Role of the LEXE Motif of Protein-primed DNA Polymerases in the Interaction with the Incoming Nucleotide*

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The LEXE motif, conserved in eukaryotic type DNA polymerases, is placed close to the polymerization active site. Previous studies suggested that the second Glu was involved in binding a third noncatalytic ion in bacteriophage RB69 DNA polymerase. In the protein-primed DNA polymerase subgroup, the LEXE motif lacks the first Glu in most cases, but it has a conserved Phe/Trp and a Gly preceding that position. To ascertain the role of those residues, we have analyzed the behavior of mutants at the corresponding F29 DNA polymerase residues Gly-481, Trp-483, Ala-484, and Glu-486. We show that mutations at Gly-481 and Trp-483 hamper insertion of the incoming dNTP. Mutants at Glu-486 are also defective in polymerization and, as mutants at Gly-481 and Trp-483, in the pyrophosphorolytic activities of φ29 DNA polymerase and compromise nucleotide insertion fidelity.

**Background:** LEXE motif is conserved among the eukaryotic DNA polymerases.

**Results:** Substitutions at LEXE residues decrease the replication and pyrophosphorolytic activities of φ29 DNA polymerase and compromise nucleotide insertion fidelity.

**Conclusion:** The results suggest a role for LEXE residues in making interactions with the incoming nucleotide.

**Significance:** Results support the proposal of a third metal ion as a general feature of the two-metal ion mechanism.

DNA replication fidelity is a key determinant of genome stability (1). Faithful synthesis of DNA depends mostly on the high intrinsic nucleotide selectivity exhibited by replicative DNA polymerases that bind correct dNTPs with about 10- to 1000-fold higher affinity than incorrect dNTPs. Such a selectivity relies on the common structure of their polymerization domains formed by the universal fingers, palm, and thumb subdomains that form a U-shaped groove responsible for dsDNA binding (1, 2). Both the binding of the incoming dNTP to the polymerization site and the presence of a complementary template base induce the rotation of the fingers toward the palm subdomain. The closure of the fingers promotes the final assembly of the hydrophobic pocket into which the nascent base pair will fit (3–8). Therefore, the high insertion fidelity displayed by DNA polymerases largely depends on the geometrical fitting between a canonical Watson-Crick base pair and this pocket (9, 10).

Once the polymerization domain has adopted the catalytically competent closed state, phosphodiester bond formation between the 3’-OH group of the growing strand and the α-phosphate of the incoming dNTP takes place. Extensive structural, kinetic, and mutational studies performed in a great variety of DNA polymerases have given sufficient experimental evidence for the two-metal ion mechanism as the one responsible for the phosphoryl transfer reaction (4, 10–16). Thus, metal ion A would lower the pKₐ value of the 3’-OH group to support its attack on the α-phosphate of the incoming dNTP, whereas metal ion B chelates the β- and γ-phosphates, stabilizing the pentacovalent transition state and facilitating the further pyrophosphate release (14). Two conserved carboxylic residues placed at the palm subdomain and belonging to conserved sequence motifs A and C coordinate these two metal ions. Although the two-metal ion mechanism for nucleotide insertion in DNA polymerases has been accepted for many years, recent crystallographic studies show the involvement of a third metal ion located between α- and β-phosphates of the incoming nucleotide in the Y family DNA polymerase η (17) and in the family X DNA polymerase β (18). In the former case, it was proposed as a transition state stabilization role for the third metal ion, whereas in pol 4 β, a potential role during pyrophosphorolysis, the reversal of the polymerization reaction has been suggested.

Bacteriophage φ29 DNA polymerase is a protein-primed DNA-dependent replicate belonging to the eukaryotic type family of DNA polymerases (family B). Like many other repli-
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**FIGURE 1. Polymerization active site of φ29 DNA polymerase.** A, proposed interaction of residue Glu-486 with the PP, moiety of the incoming nucleotide through a third metal ion. A detailed picture also shows the conserved lysine residues from the finger subdomain interacting with the phosphates of the incoming nucleotide. Catalytic metal ions A and B, indicated as green and gray spheres, respectively, are coordinated by the α- and γ-phosphates of the incoming nucleotide, the carboxylates of residues Asp-249 (motif A) and Asp-458 (motif C), shown in red sticks, and by the carbonyl group of residue Val-250 (motif A), represented as green sticks. A proposed noncatalytic metal ion C and a water molecule would mediate interactions between Glu-486 (LEXE motif) and the γ-phosphate of the nucleotide, as described to occur in RB69 DNA polymerase (5, 16, 23). Crystallographic data corresponding to φ29 DNA polymerase ternary complex are from Protein Data Bank ID code 2PYL (3). B, carbonyl group of Val-250 that interacts with the γ-phosphate of the incoming nucleotide through its coordination with metal B fits into a structural cavity formed by residues Leu-253 (from motif A) and Gly-481 and Trp-483 (belonging to the LE motif studied here). Residues and incoming nucleotide are represented as spheres.

