Three amino acid residues highly conserved in most proofreading DNA polymerases, a phenylalanine contained in the Exo II motif and a serine and a leucine belonging to the S/T/Lx, h motif, were recently shown to be critical for 3′-5′ exonucleolytic activity by acting as single-stranded DNA ligands (de Vega, M., Lázaro, J.M., Salas, M. and Blanco, L. (1998) J. Mol. Biol. 279, 807–822). In this paper, site-directed mutants at these three residues were used to analyze their functional importance for the synthetic activities of ø29 DNA polymerase, an enzyme able to start linear ø29 DNA replication using a terminal protein (TP) as primer. Mutations introduced at Phe65, Ser122, and Leu123 residues of ø29 DNA polymerase severely affected the replication capacity of the enzyme. Three mutants, F65S, S122T, and S122N, were strongly affected in their capacity to interact with a DNA primer/template structure, suggesting a dual role during both polymerization and proofreading. Interestingly, mutant S122N was not able to maintain a stable interaction with the TP primer, thus impeding the first steps (initiation and transition) of ø29 DNA replication. The involvement of Ser122 in the consecutive binding of TP and DNA is compatible with the finding that the TP/DNA polymerase heterodimer was not able to use a DNA primer/template structure. Assuming a structural conservation among the eukaryotic-type DNA polymerases, a model for the interactions of ø29 DNA polymerase with both TP and DNA primers is presented.

The linear dsDNA1 of Bacillus subtilis phage ø29 starts replication by a protein-priming mechanism (reviewed in Refs. 1 and 2) in which the viral DNA polymerase catalyzes the addition of dAMP to the hydroxyl group of Ser232 of a free TP molecule. This event occurs opposite the second 3′ nucleotide of the template, and then the initiation product (TP-dAMP) generated slides back one position to be paired to the terminal nucleotide (3). After sliding back, the same DNA polymerase molecule catalyzes the synthesis of a short elongation product (9 nucleotides) while it is still bound to the TP (transition step) (4). The incorporation of the next nucleotide forces dissociation of the TP/DNA polymerase heterodimer, starting normal DNA elongation initially coupled to strand displacement. Strand displacement synthesis proceeds from both DNA ends until the two replication forks meet. Then, the two partially replicated parental strands separate, and full-length DNA synthesis (19,285 base pairs in length) is completed.

ø29 DNA polymerase is one of the smallest replicases known (66 kDa) containing well characterized enzymatic activities (reviewed in Ref. 5). It has been included in the group of eukaryotic-type DNA polymerases based on its sensitivity to the nucleotide analogs 2-(p-n-butylnilino)dATP and N2-(p-n-butylnaphthylidGTP (6, 7), which are specific inhibitors of the eukaryotic DNA polymerase α (8), and on the presence of several regions of amino acid sequence similarity along its C-terminal portion (7, 9–12) that are responsible for 5′-3′ DNA polymerization and TP-primed initiation (11, 13–17) activities.

On the other hand, three conserved amino acid motifs, named Exo I, II, and III, have been identified in the N-terminal portion of all prokaryotic and eukaryotic DNA-dependent DNA polymerases with a proofreading activity (11, 18, 19) that invariably contain the five critical residues that were identified in Pol Iκ acting as metal ligands and responsible for the 3′-5′ exonuclease catalysis (20, 21). The proposal of an evolutionarily conserved 3′-5′ exonuclease active site (18) has been confirmed by (1) site-directed mutagenesis studies in some DNA polymerases (reviewed in Ref. 22) belonging to family A (Pol I-like) and family B (eukaryotic-type): Pol Iκ (21, 23), T7 DNA polymerase (24), ø29 DNA polymerase (Refs. 18, 25, and 26; reviewed in Ref. 5), T4 DNA polymerase (27–30), herpes simplex virus DNA polymerase type I (31–35), E. coli DNA polymerase II (36, 37), PRD1 DNA polymerase (38), Thermus aquaticus (39, 40), and cellular DNA polymerases δ (41), ε (19), and γ (42, 43) from Saccharomyces cerevisiae, and family C (DNA polymerase III), consisting of ε subunit of E.coli DNA polymerase III (44) and B. subtilis DNA polymerase III (45, 46); and (2) the recent resolution of the crystal structure of a N-terminal fragment of T4 DNA polymerase (47) and that of phage RB69 DNA polymerase (48). Both structures served to identify a lysine as an additional active site residue (30, 47, 48). The functional importance of this residue was supported by site-directed mutagenesis analysis in ø29 DNA polymerase (49) together with its invariant presence in the eukaryotic DNA polymerase family (48, 49). In addition to the residues involved in metal binding and catalysis at the 3′-5′ exonuclease active site, other residues are structurally and functionally conserved at the exonuclease domain of most proofreading DNA polymerases. Among them, ø29 DNA polymerase residues Thr15 and Asn75 of the Exo I and II motifs, respectively, were shown...
to act as ssDNA ligands, having a critical role in the stabilization of the primer terminus at the 3′-5′ exonuclease active site (50). Another highly conserved residue at the Exo II motif (Phe<sup>65</sup> in ø29 DNA polymerase) and two residues (Ser<sup>122</sup> and Leu<sup>123</sup>) belonging to the newly identified S/T/Lx,x motif were shown to act also as ssDNA ligands for 3′-5′ exonuclease (51), in agreement with the crystallographic data from T4 (47) and RB69 (48) DNA polymerases complexed with ssDNA.

