

# Chapter 2

## Improvement of $\phi$ 29 DNA Polymerase Amplification Performance by Fusion of DNA Binding Motifs

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### 1 Introduction

Most of the modern genomic, phylogenetic, and epidemiological studies rely on the amplification of tiny amounts of DNA (Demidov and Broude 2004). The DNA amplification techniques rest on the DNA synthetic properties of the DNA polymerases from both thermophilic and mesophilic organisms that have led to the development of a large variety of isothermal and temperature-cycling amplification protocols.

Although the polymerase chain reaction (PCR; Mullis and Faloona 1987) is still the most widely used methodology for DNA amplification, it has two limitations: dependence on at least a limited knowledge of the sequence to be amplified and relatively short amplicons production. Among the alternative amplification technologies developed to yield large amounts of high quality DNA for genomic studies were those based in the unique features of bacteriophage  $\phi$ 29 DNA polymerase, such as the isothermal Multiple Displacement Amplification (MDA; Dean et al. 2001, 2002).

The  $\phi$ 29 DNA polymerase is the only enzyme involved in the replication of the phage  $\phi$ 29 genome. Based on amino acid sequence similarities and its sensitivity to specific inhibitors,  $\phi$ 29 DNA polymerase was included in the eukaryotic-type family B of DNA-dependent DNA polymerases (Bernad et al. 1987). As any other DNA polymerase, it accomplishes sequential template-directed addition of dNMP units onto the 3'-OH group of a growing DNA chain, showing discrimination for mismatched dNMP insertion by a factor from  $10^4$  to  $10^6$  (Esteban et al. 1993). In addition,  $\phi$ 29 DNA polymerase catalyzes 3'-5' exonucleolysis, i.e., the release of dNMP

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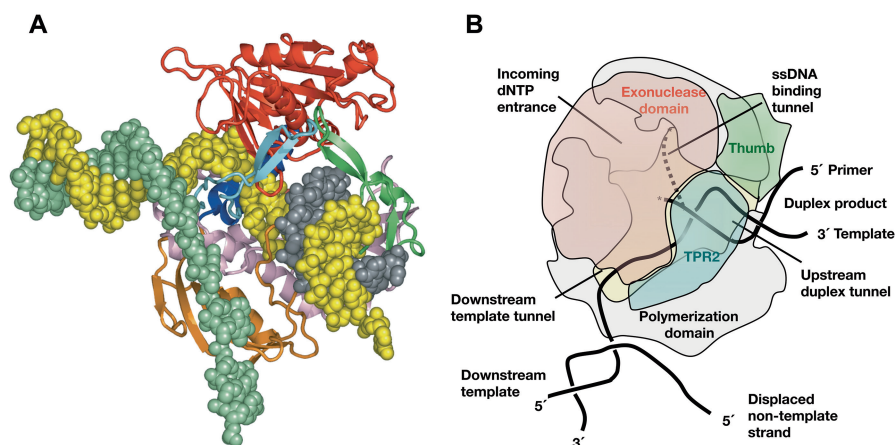
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units from the 3' end of a DNA strand (Blanco and Salas 1985), degrading preferentially a mismatched primer-terminus, and further enhancing replication fidelity by 10<sup>2</sup>-fold (Esteban et al. 1994; Garmendia et al. 1992), as it occurs in most DNA replicases.

During  $\phi$ 29 DNA replication, this 66-kDa monomeric enzyme catalyzes both the initiation and processive elongation of the complementary strand from a single binding event (reviewed by Blanco and Salas 1996). Unlike most replicative DNA polymerases whose processive DNA synthesis relies on the interaction with proteins that clamp the enzyme to DNA (for a review, see Kornberg and Baker 1992),  $\phi$ 29 DNA polymerase possesses an inherent remarkably high processivity that enables the enzyme to account for both viral replication and oligonucleotide-primed DNA replication reactions without any accessory factors. Indeed,  $\phi$ 29 DNA polymerase is capable of synthesizing >70 kb of a new DNA strand per single template-binding event before dissociating from a DNA template (Blanco et al. 1989). In addition,  $\phi$ 29 DNA polymerase is provided with strong strand displacement capacity that allows the enzyme to perform processive DNA replication through dsDNA regions in the absence of auxiliary helicase-like proteins (Blanco et al. 1989).

