersham Pharmacia. Plasmid pJLPM, a derivative of plasmid pJLw2[40] containing the ϕ29 DNA polymerase gene, was used as template for the mutagenesis reaction. After temperature cycling using *Pfu*Turbo DNA polymerase and treatment with *Dpn*I endonuclease, synthesized DNA was transformed into *Escherichia coli* XL1-blue supercompetent cells. The presence of the desired mutation and absence of others was confirmed by sequencing the entire gene. ϕ29 DNA polymerase mutants were overproduced and purified as described[40] from *E. coli* BL21(DE3) cells harbouring the corresponding recombinant plasmid.

3′-5′ exonuclease activity of ϕ29 DNA polymerase mutants

The functional importance of the above residues was first addressed by studying the 3′-5′ exonuclease activity of the different ϕ29 DNA polymerase mutants, using as substrate of the

reaction a 15mer ssDNA of heterogeneous sequence (see Legend to Figure 2). The assay was carried out in linear conditions of both time and amount of enzyme. As shown in Table I, none of the mutations significantly affected the activity of the exonuclease. The electrophoretic analysis of the ssDNA products revealed that the degradation pattern displayed by mutants P129A, P129G and Y148F was almost identical to that shown by the wild-type enzyme, the 15mer oligonucleotide being degraded processively until its length was reduced to 6mer (Figure 2A). In contrast to its nearly wild-type activity, the degradation pattern yielded by mutant Y148A was remarkably different, as it was deficient in using molecules shorter than 9mer as substrate of the exonuclease activity (Figure 2A).

As described for mutations introduced in other ssDNA ligands at the exonuclease active site, the pattern observed with ϕ29 DNA polymerase Y148A mutant respect to the size limit for processive degradation suggests differences in the stability of the DNA polymerase-ssDNA complexes related to the size of the DNA substrate[34,35,38]. Thus, binding of short substrates will exclusively rely on specific interactions with ssDNA ligands placed at or close to the active site, degradation of those substrates being much more sensitive to mutations in these residues. Contrarily, degradation defects on longer ssDNA substrates could be overcome by the potential to interact with residues of the enzyme others than those involved in 3' terminus stabilisation, as it could be the case for mutant Y148A. This hypothesis led us to analyze the ability of the mutant derivatives to hydrolyze the 5′-*p*-nitrophenyl ester of thymidine 5′-monophosphate (*p*NP-TMP) [41,42], a minimal substrate for the exonuclease activity whose binding for further hydrolysis of this non-canonical nucleotide will solely depend on those ligands responsible for the stabilisation of the 3′ terminal nucleotide of a ssDNA during exonucleolysis. The rate of hydrolysis of *p*NP-TMP catalyzed by the wild-type and mutant ϕ29 DNA polymerases was determined spectrophotometrically by continuous monitoring of the *p*-nitrophenol produced. As it can be seen in Figure 2B, mutants

P129A and P129G did not display a large reduction in their the activities (0.14 s^{-1} and 0.16 s^{-1}, respectively) relative to that of the wild-type enzyme (0.28 s^{-1}), most probably indicating a small change in the overall conformation of the catalytic site because of the single mutation introduced. In contrast, changes introduced at the Tyr148 residue strongly affected the capacity of the enzyme to hydrolyze this substrate. Thus, Y148A mutant, the most deficient in degrading short ssDNA substrates (Figure 2A), displayed a 28-fold reduction in its catalytic efficiency when acting on *p*NP-TMP. It was of significance that conservation of the aromatic ring in the Y148F mutant also prevented the proper hydrolysis of this type of substrate, exhibiting a 7-fold reduction in its efficiency (0.04 s^{-1}), in contrast to its wild-type phenotype on ssDNA. In addition to the stacking interaction between the aromatic portion of Tyr148 residue and the 3´ terminal base, crystallographic structures of ϕ29 DNA polymerase with a ssDNA at its exonuclease site suggest a potential interaction of the hydroxyl group of the Tyr with one of the phosphates of the last phosphodiester bond, placed at 2.75 Å [23] (see Figure 1). Thus, in spite of preserving the aromatic group, the low capacity exhibited by mutant Y148F to hydrolyze the minimal *p*NP-TMP substrate could be due to the loss of such interaction.