To date, in the case of ø29 DNA polymerase, none of the mutations carried out at both metal and ssDNA ligands affected the synthetic activities of the enzyme (protein-primed initiation and DNA polymerization). However, as described in this paper, a detailed biochemical characterization of various mutants at the ssDNA ligands Phe<sup>65</sup>, Ser<sup>122</sup>, and Leu<sup>123</sup> of ø29 DNA polymerase demonstrated the involvement of these residues also in primer (both DNA and TP) binding during the synthetic reactions. Based on these data, and assuming conservation of the overall structure among eukaryotic-type DNA polymerases, a three-dimensional structure prediction for the ø29 DNA polymerase/TP interaction, modeled on the crystal structure of RB69 DNA polymerase (48), is proposed.

### MATERIALS AND METHODS

**Nucleotides and Proteins**—Unlabeled nucleotides were purchased from Amersham Pharmacia Biotech. [α-<sup>32</sup>P]-dATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Restriction endonucleases were from New England Biolabs. T4 polynucleotide kinase was from Boehringer Mannheim. ø29 TP was purified as described (52). The wild-type ø29 DNA polymerase was purified from <i>E. coli</i> NP2690 cells harboring plasmid pJLw2 as described (53). ø29 DNA polymerase site-directed mutants F65Y, F65S, S122T, S122N, L123T, and L123A were purified as described (51). ø29 dsDNA binding protein and ø29 ssDNA binding protein, obtained from ø29-infected B. subtilis cells, were purified as described (54, 55).

**DNA Templates and Substrates**—Oligonucleotides 15-mer (5′-GATCAGCTGAGTAC) and 21-mer (5′-TCTATTGTACACTACTGATGC), which has a 5′-extension of six nucleotides in addition to the sequence complementary to the 15-mer, were supplied by Isgen. Oligonucleotide 15-mer was 5′-labeled with [γ-<sup>32</sup>P]-ATP and T4 polynucleotide kinase. 5′-Labeled 15-mer was hybridized to 21-mer in the presence of 0.2 mM NaCl and 0.1 mM Tris-HCl, pH 7.5, 0.4 M NaCl, and 2 μg of DNA polymerase K treatment of phage particles in the presence of SDS (56), phenol extraction, and ethanol precipitation. M13mp18 ssDNA was hybridized to the universal primer as described above, and the resulting molecule was used as a primer/template to analyze processive DNA polymerization coupled to strand displacement by ø29 DNA polymerase. Terminal protein-containing ø29 DNA (ø29 TP- DNA) was obtained as described (55).

**Replication Assay (Protein-primed Initiation plus Elongation) with ø29 TP-DNA as Template**—The incubation mixture contained, in 25 μl, 50 mM Tris-HCl, pH 7.5; 10 mM MgCl<sub>2</sub>; 20 mM ammonium sulfate; 1 mM dithiothreitol; 4% glycerol; 0.1 mg/ml BSA; 20 μM each dCTP, dGTP, dTTP, and [α-<sup>32</sup>P]-dATP (1 μCi); 0.5 μg of ø29 TP-DNA; 125 ng of purified TP; and 5 ng of either wild-type or mutant ø29 DNA polymerase. After incubation for 10 min at 30 °C, the reaction was stopped by adding 10 mM EDTA-0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns. Relative activity 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 Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M Cerenkov radiation corresponding to the excluded volume.

**Analysis of the Interaction between TP and DNA Polymerase Mutants**—The incubation mixture contained, in 150 μl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mg/ml BSA, 20 mM ammonium sulfate, 1 mM dithiothreitol, and 2 μg of DNA polymerase K. After incubation for 30 min at 4 °C, samples were loaded in 15–30% glycerol gradients (4 ml) in the presence of 50 mM Tris-HCl, pH 7.5, 20 mM ammonium sulfate, 0.2 mM NaCl, 1 mM EDTA, and 7 mM 2-mercaptoethanol and centrifuged at 4 °C for 24 h at 62,000 rpm in a Beckman SW65 rotor. Gradients were fractionated and subjected to SDS-PAGE in 12% polyacrylamide gels. After electrophoretical separation, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore) during 120 min at 100 mA and incubated with polyclonal antibodies against ø29 DNA polymerase and TP. Detection was carried out using the ECL Western blotting system (Amersham Pharmacia Biotech).