Crystallographic and biochemical studies shed light on the molecular mechanism by which  $\phi$ 29 DNA polymerase is distinctive in coupling processive and faithful polymerization to strand displacement (Kamtekar et al. 2004; Rodríguez et al. 2005). These structural studies showed  $\phi$ 29 DNA polymerase to be formed by an N-terminal exonuclease domain, containing the 3'–5' exonuclease active site, and a C-terminal polymerization domain. The latter is subdivided into the universally conserved subdomains: the palm (containing the catalytic and DNA ligand residues), the fingers (containing the dNTP ligands), and the thumb (which confers stability to the primer) (Kamtekar et al. 2004). The main difference with other family B DNA polymerases is that  $\phi$ 29 DNA polymerase contains in the polymerization domain two additional subdomains called Terminal Protein Region-1 (TPR1) and Terminal Protein Region-2 (TPR2) specifically conserved in the subgroup of DNA polymerases that initiate DNA replication using a protein as a primer (Blasco et al. 1990; Dufour et al. 2000). TPR1 lies at the edge of the palm, while TPR2, that consists of a long  $\beta$ -turn- $\beta$  conformation faces the apex of the thumb subdomain. Palm, thumb, TPR1, and TPR2, subdomains form a closed doughnut-shaped structure that encircles the upstream duplex product in a manner reminiscent of sliding clamps, endowing  $\phi$ 29 DNA polymerase with high processivity (Berman et al. 2007; Kamtekar et al. 2004; Rodríguez et al. 2005). In addition, TPR2, palm, and fingers subdomains, together with the exonuclease domain, form a narrow tunnel around the downstream template strand that forces the melting of the duplex ahead of the polymerase (strand displacement) to gain access to the polymerase active site, using the same topological mechanism as DNA helicases (see Fig. 2.1a).

Therefore, the unique fine-tuned structure of  $\phi$ 29 DNA polymerase allows the efficient coupling of processive DNA polymerization to strand displacement in a faithful way thanks to the high dNMP insertion discrimination (Esteban et al. 1993)



**Fig. 2.1** (a) Modeling processivity and strand displacement in  $\phi$ 29 DNA polymerase. The TPR2 insertion would contribute to a full encirclement of the DNA substrate, conferring a remarkable processivity, and also acts as a structural barrier, which would force the DNA strands of the parental DNA to diverge (melt). As  $\phi$ 29 DNA polymerase translocates after each polymerization cycle, the TPR2 subdomain would act as a wedge to couple polymerization to strand displacement.  $\phi$ 29 DNA polymerase subdomains are colored as follows: 3'-5' exonuclease domain in red, fingers in blue, palm in pink, thumb in green, TPR-1 in orange, and TPR2 in cyan. Modeled DNA is colored as follows: growing primer strand in grey, template strand in yellow, and displaced strand in green. Figure reproduced with permission from Rodríguez, I., Lázaro, J.M., Blanco, L., Kamtekar, S., Berman, A.J., Wang, J., Steitz, T.A., Salas, M., and de Vega, M. (2005). A specific subdomain in  $\phi$ 29 DNA polymerase confers both processivity and strand displacement capacity. *Proc Natl Acad Sci USA* 102, 6407–6412. Copyright ©2008 National Academy of Sciences, U.S.A. (b) Schematic representation of  $\phi$ 29 DNA polymerase with DNA. The upper level contains the exonucleolytic domain, TPR2 subdomain, and thumb subdomain (colored in pale red, cyan, and green, respectively). The rest of the protein is colored in grey. Asterisks mark the polymerization and exonuclease active site positions. Figure adapted with permission from Kamtekar, S., Berman, A.J., Wang, J., Lázaro, J.M., de Vega, M., Blanco, L., Salas, M., and Steitz, T.A. (2004). Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage  $\phi$ 29. *Molecular cell* 16, 609–618. Copyright ©2004 by Cell Press

and a strong intramolecular proofreading activity exhibited by the enzyme (de Vega et al. 1999; Garmendia et al. 1992). The use of single-molecule manipulation methods to study the dynamics of the partitioning mechanism in  $\phi$ 29 DNA polymerase has given the clues about how primer-terminus switches from polymerization to the exonuclease active site during the intramolecular proofreading of the polymerization errors. Briefly, the introduction of an erroneous nucleotide provokes structural distortions in the DNA helix that led first to a dissociation of the primer and template strands from the polymerization active site followed by a four to five nucleotides melting of the primer-terminus that allows its allocation at the exonuclease active site (Ibarra et al. 2009) (see also Fig. 2.1b).