Mutations at ϕ29 DNA polymerase residue Tyr148 affect ssDNA binding

The differences in processivity observed with mutant Y148A during exonucleolytic degradation of a ssDNA, the reduced hydrolysis of the *p*NP-TMP substrate observed with the two mutants at this residue, as well as the crystallographic data of ϕ29 DNA polymerase agree to a role for residue Tyr148 in ssDNA binding at the 3'-5' exonuclease active site.

To further study the ssDNA binding capacity of mutant DNA polymerases, we evaluated their ability to retard the molecule by gel shift assays. As shown in Figure 3, the wild-type enzyme produced a single retardation band, most likely corresponding to a DNA

polymerase/ssDNA complex in which the 3´-terminus was stably bound at the exonuclease active site[34]. The presence of this band absolutely depends on the absence of divalent metal ions to avoid exonucleolytic degradation of the ssDNA substrate. As expected, mutants at residue Pro129 behaved as the wild-type enzyme (Figure 3 and Table I). This result, together with those described above, allow us to rule out a ssDNA binding role for this residue, validating the last crystallographic structures of ϕ29 DNA polymerase[8]. The wild-type phenotype displayed by mutant Y148F led us to hypothesize that either the probable subtle distortion in the orientation of the aromatic group in this mutant enzyme or the disruption of the potential interaction with the last phosphodiester bond, or both, manifested when acting on the *p*NP-TMP substrate, is counteracted by the other additional contacts between the last 3´ nucleotides and the previously described ssDNA ligands[34,35,38], as well as by unspecific interactions with other DNA polymerase residues. By the contrary, removal of the aromatic ring in mutant Y148A provoked a poor ssDNA binding stability, this being the most probable cause of the exonucleolytic defects exhibited by this mutant when acting on both, short ssDNA and *p*NP-TMP substrates.

To rule out the lack of metal ions as the cause of the reduced binding of the substrate at the 3'-5' exonuclease active site of mutant Y148A, the same assay was performed in the presence of Mg^{2+} ions but using as substrate a sulfur-substituted oligonucleotide with a phosphorothioate linkage between the two 3'-terminal nucleotides to prevent its exonucleolytic degradation. Under these conditions, mutant Y148F exhibited a DNA substrate binding efficiency 10-fold lower than the wild-type enzyme (not shown). Thus, it can be concluded that amino acid residue Tyr148 of ϕ29 DNA polymerase is important for a proper and/or stable binding of ssDNA at the 3'-5' exonuclease active site.

Importance of ϕ29 DNA polymerase residue Tyr148 for exonucleolysis under

polymerisation conditions

Although ssDNA molecules are the optimal substrates for the 3'-5' exonuclease activity, as binding to the exonuclease site is not challenged by interactions with polymerisation domain residues, primer/template structures are the physiological substrates for DNA polymerases. In this case, to gain access to the exonuclease active site, primer-terminus has first to be frayed and further shuttled from the polymerisation active site to the exonuclease one. Under these conditions, stabilisation of the primer-terminus at the exonuclease site is competed by its stabilisation at the polymerisation site, contributed by residues of the polymerisation domain and by Watson and Crick pairing with the template strand. Therefore, the functional importance of ssDNA ligands at the exonuclease site would be more evident if exonuclease were assayed on a primer/template structure, as previously reported[34,35,38]. Accordingly, while the ϕ29 DNA polymerase mutant Y148A had an almost wild-type catalytic efficiency on ssDNA, its exonuclease activity on a primer/template substrate was reduced 7-fold (see Table I and Figure 4A). Therefore, the option to bind the primer at the polymerisation active site aggravates the defects in the exonucleolysis caused by the defective interaction of the 3' terminus at the exonuclease active site of Y148A mutant. On the contrary, the relative efficiency of the rest of mutants was similar to that observed on ssDNA substrates.