**DNA Gel Retardation Assay**—A 5′-labeled 15-mer/21-mer hybrid molecule was used as DNA primer/template to analyze the interaction with either wild-type or mutant ø29 DNA polymerases. The incubation mixture, in a final volume of 20 μl, contained 12 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, 20 mM ammonium sulfate, 0.18 ng of the 15-mer/21-mer molecule, and 5 ng of either wild-type or mutant ø29 DNA polymerase. To analyze the interaction of wild-type ø29 DNA polymerase/TP heterodimer with DNA primer/template and ssDNA, the heterodimer was preformed by incubating 40 ng of wild-type DNA polymerase with 20 ng of TP, in the presence of 12 mM Tris-HCl, pH 7.5, and 20 mM ammonium sulfate. After incubation for 30 min at 4 °C, either the heterodimer, 40 ng of wild-type ø29 DNA polymerase, or 20 ng of TP were incubated with either 0.18 ng of the 15-mer/21-mer or with 0.075 ng of 15-mer molecule, under the conditions described above. When ssDNA was used as substrate, MgCl<sub>2</sub> was omitted and MnCl<sub>2</sub> was used instead of MgCl<sub>2</sub>, and the incubation was maintained for 90 min at 30 °C. The reactions were stopped by addition of 0.5% SDS, 0.1% SDS, filtered through Sephadex G-50 spin columns, and the resulting molecules were loaded onto a 12% polyacrylamide gel. After electrophoretical separation, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore) during 120 min at 100 mA and incubated with polyclonal antibodies against ø29 DNA polymerase and TP. Detection was carried out using the ECL Western blotting system (Amersham Pharmacia Biotech).
DNA. Quantitation of the binding capacity of the wild-type ø29 DNA polymerase versus its mutant derivatives was carried out by densitometry of the retarded band.

**Polymerization of the Wild-type ø29 DNA Polymerase/TP Heterodimer on a Short Primer/Template Structure**—The hybrid molecule 15-mer/21-mer can be used as a substrate for DNA-dependent DNA polymerization, because it contains a 6-nucleotide-long 5'-protruding end. The incubation mixture contained, in 12.5 μl of Tris-HCl, pH 7.5; 10 mM MgCl₂; 0.18 ng of 5'-labeled 15-mer/21-mer; 400 nM each of the four dNTPs; and either 40 ng of wild-type ø29 DNA polymerase; 20 ng of TP, or 60 ng of the DNA polymerase/TP complex (preformed as indicated above). After incubation for 2 min at 4 °C (to guarantee the stabilization of the interaction between TP and DNA polymerase), the reaction was stopped by adding EDTA up to 10 mM. Samples were analyzed by 8 M urea-20% PAGE and autoradiography. Polymerization is detected as an increase in the size (15-mer) of the 5'-labeled 15-mer/molecular band.

3'-5' **Exonuclease Assay of the Wild-type ø29 DNA Polymerase/TP Heterodimer**—The incubation mixture contained, in 12.5 μl of 12 mM Tris-HCl, pH 7.5; 10 mM MgCl₂, and either 40 ng of the wild-type ø29 DNA polymerase, 20 ng of TP, or 60 ng of the DNA polymerase/TP heterodimer (preformed as indicated above). 0.075 ng of a 5'-labeled oligonucleotide (15-mer) was used as ssDNA substrate. Samples were incubated for 2 min at 4 °C (to preserve the stabilization of the TP/DNA polymerase heterodimer) and quenched by adding 3 μl of sequencing gel loading buffer. Reactions were analyzed by 8 M urea-20% PAGE and autoradiography.

**RESULTS**

The **Exo II and (S/T)Lx₂ Motifs of Protein-primed DNA Polymerases**—Fig. 1 shows a multiple alignment of two amino acid motifs, highly conserved in all DNA-dependent DNA polymerases endowed with a proofreading activity, but restricted here to the subgroup of eukaryotic-type DNA polymerases that use a protein-priming mechanism and to RB69 DNA polymerase, the crystal structure of which has been recently solved (48). The first block of amino acid similarity (originally called the Exo II motif; Ref. 18) can be defined by the consensus sequence hhxANx₂₋₃(F/Y)Dx₂Ahx. An aromatic residue (His in most cases) precedes the almost invariant Asn residue involved in ssDNA binding, both by crystallographic analysis (47, 48, 61) and site-directed mutagenesis studies (50). Similarly, the critical Asp residue of the Exo II motif is always preceded by a Phe or a Tyr residue. The second segment, defined by the consensus sequence (S/T)Lx₂Ahx (51), can be extended to hxxSLx₂Ahx among the group of TP-primed DNA polymerases. In this group, a Ser residue is present in 14 of 17 DNA polymerases, and Leu is invariably present in all the sequences reported.