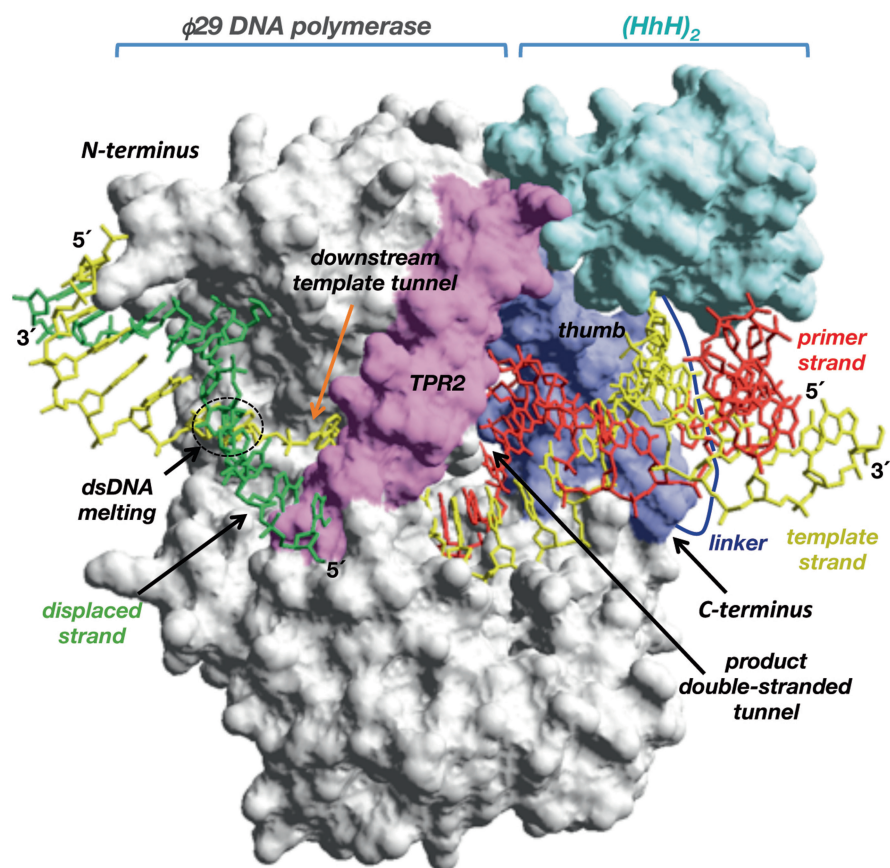
The abovementioned properties led to envisage  $\phi$ 29 DNA polymerase as an ideal tool to achieve strand displacement amplification, overcoming two frequent limitations in the amplification procedures, fidelity of synthesis and length of the amplified

products. In a simple setup, the ability of  $\phi 29$  DNA polymerase to use primed circular ssDNA as template allows rolling circle DNA amplification (RCA), producing very long single-stranded concatemeric DNA molecules containing more than ten copies of the original circular template (Blanco et al. 1989). In perhaps one of the most efficient procedures for isothermal dsDNA amplification, developed by Amersham Biosciences/Molecular Staging (Dean et al. 2001, 2002),  $\phi 29$  DNA polymerase is combined with random hexamer primers to achieve isothermal and faithful  $10^4$  to  $10^6$ -fold amplification via strand displacement of picograms of circular plasmids [Templiphi™ (www.gehealthcare.com)] and of 10 ng of genomic DNA [Genomiphi™ (www.gehealthcare.com) and Repli-G® (www1.qiagen.com)], yielding high quality amplification products that can be either digested or sequenced directly without further purification steps.

The use of random hexamer primers in those methodologies makes unnecessary any previous knowledge of the sequence. Therefore, any DNA is susceptible to be amplified by  $\phi 29$  DNA polymerase, that renders large DNAs of hundreds of kbs. In addition, Multiply primed Rolling Circle Amplification (MRCA) has been shown to be the most powerful technology to amplify circular templates of variable size required for genome sequencing (Dean et al. 2001), detection of circular viral genomes, genotyping of single nucleotide polymorphisms (Qi et al. 2001), whole genome analysis of non-cultivable viruses (Johne et al. 2009), detection and identification of circular plasmids in zoonotic pathogens (Xu et al. 2008), and for the description of new metagenomes (López-Bueno et al. 2009). The combination of plasmid amplification with subsequent direct sequencing represents a simple, fast, and low-cost method to get results comparable to those obtained with the use of other methods (Maruyama et al. 2009).

## 2 Design of $\phi 29$ DNA Polymerase Fusions

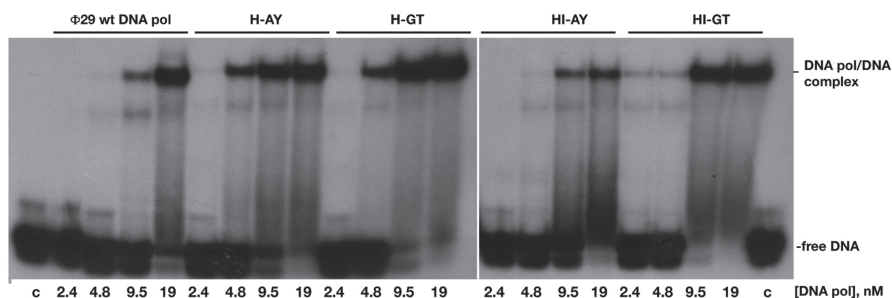
Notwithstanding the robustness of  $\phi 29$  DNA polymerase, it would be desirable to further improve its amplification efficiency. Our goal was to render the enzyme with a better DNA binding. To this end, and based in the previous studies carried out with *Taq* and *Pfu* DNA polymerases (Pavlov et al. 2002, 2004), we fused the *Methanopyrus kandleri* Topo V (HhH)<sub>2</sub> domain H (residues 696–751) or H and I [residues 696–802; (28–31)] to the C-terminus of  $\phi 29$  DNA polymerase just at the exit of the upstream dsDNA product to allow potential interaction of the (HhH)<sub>2</sub> domains with the growing dsDNA without compromising the strand displacement capacity (see in Fig. 2.2a structural model of a (HhH)<sub>2</sub> domain linked to the C-terminus of  $\phi 29$  DNA polymerase; de Vega et al. 2010). The DNA binding domains were not fused to the N-terminus of the enzyme to prevent potential impairments of coupling polymerization to the DNA unwinding ability of the enzyme, as melting of the DNA should take place near this domain. The (HhH)<sub>2</sub> domains were fused to the polymerase through the flexible linkers Gly-Thr-Gly-Ser-Gly-Ala (GTGSGA), a variant of the linker used to fuse the single-stranded DNA binding protein of bacteriophage RB69