The above result led us to speculate that the wild-type equilibrium between the exonuclease and polymerisation activities should be altered in mutant Y148A, due to its low exonuclease activity. To evaluate this hypothesis, we studied the functional coupling between synthesis and degradation on the primer/template molecule as a function of dNTPs concentration (see Figure 4B). Without nucleotides, the only bands that could be detected corresponded to primer degradation products due to the 3´-5´ exonuclease activity. As the concentration of the unlabelled dNTPs provided was increased, the exonuclease activity was

progressively competed by the polymerisation one, and net dNMPs incorporation was observed as an increase in the size of the labelled primer, allowing us to define the dNTP concentration to obtain an efficient elongation for each mutant derivative (exo/pol ratio). As expected, none of the mutations interfered with the polymerisation reaction. As shown in Table I and Figure 4B, the wild-type enzyme, as well as mutants P129A, P129G and Y148F, required 25 nM and 25-50 nM dNTPs to give rise to +1 and +5 elongation products, respectively. By the contrary, mutant protein Y148A required a concentration of dNTP 17- and 8-fold lower than that of the wild-type enzyme to give the +1 and +5 products, respectively (see Table I and Figure 4B).

All these results allow us to conclude a critical role in primer-terminus stabilisation at the 3'-5' exonuclease active site for Tyr148 under polymerisation conditions, as deduced from the favoured polymerisation observed with mutant Y148A.

The 3'-5' exonuclease activity of replicative DNA polymerases prevents the fixation of polymerisation errors by the efficient proofreading of misincorporated nucleotides. Therefore, the alteration of the delicate equilibrium between the synthetic and degradative activities shown by mutant Y148A could compromise the editing function of the polymerase. To address this issue, the ability to promote stable incorporation of mismatched nucleotides during polymerisation by mutants at residue Tyr148 was studied by analyzing the insertion of dAMP opposite non-complementary positions. As shown in Figure 4C, the wild-type DNA polymerase did not produce stable misincorporation, with dAMP insertion occurring only opposite to complementary positions 16 and 17. In the case of mutant Y148F, dAMP was not stably misincorporated at non-complementary positions, as it could be predicted based on its wild-type exo/pol balance. The higher intensities of the bands corresponding to positions 16 and 17 are probably due to the slightly reduced (75%) exonuclease activity displayed by this mutant on primer/template molecules. In the case of mutant Y148A, the appearance of label

at positions 19 and 20 indicates that misincorporation of dAMP and further elongation took place even at the lowest dATP concentration (125 nM). Quantification of the misincorporation produced showed that, in agreement with a reduction of the exo/pol ratio, the fidelity of DNA synthesis was reduced 13-fold. Under these conditions, the exonuclease deficient ϕ29 DNA polymerase double mutant D12A/D66A[2] showed a 20-fold reduction in fidelity with respect to the wild-type enzyme. Therefore, the presence of the next correct nucleotide, together with the altered ssDNA binding capacity of the Y148A mutant, decrease its proofreading efficiency by favouring extension of polymerisation errors, reducing the fidelity of the DNA synthesis.

The crystallographic resolution of the structure of many proofreading DNA polymerases has shown how their exonuclease domain contains a deep crevice of narrow dimensions, formed by the ssDNA ligands[4,9,10,23,26,43] conserved among DNA polymerases from families A, B and C[2,34,35,44,45], along which the primer-terminus is properly stabilized and oriented at the catalytic site for further hydrolysis. Such a cleft is usually capped by an aromatic residue that packs against the 3' terminal base (as ϕ29 DNA polymerase residue Tyr148, this article). Contrarily to other ssDNA ligands, this aromatic residue is not conserved in the primary structure among the different families[7]. Thus, crystallographic structures of phage T7 DNA polymerase[26] and *E. coli* Pol Ik[4,43], both belonging to family A, show the non-conserved residues Tyr473 and Trp160 as those capping the ssDNA crevice, respectively (see Figure 5). Although it has been shown here that homologs to ϕ29 DNA polymerase residue Tyr148 are conserved in the Kx$_2$*h*x*A* motif of many family B DNA polymerases (see Figure 1), several members contain an aliphatic residue in the corresponding position. Thus, bacteriophage RB69 DNA polymerase has a glycine instead of the expected aromatic residue. However, inspection of the editing complex of this DNA polymerase[28] shows how in this case Phe123, very close to the Exo I motif[9], forms the lid of the ssDNA binding groove (see Figure 5).