By site-directed mutagenesis in ø29 DNA polymerase, we have recently shown that the Phe residue of the Exo II motif, and the Ser and Leu residues of the hxxSLx₂Ahx motif are involved in ssDNA binding during 3'-5' exonuclease (51). Here we study the importance of these three latter residues for the other specific functions intrinsic to the mechanism used by ø29 DNA polymerase to replicate the viral genome. Six ø29 DNA polymerase mutant derivatives (F65Y, F65S, S122T, S122N, L123T, and L123A), overexpressed and purified as described (51), were analyzed using a variety of in vitro assays corresponding to the different stages of the TP-primed ø29 DNA replication mechanism.

**Protein-primed TP-DNA Replication with the Mutant DNA Polymerases**—ø29 DNA replication involves TP-primed initiation at both termini, a special activity of ø29 DNA polymerase, that catalyzes the template-directed formation of a covalent complex between the viral TP and 5'-dAMP (initiation step) and the subsequent elongation (via strand displacement) of the initiation complex to produce full-length ø29 DNA (reviewed in Refs. 1 and 2).

Using a minimal replication system based on ø29 TP-DNA, ø29 DNA polymerase, and ø29 TP (62), the efficiency displayed by mutants F65S, S122T, and S122N was reduced 10-, 4-, and 4-fold, respectively, (see Table I) relative to that of the wild-type polymerase, although in all cases, elongation reached unit-length ø29 TP-DNA (Fig. 2A). Indeed, when such an assay...
The different activity assays were carried out as described under "Materials and Methods.”

| Parameter assayed                      | Substrate(s)          | ø29 DNA polymerase |
|----------------------------------------|-----------------------|-------------------|
| DNA replication                        | TP, dNTP              | wt | F65Y | F65S | S122T | S122N | L123T | L123A |
| TP-DNA amplification                   | TP, dNTP              | 100 | 76   | 10   | 26   | 26    | 60    | 85    |
| Primed M13 DNA replication             | dNTP                  | 100 | 105  | 1.2  | 9    | 3.2   | 30    | 109   |
| TP-DNA initiation                       | TP, dATP              | 100 | 74   | 25   | 55   | 45    | 83    | 83    |
| TP-DNA amplification                   | TP, dATP              | 100 | 72   | 28   | 51   | 21    | 59    | 95    |
| Enzyme/DNA binding                     | dsDNA (15-mer/21-mer) | 100 | 40   | 26   | 48   | 8     | 57    | 66    |

* Numbers indicate the percentage of activity relative to the wild-type enzyme obtained from several experiments. wt, wild-type.

b Analyzed by gel retardation assays on low ionic strength polyacrylamide gels.

**Fig. 2.** ø29 TP-DNA replication and amplification by point mutants of ø29 DNA polymerase. A, replication of ø29 TP-DNA. The assay was carried out as described under “Materials and Methods,” in the presence of 5 ng of either wild-type or mutant ø29 DNA polymerase. After incubation for 10 min at 30 °C, relative activity values were calculated (see Table I), and the length of the synthesized DNA was analyzed by alkaline agarose gel electrophoresis. The migration position of unit-length ø29 DNA is indicated. B, amplification of ø29 TP-DNA. The assay was carried out as described under “Materials and Methods,” in the presence of 5 ng of ø29 TP-DNA, 5 ng of either wild-type or mutant ø29 DNA polymerases, 5 ng of TP, and 10 μg of each ø29 dsDNA binding protein and ø29 ssDNA binding protein. After 90 min of incubation at 30 °C, the reaction was stopped with 10 mM EDTA. The relative activity values were calculated (see Table I), and the length of the synthesized DNA was analyzed by alkaline agarose gel electrophoresis.

ø29 TP-DNA amplification products were detected with these three mutant ø29 DNA polymerases (Fig. 2B; see Table I).

**Strand Displacement Capacity of Mutant ø29 DNA Polymerases**—As mentioned above, ø29 DNA polymerase has to couple processive DNA synthesis to strand displacement to efficiently replicate ø29 TP-DNA. In order to analyze whether the defects in replicating ø29 TP-DNA that were exhibited by ø29 DNA polymerase mutants F65S, S122T, and S122N were specifically due to a defective strand displacement capacity, we performed a primed-M13 replication assay in which ø29 DNA polymerase starts polymerization from the 3'-OH group of a DNA oligonucleotide. The first replication round does not require strand displacement, but once it is completed, the polymerase encounters the 5'-end of the primer; the next rounds of replication (rolling circle-type) require, after this point, an active strand displacement. As shown in Fig. 3, the size of the replication products obtained with the mutant ø29 DNA polymerases (severalfold the length of the M13 DNA template) reveals that they are not specifically affected in strand displacement synthesis. However, the global efficiency displayed by mutants F65S, S122N, and S122T was reduced 4-, 2.2- and 1.7-fold, respectively, with respect to that of the wild-type enzyme (see Table I). Such a defect could be pointing to a more general defect in the interaction with DNA (see below).