**Fig. 2.2** Modeling of the fusion DNA polymerase. The figure represents the structural model of a (HhH)<sub>2</sub> domain (colored in cyan) joint through a linker peptide (in dark blue) to the C-terminus of  $\phi 29$  DNA polymerase (colored in grey).  $\phi 29$  DNA polymerase TPR2 insertion and thumb subdomains are colored in pink and dark blue, respectively. The modeled primer, template, and displaced strands are colored in red, yellow, and green, respectively. The upstream tunnel that encircles the newly synthesized dsDNA, the downstream template tunnel as well as the N- and C-termini of  $\phi 29$  DNA polymerase are indicated. Figure reproduced with permission from de Vega, M., Lazaro, J.M., Mencia, M., Blanco, L., and Salas, M. (2010). Improvement of  $\phi 29$  DNA polymerase amplification performance by fusion of DNA binding motifs. *Proc Natl Acad Sci USA* 107, 16506–16511

to its cognate DNA polymerase (Sun et al. 2006) or Ala-Tyr-Val-Asp-Gly-Ala (AYVDGA; Pavlov et al. 2002) to guarantee the structural and independent folding of the enzyme and the DNA binding domains. Such protein engineering yielded the fusion polymerases  $\phi 29$  H-GT and  $\phi 29$  HI-GT (originally named  $\phi 29$ -H and  $\phi 29$ -HI; de Vega et al. 2010), as well as  $\phi 29$  H-AY and  $\phi 29$  HI-AY. Here, H and I stand for Topo V domains H and I, respectively; whereas, GT and AY stand for the GTGSGA and AYVDGA linkers, respectively.





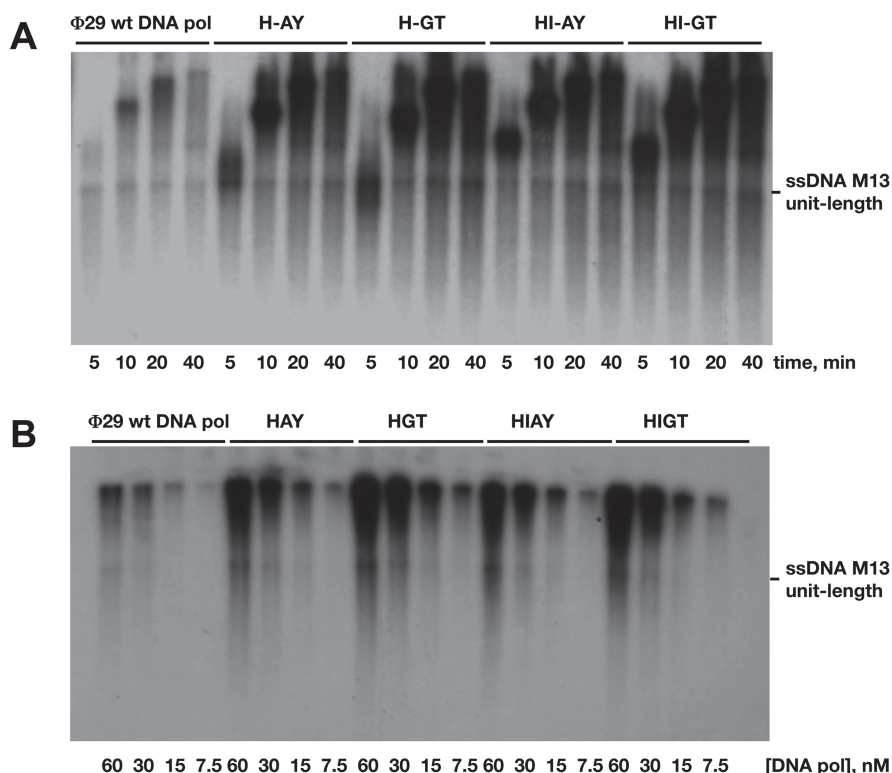
**Fig. 2.3** Gel retardation of primer/template DNA molecules by  $\phi 29$  wild-type and fusion DNA polymerases. The 5'-labeled hybrid molecule 15-mer/21-mer (dsDNA) was incubated with wild-type  $\phi 29$  DNA polymerase or with the indicated fusion DNA polymerase, under the conditions described in Sect. 7. After gel electrophoresis, the mobility of free dsDNA and the polymerase-DNA complex was detected by autoradiography