Altogether, these observations lead us to conclude that proofreading DNA polymerases have found different solutions to place the required aromatic group to stack against the 3' terminus of the primer strand at the exo site. The results shown here on the one hand indicate the importance of this residue in the stabilisation of the primer-terminus at the exonuclease active site, a requisite to guarantee the fidelity of the polymerisation process and, on the other hand, complete the biochemical analysis of the residues that form the exonuclease site of proofreading DNA polymerases by using ϕ29 DNA polymerase as model of study.

Mutation at ϕ29 DNA polymerase residues Pro129 and Tyr148 do not alter coupling between DNA polymerisation and strand displacement

ϕ29 DNA polymerase is endowed with an intrinsic characteristic that differentiates this enzyme from most replicases, the efficient coupling of DNA polymerisation to strand displacement, precluding the need for DNA unwinding proteins as helicases during replication of the viral genome[46]. The crystallographic structures of ϕ29 DNA polymerase as well as biochemical studies have given the clues about the molecular bases of how the strand displacement is performed[23,47,48]. Thus, besides the palm, thumb and fingers subdomains, the polymerisation domain of ϕ29 DNA polymerase is also contributed by an additional subdomain called TPR2 that corresponds to a protein region specifically present in protein-primed DNA polymerases[23,49]. This subdomain, together with the fingers, palm, and exonuclease domain, form a narrow tunnel through which the template strand is threaded to reach the polymerisation active site. The fact that deletion of this subdomain prevented the strand displacement capacity of ϕ29 DNA polymerase, led to propose that the TPR2 subdomain could act as a molecular ‘‘wedge’’ to separate the parental DNA strands as the polymerase moves along the DNA during polymerisation, thus conferring a helicase-like function on the DNA polymerase [48].

To analyze whether the mutations introduced at residues Pro129 and Tyr148 of ϕ29 DNA polymerase affected the catalysis of DNA polymerisation coupled to strand displacement, a primed M13 DNA replication assay was performed. Under these conditions, polymerase starts replication from the 3´-OH group of a short hybridized primer, requiring strand displacement for ongoing polymerisation once the 5´-terminus is reached. As shown in Figure 6 (see also Table I), none of the mutant enzymes was specifically affected in performing strand displacement, giving elongation products longer than unit length at a wild-type rate.

These results allow us to rule out any role for residues Pro129 and Tyr148 in the strand displacement capacity of the viral enzyme. In agreement with these results, none of the mutant DNA polymerases was affected in performing the protein-primed replication of the ϕ29 genome (not shown)

It had been shown that all the mutations introduced at the catalytic residues corresponding to the Exo I, Exo II, Exo III and Kx$_2$*h*x*A* motifs of ϕ29 DNA polymerase, responsible for the 3'-5' exonuclease activity, in addition to inactivating the 3'-5' exonuclease activity, severely affected the rate of DNA synthesis coupled to strand displacement[5-7]. By the contrary, the fact that none of the mutations introduced at the ssDNA ligand residues at the exonuclease active site affected the strand displacement capacity of the polymerase[34,38,39], led to the proposal that the dual role in 3'-5' exonuclease and strand displacement was restricted to the metal ligands, such as Asp12 and Glu14 of the Exo I motif (DxE), Asp66 of the Exo II motif (Nx$_{2-3}$F/YD), Asp169 of the Exo III motif (Yx$_3$D) and Lys143 of the Kx$_2$*h*x*A* motif. To conciliate these results with the crystallographic data, that appear to rule out contacts between the displaced strand and the exonuclease active site, it could be hypothesized that the absence of metal ions at the exo site affects the proper orientation of the TPR2 subdomain of ϕ29 DNA polymerase, directly involved in coupling polymerisation to strand displacement. Thus,