**TP-dAMP Formation (Initiation of ø29 DNA Replication)**—To start ø29 DNA replication, the DNA polymerase has to form an heterodimer with a free TP molecule to catalyze the template-directed insertion of dAMP onto the hydroxyl group of Ser232 of the TP, i.e. the initiation reaction. Surprisingly, mutants F65S and S122N displayed a significantly reduced (3- and 4-fold, respectively) TP-primed initiation capacity (Fig. 4 and Table I). Because the initiation of ø29 DNA replication is a template-directed event (3), the initiation defect could be the consequence of a weak affinity for the template DNA. This possibility could be tested because ø29 DNA polymerase can catalyze the deoxynucleotidylation of TP in the absence of template (63). Under these conditions, the activity of the mutant derivatives of ø29 DNA polymerase was similar to or even lower than (in the case of mutant S122N) that corresponding to a templateless TP-dAMP formation (Fig. 4 and Table I). Thus, the low initiation capacity displayed by mutants points to a more specific defect, i.e. the interaction with the primer protein.

The ability of mutants F65S and S122N to interact with the TP was tested by using an interference assay (see under “Materials and Methods”) in which wild-type and mutant polymerases compete for TP. As a control, ø29 DNA polymerase mutant D249E, which is catalytically inactive but has an intact capacity to interact with TP, was used (15). As expected (shown in Fig. 5), the inhibition profile obtained with this mutant paralleled the theoretical one. Contrarily, the wild-type enzyme was poorly competed by F65S and S122N mutant polymerases, probably reflecting a defective interaction with TP, and only mutant F65S was able to interfere the wild-type activity,
but only using a high ratio of mutant versus wild-type enzyme (see Fig. 5).

To confirm these results, the interaction of these mutant DNA polymerases with TP was directly analyzed by ultracentrifugation, as described under “Materials and Methods.” Western blot analysis was used to identify the peaks corresponding to a TP/DNA polymerase heterodimer (97 kDa) and the free monomers of TP (31 kDa) and DNA polymerase (66 kDa). As can be observed in Fig. 6, whereas the wild-type polymerase formed an heterodimer with TP, DNA polymerase mutant S122N and TP eluted separately as monomers (Fig. 6B), indicating a TP binding defect. Under these assay conditions, mutant F65S was able to form a complex with TP (not shown), suggesting that the interaction defect is not as drastic as that displayed by mutant S122N.

Affinity of Mutant ø29 DNA Polymerases for Primer/Template Structures—The affinity for primer/template DNA molecules of wild-type and ø29 DNA polymerase mutants at residues Phe65, Ser122, and Leu123 was directly studied using gel retardation assays, as described under “Materials and Methods.” Under these conditions, the wild-type ø29 DNA polymerase produces a single retardation band using a labeled hybrid 15-mer/21-mer molecule (see Fig. 7) that has been interpreted as an enzyme-DNA complex competent for polymerization (16).

As also shown in Fig. 7, ø29 DNA polymerase mutant L123A had an affinity similar to that of the wild-type for primer/template structures; mutants F65Y and L123T showed a reduced binding efficiency; and mutants F65S, S122T, and
S122N were unable to produce any DNA retardation (see Table I). Therefore, although Phe65, Ser122, and Leu123 residues have been described as ssDNA ligands, stabilizing and orienting the primer terminus during 3'→5' exonucleolysis (51), it is apparent that mutations introduced at such positions also affect the stabilization of the DNA during polymerization.