### 3 DNA Polymerase Fusions Show a Higher DNA Binding

The first question that had to be addressed was whether the fusion of the  $(\text{HhH})_2$  domains to  $\phi 29$  DNA polymerase enhanced its DNA binding capacity. To this end DNA gel retardation assays were performed, using as substrate a labeled primer/template hybrid (15-mer/21-mer) DNA molecule. As shown in Fig. 2.3, the wild-type  $\phi 29$  DNA polymerase produced a single retardation band using the labeled hybrid molecule (see Fig. 2.3) that has been interpreted as an enzyme–DNA stable complex competent for polymerization (Méndez et al. 1994), and whose intensity depends on the concentration of enzyme added. Except for DNA polymerase HI-AY, the H-GT, H-AY, and HI-GT fusion enzymes showed a higher DNA binding capacity than the wild-type enzyme. Therefore, the aim of improving the DNA binding ability of  $\phi 29$  DNA polymerase by adding the  $(\text{HhH})_2$  domains H and HI from Topo V to its C-terminal end was successfully accomplished (de Vega et al. 2010).

### 4 Fusion of the $(\text{HhH})_2$ Domains to the C-Terminal End of $\phi 29$ DNA Polymerase Significantly Improved Its Polymerization Potential

As mentioned above, the amplification potential of  $\phi 29$  DNA polymerase relies on the simultaneous binding and translocation of the primer, template, and displaced strands through different regions of the polymerization domain (see Fig. 2.1) that guarantees processive polymerization coupled to strand displacement. Fusion of DNA binding motifs to a DNA polymerase with the aim to improve its polymerization capacity required that the enhanced binding conferred by the additional protein/DNA contacts did not halt translocation of the enzyme along the DNA. One of the assays to determine whether fusion of the  $(\text{HhH})_2$  domains affected the singular properties of  $\phi 29$  DNA polymerase is rolling circle replication (RCR) using as

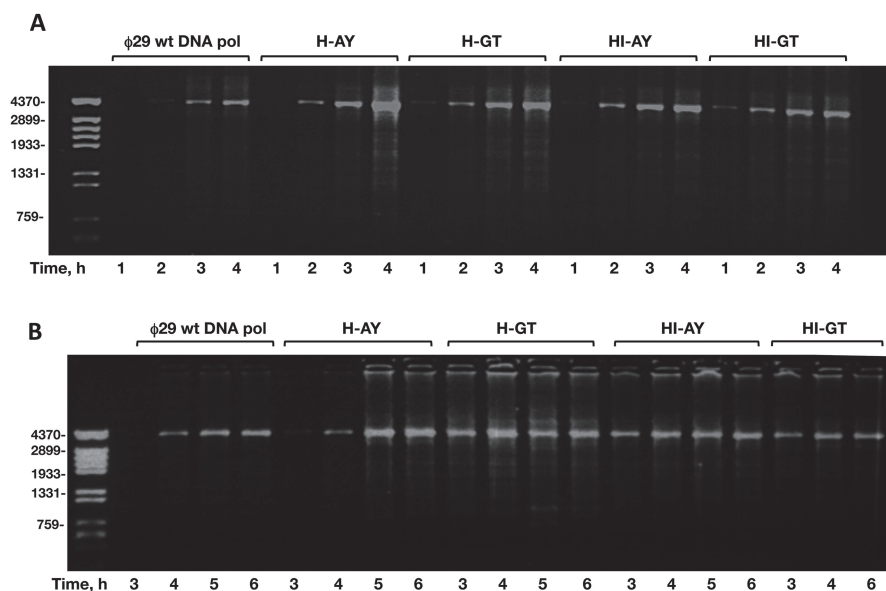


**Fig. 2.4** (a) Strand displacement-coupled M13 DNA replication by  $\phi 29$  wild-type and fusion DNA polymerases. Replication of singly primed M13 DNA was carried out as described in Sect. 7 using 30 nM of  $\phi 29$  wild-type or fusion DNA polymerases. The position of unit-length M13 DNA is shown at the right. (b) Processive synthesis by  $\phi 29$  wild-type and fusion DNA polymerases. The assay was performed as described in Sect. 7 in the presence of 4.3 nM of singly primed M13 DNA and decreasing concentrations of the indicated DNA polymerase. Lanes in part b of the figure and corresponding to the wild-type, H-GT, and HI-GT DNA polymerases are reproduced with permission from de Vega, M., Lazaro, J.M., Mencia, M., Blanco, L., and Salas, M. (2010). Improvement of  $\phi 29$  DNA polymerase amplification performance by fusion of DNA binding motifs. *Proc Natl Acad Sci USA* 107, 16506–16511

substrate singly primed circular ssDNA of bacteriophage M13, as both processivity and strand displacement can be simultaneously analyzed. As it is shown in Fig. 2.4a, whereas the replication rate displayed by the fusion DNA polymerases was similar to that of  $\phi 29$  DNA polymerase, as deduced from the length of the products synthesized, the increase in the total amount of DNA yielded by DNA polymerases H-AY, H-GT, and HI-GT with respect to the wild-type DNA polymerase (four-, five- and sevenfold, respectively) reflected a meliorated usage of the primer/template junctions, agreeing with their improved DNA binding (de Vega et al. 2010). In addition, since decreasing enzyme/DNA ratios did not alter the length of the elongation products made by the four DNA polymerase variants, it could be concluded that addition of the  $(\text{HhH})_2$  motifs to the DNA polymerase did not affect the intrinsic processivity of the enzyme (Fig. 2.4b) (de Vega et al. 2010).