it has been reported that the exo site of ϕ29 DNA polymerase is formed not only by residues from the exo domain, but also by residues belonging to the tip of the thumb subdomain, as residue Leu567[23,38]. Therefore, the lack of metal ions in mutant polymerases at the catalytic residues could disassemble the normal interaction between the thumb subdomain and the exonuclease domain. As the apexes of thumb and TPR2 subdomains are in contact, a subtle distortion in the thumb subdomain could cause an inappropriate orientation of the TPR2 subdomain.

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Legends to Figures

Figure 1. Left; Multiple amino acid sequence alignment of the Kx_2hxA motif present in the 3′-5′ exonuclease domain of family B (eukaryotic-type) DNA polymerases. DNA polymerase nomenclature and sequence references are compiled in[45], with the exception of linear mitochondrial plasmid DNA polymerase from *Morchella conica* (pMC3-2; GenBank accession number **CAA45364**); *Pyrodictium occultum* DNA polymerase II (Pol II; GenBank accession number **BAA07579**); phage GA-1 DNA polymerase (GA-1; GenBank accession number **NP_073685**); African swine fever virus DNA polymerase (ASFV; GenBank accession number **NC_001659**); Chlorella virus DNA polymerase (CHV; GenBank accession number **M86837.1**); *Bombyx mori* nuclear polyhedrosis virus DNA polymerase (BmMnPV; GenBank accession number **NC_001962**); Guinea pig cytomegalovirus DNA polymerase (GPCMV; GenBank accession number **NC_011587.1**); Pseudorabies virus DNA polymerase (PRV1; GenBank accession number **L24487**); *Methanococcus voltae* DNA polymerase (M.volt.; GenBank accession number **L33366**); *Saccharomyces cerevisiae* DNA polymerase epsilon (ε (Sc); GenBank accession number **NP_014137.1**); linear plasmid pAL2-1 DNA polymerase from *Podospora anserina* (pAL2-1; GenBank accession number **X60707**); linear mitochondrial plasmid DNA polymerases from *Gelasinospora* sp. (GenBank accession number **S62752**), *Brassica napus* (GenBank data bank/a.n. **NP862323**), *Flammulina velutipes* (GenBank accession number **BAB13496**), *Pichia kluyveri* (GenBank accession number **CAA72340**), and *Porphyra purpurea* (GenBank accession number **NP_049297**); and *Streptococcus pneumoniae* phage Cp-1 DNA polymerase (GenBank accession number **Q37989**). Numbers between slashes indicate the position of the first amino acid residue aligned relative to the N-terminus of each DNA polymerase. The highly conserved Lys, the hydrophobic residue (*h*), and the aromatic one (*A*) of the Kx_2hxA motif are indicated in red letters over a black background, by a grey box, and by yellow letters over

a black background, respectively. The conserved aromatic residue (Phe, His or Trp) is indicated in yellow letters over a black background. ϕ29 DNA polymerase residue Tyr148, studied here, is indicated with an asterisk. **Right; *Stabilisation of ssDNA at the 3´-5´ exonuclease active site of bacteriophage ϕ29 DNA polymerase*.** The crystallographic data corresponding to the ϕ29 DNA polymerase with a pentanucleotide bound at its 3´-5´ exonuclease active site are from Protein Data Bank ID code 1XHZ[23]. For clarity, only the 3´-5´ exonuclease site is presented. Upper panel: the side chains of residues T15 (Exo I motif), N62 and F65 (Exo II motif), and L567 (from the thumb subdomain) have been previously shown to be involved in making contacts with ssDNA[23,34,35,38]. Figure also shows the putative ssDNA ligand residues P129 (from the (S/T)Lx$_2$*h* motif, coloured in light pink) and Y148 (belonging to the Kx$_2$*h*x*A* motif, coloured in yellow), studied in this work. Lower panel: detailed view of the interactions between Y148 and the 3´ terminal nucleotide. Figures was made by using the Swiss-PdbViewer program (http://www.expasy.org/spdbv/) and further rendering with Pymol (http://www.pymol.org).