The defective interaction displayed by mutants F65S and S122N with both TP and DNA primers could indicate that they occupy a common region of the ø29 DNA polymerase. To address this question, we analyzed whether the binding of TP is compatible with the binding of a primer terminus by the same DNA polymerase molecule. As shown by gel retardation assays (Fig. 8A, left panel), the capacity of the DNA polymerase and the TP to bind DNA is lost after formation of an heterodimer. In addition, the intrinsic DNA binding capacity of TP (52) is reduced after the formation of the heterodimer with ø29 DNA polymerase. In this assay, a significant portion of the labeled oligonucleotide remained single-stranded (see the legend to Fig. 8A). Because metal ions are included in this assay, the observed variation in the amount of this material is due to 3'→5' exonucleolysis by ø29 DNA polymerase. In agreement with these data, formation of a TP/DNA polymerase heterodimer prevented both exonucleolysis and polymerization on a DNA primer strand (Fig. 8A, right panel), even upon addition of a
FIG. 8. A, binding of TP to the DNA polymerase prevents binding and polymerization on DNA primer/template structures. The assay was carried out as described under "Materials and Methods," using 5'-labeled 15-mer/21-mer synthetic hybrid, in the presence of either 40 ng of wild-type ø29 DNA polymerase, 20 ng of TP, or 60 ng of TP/DNA polymerase complex. Samples were analyzed by gel electrophoresis in the conditions described by Méndez et al. (16). The bands corresponding to the free dsDNA and to the dsDNA complexed with either DNA polymerase or TP are indicated. The fastest migrating band correspond to a portion of nonhybridized labeled ssDNA. The right panel shows the inhibition of the polymerization capacity of ø29 DNA polymerase by addition of TP. The assay was carried out as described under "Materials and Methods," using the same conditions described above, incubating for 2 min at 4 °C in the presence of 400 nM each of the four dNTPs. Samples were analyzed by 8 M urea-20% polyacrylamide gel electrophoresis and autoradiography. Polymerization is detected as an increase in the size (15-mer) of the 5'-labeled primer. B, binding of TP to the DNA polymerase prevents binding and 3'-5' exonuclease on ssDNA. The binding assay (left panel) was carried out under the same conditions described in A but using 5'-labeled 15-mer as ssDNA substrate. The bands corresponding to free ssDNA and to the DNA polymerase/ssDNA complex are indicated. 3'-5' exonuclease of ssDNA (right panel) was carried out as described under "Materials and Methods," by adding Mg2+ ions and incubating for 2 min at 4 °C. Samples were analyzed by 8 M urea-20% polyacrylamide gel electrophoresis and autoradiography. The position of different degradation intermediates of the 15-mer substrate is indicated. Abbreviations used are as follows: DNA pol, wild-type ø29 DNA polymerase; DNA pol/TP, wild-type ø29 DNA polymerase/TP heterodimer.

high concentration of dNTPs (400 nM). These results support our hypothesis of a unique primer binding site to be non-simultaneously occupied by TP or a DNA primer. We also analyzed whether binding of TP, which appears to require Ser122, interferes with the binding of ssDNA at the 3'-5' exonuclease active site of ø29 DNA polymerase. As shown in Fig. 8B (left panel), the retardation band obtained with the DNA polymerase alone, which has been interpreted as a stable interaction of ssDNA at the 3'-5' exonuclease active site (50), is not produced when a DNA polymerase/TP heterodimer is used. This suggests that TP binding occludes the access to the 3'-5' exonuclease binding cleft. In addition, when Mg2+ ions were added to activate exonuclease, less than 25% of the ssDNA molecules were degraded by the heterodimer in comparison with the ø29 DNA polymerase alone. Such abortive products could be detected by using a 3'-5' exonuclease deficient ø29 DNA polymerase (4, 25, 26). It was interesting to study the transition step of mutants F65S and S122N, because both have a strongly reduced 3'-5' exonuclease activity (51) and a defective TP interaction (this paper). Thus, truncated elongation assays were carried out using a high amount of both wild-type or mutant DNA polymerase, to favor the initiation reaction, i.e. TP-dAMP formation (see under “Materials and Methods”). Providing dATP as the only nucleotide, the wild-type enzyme gave rise to TP-dAMP and TP-(dAMP)2 products, as expected, taking into account its exonucleolytic activity reported to occur on the third nucleotide added to the TP (64), whereas mutant F65S was able to produce up to TP-(dNMP)4 products (Fig. 9). With dATP, dGTP, and dTTP, elongation with the wild-type polymerase mainly occurred up to TP-(dNMP)4, which is the expected size when replication starts from the left origin (see sequence in Fig. 9). Under these conditions, mutant F65S carried out transition more efficiently than the wild-type enzyme. This apparent stimulation of the transition capacity of mutant F65S could be explained considering its strongly reduced 3'-5' exonuclease activity (51). However, when the four nucleotides were provided, to allow the DNA polymerase to fully replicate ø29 TP-DNA, mutant F65S still gave rise to abortive transition products up to TP-(dNMP)8, not detected with the wild-type enzyme (Fig. 9). It has been
shown that during insertion of the first nine nucleotides, ø29 DNA polymerase remains bound to TP as an heterodimer (4), but both proteins dissociate once the next nucleotide has been incorporated. When this rate-limiting step has been completed, the stability of the DNA polymerase depends exclusively on the interactions with the DNA. Thus, it is tempting to speculate that the transition abortive bands appearing with the F65S mutant are produced as a consequence of its poor DNA binding capacity.

When either dATP alone or dATP, dGTP, and dTTP were provided, mutant S122N gave rise to a similar proportion of TP-(dAMP)_2 versus TP-dAMP products relative to the wild-type (Fig. 9), despite the fact that it has a very reduced 3'-5' exo- nuclease activity (51). Therefore, the polymerization advantage expected as a consequence of a decreased 3'-5' exo- nuclease activity in mutant S122N is probably counteracted by the defective interaction with TP displayed by this mutant that reduces the yield of all TP-(dNMP)_n intermediates requiring a stable TP/DNA polymerase interaction. On the contrary, when the four nucleotides were provided, those TP-(dNMP)_n intermediates that completed the transition stage, could be elongated via a facilitated TP/DNA polymerase dissociation, producing a good yield of fully replicated ø29 TP-DNA molecules. In agreement with that, and despite a reduction in its DNA binding capacity, mutant S122N did not produce the aborted transition products observed with mutant F65S.