## 5 DNA Polymerase Fusions Show an Increased Multiply Primed Rolling Circle Amplification Efficiency

As noted above, both the high processivity and the strand displacement capacities of  $\phi$ 29 DNA polymerase were the basis for the development of one of the most efficient procedures for isothermal dsDNA amplification of circular plasmids, named MRCA. In this method,  $\phi$ 29 DNA polymerase combined with random hexamer primers yields isothermal and faithful  $10^6$ -fold amplification of picograms of circular plasmids via a strand displacement mechanism (Dean et al. 2001, 2002). Because of the large reduction ( $\sim 10^5$ -fold) in the input of DNA molecules used in MRCA assays compared to RCR assays described above, an increase in the efficiency of RCA by the fusion DNA polymerases could not be readily anticipated. In this sense, our previous results with a  $\phi$ 29 DNA polymerase containing a fused (His)<sub>6</sub> tag at its C-terminus showed that despite of its efficiency during RCR, it was unable to yield detectable amplification products during MRCA (J.M. Lázaro, M. de Vega, and M. Salas, unpublished results). Therefore, efficiency of  $\phi$ 29 DNA polymerase fusions was tested in MRCA assays with 1 pg (Fig. 2.5a) and 10 fg (Fig. 2.5b) of a 4.2-kb-long dsDNA circular plasmid and 3'-protected random hexamer primers.



**Fig. 2.5** Multiply primed Rolling Circle Amplification of plasmidic DNA by  $\phi$ 29 wild-type and fusion DNA polymerases. The assay was performed as described in Sect. 7, in the presence of 1 pg (**a**) or 10 fg (**b**) of plasmidic DNA as input and 50 nM of  $\phi$ 29 DNA polymerase or the indicated fusion DNA polymerase. At the left, linear DNA fragments obtained after digesting  $\phi$ 29 DNA with *Hind*III, used as DNA length markers. Lanes in part **a** of the figure and corresponding to the wild-type, H-GT, and HI-GT DNA polymerases are reproduced with permission from de Vega, M., Lázaro, J.M., Mencia, M., Blanco, L., and Salas, M. (2010). Improvement of  $\phi$ 29 DNA polymerase amplification performance by fusion of DNA binding motifs. *Proc Natl Acad Sci USA* 107, 16506–16511



As shown in Fig. 2.5a, whereas  $\phi$ 29 DNA polymerase required 3 h to give a detectable product of amplification reaction of 1  $\mu$ g of plasmid, fusion DNA polymerases rendered a perceptible amplification product from 1 to 2 h, with the total yield of amplified products at the end of the amplification reaction time being three to fourfold higher than that obtained with the wild-type enzyme (de Vega et al. 2010). With all the five DNA polymerases (four designed fusions and the wild-type enzyme), more than 80 % of the amplified DNA was linearized after digestion with the endonuclease *Eco*RI rendering the corresponding 4.2-kb dsDNA fragment and indicating that the amplification products were tandem repeats of the original plasmid. In the presence of 10  $\mu$ g of input plasmid, DNA polymerases H-GT, HI-AY, and HI-GT produced detectable amplification products 1 h before the wild-type  $\phi$ 29 DNA polymerase, the final amount of the amplicons being at least twice as high (see Fig. 2.5b). Analysis of the sequence of 4918 nonoverlapping nucleotides from the amplicons showed that the fusion DNA polymerases performed faithful DNA amplification as no polymerization errors were detected, with the exception of the HI-AY fusion protein that gave rise to a G to T transversion (de Vega et al. 2010; Salas et al. 2013).

## 6 Concluding Remarks

Altogether, these results indicate that (1) the (HhH)<sub>2</sub> domains do not interfere with the proper placement of the primer/template junction at the polymerization active site of the designed fusions, and also do not impede their strand displacement ability, (2) the enhancement in DNA binding described above does not halt translocation of the engineered enzymes along DNA, therefore not affecting the processivity of the enzyme, (3) as a result, the presence of HhH domains at the  $\phi$ 29 DNA polymerase C-terminus in the fusion proteins provides their superior RCR/MRCA performance that could be mainly explained by their enhanced interaction with DNA, most likely affecting recognition/stabilization of the primer/template junction (de Vega et al. 2010). It is worth to note that the protein fusions described here endow  $\phi$ 29 DNA polymerase with a higher amplification efficiency of whole genomic DNA, too (de Vega et al. 2010).