Figure 2. 3´-5´exonucleolytic activity of point mutants of ϕ29 DNA polymerase. (a) *Exonuclease activity on ssDNA*. The incubation mixture contained, in a final volume of 12.5 µl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA and 10 mM $MgCl_2$. As substrate, 1.2 nM of 5'-labelled 15mer (5´ GATCACAGTGAGTAC) was used as ssDNA substrate. The amount of DNA polymerase added (6 nM) was adjusted to obtain linear conditions. Samples were incubated at 25ºC for the indicated times and quenched by adding 3 µl of gel loading buffer. Reactions were analyzed by electrophoresis in 8 M urea-20% polyacrylamide gels and densitometry of the autoradiograph. Total degradation was obtained by calculating the number of catalytic events giving rise to each degradation product. From these data, the activity of each mutant derivative (indicated in Table I) was calculated relative

to the wild-type ϕ29 DNA polymerase. The position of different degradation intermediates of the 15mer substrate (15mer) is indicated. Mutations are indicated by the original residue (in single letter notation), its position, and the replacing amino acid (i.e. P129A: Pro129 to Ala). **(b)** *Hydrolysis of pNP-TMP.* The incubation mixture contained, in 300 µl, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 1 mM $MnCl_2$, 3 mM *p*NP-TMP (dissolved in 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl), and 17 µM of either the wild-type or the indicated mutant ϕ29 DNA polymerase. Hydrolysis was studied by monitoring *p*-nitrophenol production at 420 nm with a Hitachi U-2000 spectrophotometer at 25 ºC, as described[42]. Production of *p*-nitrophenol was plotted against time and adjusted to a rectangular hyperbola by least-squares nonlinear regression, using the Kaleidagraph 3.6.4 software. Linearity in the production of *p*-nitrophenol was obtained in the 5-100 sec time range. Slopes obtained by linear regression adjustments of those points allowed us to calculate the activity for the hydrolysis of the phosphoester bond (s^{-1}).

Figure 3. Binding stability of ssDNA at the 3´-5´ exonuclease site of either the wild-type or mutant ϕ29 DNA polymerases. The incubation mixture contained, in a final volume of 20 µl, 12 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulphate, 0.1 mg/ml BSA, 1.2 nM of the 5'-labelled 15mer and 0.5 nM of either the wild-type or mutant ϕ29 DNA polymerases. After incubation for 5 minutes at 4 ºC, the samples were subjected to electrophoresis in precooled 4% (w/v) polyacrylamide gels (80:1, monomer:bis) containing 12 mM Tris-acetate (pH 7.5) and 1 mM EDTA, and run at 4ºC in the same buffer at 8 V/cm[50]. After autoradiography, ϕ29 DNA polymerase/DNA stable interaction was detected as a shift (retardation) in the migrating position of the labelled DNA, and quantified by densitometry of the autoradiograms corresponding to different experiments (see also Table I).