DISCUSSION

ø29 DNA polymerase shares with other eukaryotic-type DNA polymerases (family B), Pol I-like DNA polymerases (family A), and Pol III DNA polymerases (family C) several regions of amino acid similarity at their 3'-5' exo- nuclease domain (11, 12, 18, 19, 49, 51). Three of these regions, the so-called Exo I, II, and III motifs (18), contain the five catalytic amino acid residues acting as metal ligands, as they were originally identified by crystallographic studies of Pol Iκ (20). The predictions of an evolutionarily conserved 3'-5' exo- nuclease active site has been confirmed with the resolution of the crystal structure of an N-terminal fragment of T4 DNA polymerase (47) and that of phage RB69 DNA polymerase complexed with ssDNA and divalent metal ions (48). Recently, a highly conserved aromatic (Phe or Tyr) residue, located at the Exo II motif, and a Ser and
FIG. 10. Three-dimensional structure prediction for TP/DNA polymerase interaction. This prediction is based on the structure of bacteriophage RB69 DNA polymerase (18), the proofreading and polymerization domains of which are homologous to those of ø29 DNA polymerase. A, amino acid residues 1–102 are uncolored because they are not present in ø29 DNA polymerase. Residues 103–339, conforming the 3'-5' exonuclease domain, are shown in blue. The 5'-3' polymerization domain, formed by amino acids 340–903, is depicted in green. In our model, the terminal protein (TP), in gray, which acts as an initiation primer for ø29 DNA polymerase, is predicted to bind the cleft formed by the thumb, palm and fingers. The polymerization catalytic core formed by RB69 DNA polymerase residues Asp411, Asp456, and Asp458 (Asp250, Asp456, and Asp458 of ø29 DNA polymerase) is shown as red spheres, whereas those residues involved in primer terminus stabilization at the polymerization active ø29 DNA Polymerase Ser^{122} Mediates Interaction with TP
a Leu residue that form the S/TLx₂h motif, located between the Exo II and Exo III motifs (51), were shown to be functionally important for a stable interaction with ssDNA during proofreading of DNA polymerization errors (51). In this study, we analyzed the importance of these three ø29 DNA polymerase residues for the synthetic activities, i.e. protein priming and DNA polymerization.

As was mentioned above, the first step of ø29 DNA replication requires the interaction between a DNA polymerase molecule and a free molecule of TP and the further recognition of the origins of replication, located at either DNA terminus, by the heterodimer. Initiation of replication occurs by the covalent linkage of DAMP to the hydroxyl group of Ser²⁹² of TP, in a reaction catalyzed by ø29 DNA polymerase (reviewed in Refs. 1 and 2) and directed by the second nucleotide at the 3′-end of the template. The first nucleotide is recovered by a sliding back mechanism (3), and the DNA polymerase remains bound to the TP until 9 dNMP residues have been incorporated (transition step) (4). Afterward, dissociation of DNA polymerase and TP takes place, and elongation occurs coupled to strand displacement, giving rise to fully replicated ø29 DNA molecules.

Interestingly, F65S mutant and those changes introduced at Ser²⁹² residue displayed a low efficiency in replicating and amplifying ø29 TP-DNA. Such a defect was not due to a defective strand displacement as deduced from their capacity to carry out rolling circle elongation using primed M13 DNA as template, strongly supporting the hypothesis that only those residues acting either directly or indirectly as metal ligands at the 3′-5′ exonuclease active site are involved in strand displacement (25, 26, 49, 50).

The analysis of the protein-primed initiation step revealed that mutant polymerases F65S and S122N were very affected in carrying out such an activity, both in the absence and in the presence of TP-DNA. This defect could be directly related with either a moderately (F65S mutant) or highly (S122N mutant) decreased capacity to form a stable TP/DNA polymerase heterodimer. Interestingly, mutants F65S, S122T, and S122N also displayed a poor capacity to interact with a DNA primer/template structure. The alteration of the capacity to interact with these two different primer structures (TP and DNA) as a consequence of the mutations introduced was apparent when the transition between protein and DNA priming, inherent to the ø29 DNA replication mechanism, was studied. Thus, mutant F65S was able to produce an efficient extension of the TP-DAMP product in the transition stage. However, its reduced capacity to interact with the newly created DNA primer leads to abortive transition products. Contrarily, the poor capacity to maintain the interaction with TP displayed by mutant S122N makes it difficult to reach the transition stage. At this point, a weakened TP/DNA polymerase interaction would facilitate entrance into the elongation stage. Little is known about the regions of the DNA polymerase that are making contacts with the TP. To date, only the conserved motif Tx₂GR of ø29 DNA polymerase, the counterpart of which in Pol Iκ (16) and RB69 (48) forms part of the palm subdomain at the polymerization domain, has been involved in interaction with TP (16) (see the following section). On the other hand, the fact that the C-terminal domain (polymerization domain) is unable to interact stably with the TP by itself, dropping up to 1000-fold its initiation capacity with respect to that of the complete enzyme (65), suggested that the N-terminal domain (3′-5′ exonuclease domain) should contribute to TP binding. Here, we provide the first direct evidence involving a single residue of the N-terminal domain (Ser²⁹² of the hxy₂SLx₂h motif) of ø29 DNA polymerase, in TP binding.