Also note that so far the amplification efficiency of new fusion proteins has been tested only with large chunks of DNA. However, mostly short circular DNAs serve as probes in the RCA-based diagnostics and/or as templates in the RCA-based therapeutics. And though it would be reasonable to assume the superior amplification of smaller circular probes by the designed fusions, this should be directly proved in future studies.

## 7 Materials and Methods

### 7.1 Generation of $\phi$ 29 DNA Polymerase Fusions

To make the fusion proteins  $\phi$ 29 HI-AY and HI-GT, a DNA fragment containing the residues that code for the (HhH)<sub>2</sub> domains H (56 amino acids) and I (51 amino acids) of the Topoisomerase V from *M. Kandleri* (GenBank code AF311944 and

Pavlov et al. 2002) was synthesized by the GenScript Corporation and cloned between the *EcoRV* sites of commercial vector pUC57 as described (de Vega et al. 2010; Salas et al. 2013). The resulting plasmid pUC57-HhH was used as template for amplifying a DNA fragment encoding the domains H and I by PCR. Therefore, the primer 1 5'-GGCGGGATCCTTAATGATGATGATGATGATGGCC, together with the primers 2 5'-GCGTATGATGTGGGCGCCGG or primer 3 5'-GGCAC CGGCTCTGGCGCCTGGAAAGAATGGCTGGAACG, gave the DNA I and II fragments of 369 bp, respectively. In addition to a *KasI* site introduced by both primers, primer 2 also introduced the sequence encoding the AYDVGA connector (Pavlov et al. 2002), whereas the primer 3 introduced the nucleotide sequence encoding the GTGSGA connector previously described (Sun et al. 2006). The primer 1 contained the sequence encoding the six histidine residues followed by a stop codon and a *BamHI* site.

At the same time, a pJLW2 plasmid derivative (Lázaro et al. 1995) containing the gene encoding the  $\phi$ 29 DNA polymerase (572 amino acids) was used as a template for a PCR reaction carried out with the primer 4 (5'-CCGTCTCCGGG AGCTGCATGTG) including a 5' *HindIII* site and the primers 5 (5'-GGCGCCCA CATCATACGCTTTGATTGTGAATGTGTCATCAACC) or 6 (5'-GGCGCCAGA GCCGGTGCCTTTGATTGTGAATGTGTCATCAACC), to obtain the fragments III and IV of 1757 bp, respectively. The fragments III and IV will therefore contain the DNA encoding the  $\phi$ 29 DNA polymerase followed by the peptide AYDVGA (fragment III) and GTGSGA (fragment IV) sequences which also include a *KasI* site. The fragments I–IV were purified in 0.7 % agarose gels and were then digested with *KasI*. The digested DNA fragments I and III, and II and IV were ligated with the T4 DNA ligase to obtain a linear DNA of 2108 bp encoding the fusion protein HI-AY (Fragment V) and HI-GT (Fragment VI), respectively. The ligated products were purified in 0.7 % agarose gels and were then digested with the *BamHI* and *HindIII* endonucleases. The digested products were purified by electrophoresis in agarose gels. The fragments V and VI were cloned in the vector pT7-4 (Tabor and Richardson 1985). The fusion proteins HI-AY and HI-GT were used as the template to construct the fusion polymerases H-GT and H-AY, respectively, by inserting a stop codon after the H fragment of TopoV by means of the QuikChange® (Stratagene) directed mutagenesis kit. The confirmation of the DNA sequence and the absence of additional mutations was carried out by means of sequencing the entire gene. The fusion DNA polymerases were expressed in BL21(DE3) cells of *E. coli* that housed the cloned chimeric gene in a pJLW2 plasmid derivative, and were purified essentially as described in Lázaro et al. (1995).

## 7.2 DNA Binding Capacity of the Fusion DNA Polymerases

Oligonucleotides 15-mer (5'-GATCACAGTGAGTAC) and 21-mer (5'-TCTATTGTACTCACTGTGATC), the latter with a 5'-extension of six nucleotides in addition to the sequence complementary to the 15-mer, were supplied

by Isogen. Oligonucleotide 15-mer was 5'-labeled with [ $\gamma$ - $^{32}$ ]ATP and T4 polynucleotide kinase. 5'-labeled 15-mer was hybridized to 21-mer in the presence of 0.2 M NaCl and 60 mM Tris-HCl, pH 7.5. The resulting 5'-labeled 15-mer/21-mer hybrid molecule was used as DNA primer/template to analyze the interaction with the wild-type and the indicated fusion  $\phi$ 29 DNA polymerases. The incubation mixture contained, in a final volume of 20  $\mu$ L, 12 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM ammonium sulfate, 0.1 mg/mL BSA, 1 nM of the 15-mer/21-mer molecule and the indicated concentrations of the corresponding enzyme. After incubation for 5 min at 30 °C, the samples were subjected to electrophoresis in 4% (w/v) polyacrylamide gels (80:1, monomer:bis), containing 12 mM Tris-acetate, pH 7.5, and 1 mM EDTA, and run at room temperature in the same buffer at 8 V/cm. After autoradiography, the polymerase complexed with dsDNA was detected as a mobility shift (retardation) in the migrating position of the labeled DNA.