Figure 4. Involvement of ϕ29 DNA polymerase residues P129 and Y148 in the exonucleolysis of a primer-terminus. (a) *3'-5' Exonuclease assay.* The incubation mixture contained, in a final volume of 12.5 µl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA and 10 mM $MgCl_2$. As substrate, 1.2 nM of the primer/template molecule (5′-GATCACAGTGAGTAC/5′-TCTATTGTACTCACTGTGATC) (15mer/21mer double-stranded DNA; dsDNA) was used. The amount of DNA polymerase added (6 nM) was adjusted to obtain linear conditions. Samples were incubated at 25ºC for the indicated times and quenched by adding EDTA up to a final concentration of 10 mM. Reactions were analyzed by electrophoresis in 8 M urea-20% polyacrylamide gels and densitometry of the autoradiograph. Total degradation was obtained by calculating the number of catalytic events giving rise to each degradation product. From these data, the catalytic efficiency of each mutant derivative (indicated in Table I) was calculated relative to wild-type ϕ29 DNA polymerase. **(b)** *DNA polymerase/exonuclease (pol/exo) coupled assay.* The dsDNA molecule described above contains a six nucleotides 5'-protruding end and can also be used as substrate for DNA-dependent DNA polymerisation. The incubation mixture contained, in 12.5 µl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 4% (v/v) glycerol, 0.1 mg/ml BSA, 1.2 nM of 5′-labelled dsDNA, 30.3 nM of wild-type or mutant ϕ29 DNA polymerases and the indicated increasing concentrations of the four dNTPs. After incubation for 10 minutes at 25ºC, the reaction was stopped by adding EDTA up to a final concentration of 10 mM. Samples were analyzed by electrophoresis in 8 M urea-20% polyacrylamide gels and autoradiography. Polymerisation or 3'-5'-exonucleolysis is detected as an increase or decrease, respectively, in the size (15mer) of the 5'-labelled primer. **(c)** *Misincorporation during DNA replication by site-directed mutants at residue Tyr148 of ϕ29 DNA polymerase.* Conditions were essentially as described above for the polymerisation/exonuclease coupled assay on the primer/template molecule, but in this case, increasing concentrations of only

dATP, complementary to the positions 16, 17, 19 and 21 of the template strand was added. To prevent exonucleolytic degradation of the primer-terminus, 25 μM dCTP was added. After incubation for 5 minutes at 30ºC, samples were analyzed by 8 M urea-20% polyacrylamide gel electrophoresis. After autoradiography, misinsertion of dAMP at non-complementary positions is observed as the appearance of extension products of the 5'-labelled primer (15mer) larger than the correct 17mer extension product. The misincorporation produced in each case, expressed as the ratio 18mer + 19mer/17mer + 18mer + 19mer, was determined by densitometry of the autoradiogram.

Figure 5. An aromatic residue stacks against the 3' teminus of ssDNA at the exonuclease active site of proofreading DNA polymerases. The 3' terminal base of the primer-terminus and the indicated aromatic residue are shown as green and either magenta, orange or blue spheres, respectively. Crystallographic data of *E. coli* PolIk editing-mode complex are from Protein Data Bank code 1kln[25]. Determination of the aromatic group capping the ssDNA crevice of the 3'-5' exonuclease domain of bacteriophage T7 DNA polymerase was performed by making an structural alignment of the exonuclease domains of *E. coli* PolIk editing complex [25] and T7 DNA polymerase (PDB ID code 1T7P[26]) by using the Swiss PdbViewer program (http://www.expasy.org/spdbv/) Coordinates of the RB69 polymerase editing-mode complex were from PDB code 1CLQ[30]. Figures were rendered with the Pymol software (http://www.pymol.org).

Figure 6. Strand displacement coupled to M13 DNA replication by mutant ϕ29 DNA polymerases. The reaction mixture contained in a final volume of 25 μl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 10 mM $MgCl_2$, 40 μM of each of the four dNTPs, [α-^{32}P]dATP (1 μCi), 4.2 nM of singly-primed M13 ssDNA and 16.8 nM of

either wild-type or mutant ϕ29 DNA polymerases. After incubation for the indicated times at 30ºC, the reaction was stopped by 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 relative activity values (see Table I). For size analysis, the labelled DNA was denatured by treatment with 0.7 M NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels as described[51] and further aurtoradiography. The indicated migration position of unit-length M13 DNA (7250 bp) was detected by ethidium bromide staining.