The interaction of ø29 DNA polymerase with a TP primer was modeled. Attempts to obtain ø29 DNA polymerase crystals adequate for x-ray diffraction analysis have not been successful so far. Therefore, our working models are based on extrapolation to the resolved three-dimensional structure of other DNA polymerases from family A (Pol Iκ (66, 67), Thermococcus aquaticus DNA polymerase (68, 69), Bacillus stearothermophilus DNA polymerase (70, 71), and T7 DNA polymerase (72)) and from family B (RB69 DNA polymerase (48)). All of them showed a similar bimolecular organization, with an N-terminal domain containing the 3′-5′ exo and transition stages of ø29 DNA replication. Afterward, during the elongation stage, the same cleft serves to hold the DNA primer/template structures, where residue Ser²⁹² seems to act as a DNA ligand during both DNA synthesis and proofreading of mismatched primer termini. The code color used is as described for A.

**site, Thr⁵⁰⁴ and Gln⁹⁹** (Thr⁵⁰⁴ and Arg⁴⁹⁸ of ø29 DNA polymerase) are shown in dark blue. RB69 DNA polymerase residues Phe⁴³⁴, Ser⁴⁹⁹, and Leu⁴⁹⁹, homologous to the ø29 DNA polymerase residues Phe⁶⁵, Ser¹²², and Leu¹²², studied in this paper, are shown in magenta, yellow, and orange, respectively. B, in this scheme, we propose that a unique primer binding site is initially occupied by the TP primer molecule during the initiation and transition stages of ø29 DNA replication. Afterward, during the elongation stage, the same cleft serves to hold the DNA primer/template structures, where residue Ser¹²² seems to act as a DNA ligand during both DNA synthesis and proofreading of mismatched primer termini. The code color used is as described for A.
the other hand, the positioning of the TP to reach the catalytic site (formed by the aspartate residues of motifs DxxSxLYP and YGDtDS of eukaryotic-type DNA polymerases (11), depicted as red spheres in Fig. 10A, outlining the portion of the polymerization active site and their close vicinity to the TTP GR motif), probably occludes the entrance to the ssDNA binding cleft (the length of which can bury the four 3′-terminal nucleotides of a ssDNA (48) at the 3′-5′ exonuclease domain (depicted in blue in Fig. 10). Such an occlusion would explain the incapacity of the DNA polymerase to bind a ssDNA 15-mer once it forms a heterodimer with a TP molecule. This fact could be physiologically relevant, because the heterodimer would prevent the exonucleolytic degradation of the 3′-ends of e29 TP-DNA by e29 DNA polymerase before replication starts.

The Ph residue of the Exo II motif (Ph212 in RB69 DNA polymerase, represented in magenta spheres in Fig. 10) is located at the bottom of the 3′-5′ exonuclease cleft and, therefore, far enough to preclude direct contacts with the primer structure (TP or DNA). The defective TP binding displayed by a mutant at the homologous residue (Ph65) of e29 DNA polymerase, represented in this paper) is located 25 Å away from the polymerization active site, although we cannot rule out the possibility that the structure of the former is first stabilized at the exonuclease active site by the ssDNA ligands, somehow mimicking the proofreading mode of the latter. When the e29 polymerase is bound to a primer/template structure, the 3′-end of the TP can be specifically relevant, because the heterodimer would prevent the exonucleolytic degradation of the 3′-ends of e29 TP-DNA by e29 DNA polymerase before replication starts.

RB69 DNA polymerase residue Ser122 (shown in yellow in Fig. 10), the counterpart of Ser122 of e29 DNA polymerase (this paper) is located 25 Å away from the polymerization active site, but facing the primer binding cleft. This situation would allow the ssDNA to be bound to a primer/template structure, the 3′-end of the former is first stabilized at the exonuclease active site by the ssDNA ligands, somehow mimicking the proofreading mode following misincorporation. Indeed, under gel retardation assay conditions, analysis of the retarded band revealed exonucleolytic degradation of the primer (17).

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