### 7.3 Rolling Circle Replication Assay

The M13mp18 ssDNA was hybridized with the universal primer (5'-GTTTTCCCAGTCACGAC) in the presence of 0.2 M NaCl and 60 mM Tris-HCl, pH 7.5 and the resulting molecule was used as a primer/template to analyze the polymerization of processive DNA coupled to the strand displacement by the fusion DNA polymerases. The incubation mixture contained in 25  $\mu$ L, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/mL BSA, 40  $\mu$ M dCTP, dGTP, dTTP and [ $\alpha$ - $^{32}$ P]dATP (1  $\mu$ Ci), 4.3 nM of single primed M13mp18 ssDNA, and 30 nM of the wild-type or fusion  $\phi$ 29 DNA polymerase. After incubating for the indicated times at 30 °C, the reactions were stopped by the addition of 10 mM EDTA-0.1% SDS, and the samples were filtered through Sephadex G-50 columns. The synthesized DNA was calculated from the Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M NaOH and was subjected to electrophoresis in 0.7% alkaline agarose gels. After the electrophoresis, unit-length M13mp8 ssDNA was detected by staining with ethidium bromide, and the gels were then dried and autoradiographed.

### 7.4 Processive Polymerization by the Fusion DNA Polymerases

The processivity of the fusion DNA polymerases was analyzed with different proportions of enzyme/DNA. The incubation mixture contained, in 25  $\mu$ L, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/mL BSA, 40  $\mu$ M dCTP, dGTP, dTTP, and [ $\alpha$ - $^{32}$ P]dATP (1  $\mu$ Ci), 4.3 nM of single primed

M13mp18 ssDNA, and the indicated decreasing concentrations of the wild-type or fusion  $\phi$ 29 DNA polymerases. After incubating for 20 min at 30 °C, the reactions were stopped by the addition of 10 mM EDTA-0.1 % SDS, and the samples were filtered through Sephadex G-50 columns. For size analysis, the labeled DNA was denatured by treatment with 0.7 M NaOH and was subjected to electrophoresis in 0.7 % alkaline agarose gels. The processivity of the polymerization was evaluated by analyzing the length of the replication products with decreasing proportions of DNA polymerase/DNA.

### 7.5 *Multiply Primed RCA*

The incubation mixture contained, in 12.5  $\mu$ L, 37 mM Tris-HCl, pH 7.5, 0.025 % Tween-20, 50 mM KCl, 45 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM  $\text{MgCl}_2$ , 50  $\mu$ M of 3'-protected random hexamers, 500  $\mu$ M dNTPs, and the indicated amount of plasmid DNA (4.2 kbp) as input. To denature DNA, samples were incubated for 3 min at 95 °C and afterwards chilled on ice for 5 min. Reactions were started by adding 50 nM of  $\phi$ 29 DNA polymerase or the corresponding fusion DNA polymerase. After incubation at 30 °C for the indicated times, reactions were stopped by incubating the samples for 10 min at 65 °C. One microliter of each reaction was digested with *Eco*RI and further analyzed by electrophoresis in 0.7 % agarose gels. After electrophoresis, the amplified DNA was detected by ethidium bromide staining.

### 7.6 *Measurement of the Accuracy of the Fusion DNA Polymerases*

The protocol used is as described (de Vega et al. 2010; Salas et al. 2013). Thus, samples from the experiment corresponding to multiply primed RCA of plasmidic DNA by the wild-type and fusión  $\phi$ 29 DNA polymerases were taken (3  $\mu$ L) and mixed with 17  $\mu$ L of restriction mix (2  $\mu$ L New England Biolabs (NEB) 10 $\times$  *Eco*RI Buffer, 0.5  $\mu$ L NEB *Eco*RI enzyme [10 units], and 14.5  $\mu$ L  $\text{H}_2\text{O}$ ) to get linear monomers of amplified plasmid. After incubation for 1 h at 37 °C, DNA was purified through Qiagen Gel-Extraction Kit Columns and eluted in 30  $\mu$ L of buffer 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. Ten microliters of each of the eluted samples were religated by mixing with 2  $\mu$ L NBE 10 $\times$  Ligase Buffer, 8  $\mu$ L  $\text{H}_2\text{O}$ , and 0.5  $\mu$ L NEB Ligase (200 units). After overnight incubation at 16 °C, 2  $\mu$ L of each of the ligation reactions were transformed onto chemically competent *E. coli* cells XL-1 Blue. About 1000 transformants were obtained using each of the amplified samples, whereas no transformant was obtained from control samples that contained the initial amount of plasmid pT7-4 and underwent the same procedure as the amplified samples. Two clones from each transformation were selected and the plasmid was prepared and sequenced according to standard procedures. In total, 4918 nonoverlapping nucleotides were sequenced for each polymerase.

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