**EXPERIMENTAL PROCEDURES**

**Nucleotides and DNAs—**Unlabeled nucleotides and [α-32P]dATP (3000 Ci/mmol) were supplied by Amersham Biosciences. Oligonucleotides were obtained from Invitrogen. Oligonucleotides 15-mer (5'-GATCAGTGTAGTC), 21-mer (5'-TCTATTGTACTCTGTGATC), and 21-mer-A (5'-TCTATGTCCTAGTCGATC) were purified electrophoretically on 8 M urea, 20% polyacrylamide gels. The 15-mer oligonucleotide was 5'-labeled with [γ-32P]ATP and T4 polynucleotide kinase and further hybridized to the 21-mer and 21-mer-A oligonucleotides in the presence of 0.2 M NaCl and 50 mM Tris-HCl, pH 7.5, resulting in a primer-template molecule that can be used in the coupled DNA polymerization/exonuclease assay. M13mp18 single-stranded DNA was hybridized to the universal primer 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 processive DNA polymerization coupled to strand displacement by φ29 DNA polymerase variants. Terminal protein-containing φ29 DNA (φ29 TP-DNA) was obtained as described (25).

**Proteins—**Phage T4 polynucleotide kinase was obtained from New England Biolabs. Wild-type φ29 DNA polymerase was purified from *Escherichia coli* BL21(DE3) cells harboring plasmid pJLPM (a derivative of pT7-4w2), as described (26). φ29 TP was purified as described (27).

**Site-directed Mutation and Expression of Mutants of φ29 DNA Polymerase—**The DNA polymerase mutants were obtained using the QuikChange site-directed mutagenesis kit provided by Stratagene, using as template a recombinant derivative of plasmid pT7-4 (28) containing the viral gene 2 that encodes the wild-type φ29 DNA polymerase (26) or plasmid pT7-3 (28) harboring the φ29 DNA polymerase exonuclease-deficient mutant D12A/D66A (29). The presence of the desired...

Cative polymerases, besides the 5′–3′ synthetic activity, in the same polypeptide chain it contains a 3′–5′-exonuclease that proofreads polymerization errors (19, 20). The polymerase forms a heterodimer with the phage terminal protein (TP) to initiate DNA replication at the origins, located at both ends of the linear genome. Thus, the polymerase catalyzes the formation of a covalent bond between the initiator dAMP and the hydroxyl group of Ser-232 of the primer TP (21) in the so-called initiation reaction. Once the TP-DAMP initiation product is formed, the same DNA polymerase catalyzes processive chain elongation via a strand displacement mechanism to fulfill TP-DNA replication (22).

X-ray crystal structure of φ29 DNA polymerase ternary complex has shown that metal ions A and B are coordinated not only by the α- and γ-phosphates of the incoming nucleotide and the catalytic residues Asp-249 (motif A) and Asp-458 (motif C), but also by the carbonyl group of Val-250 (motif A) (3), which is highly conserved in the protein-primed subgroup of DNA polymerases (see Fig. 1A). In addition, multiple sequence alignments identified a conserved Glu residue (Glu-486 in φ29 DNA polymerase) placed at the polymerization active site of family B DNA polymerases. This residue has been originally proposed as a new indirect ligand of a noncatalytic metal ion C in RB69 DNA polymerase (5, 16, 23), and it is forming part of the so-called LEXE motif (24).

The aim of this work was to ascertain the role of residues belonging to the LEXE motif of family B DNA polymerases during the polymerization reaction, using as a model the φ29 DNA polymerase. The biochemical analyses of φ29 DNA polymerase mutants at residues Gly-481, Trp-483, Glu-486 (see Fig. 1B), and Ala-484 suggest a role for this motif in establishing indirect contacts with the incoming dNTP at the polymerization active site and support the presence of a third metal ion at the catalytic site.
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Mutation and the absence of additional ones were determined by sequencing the entire gene. DNA polymerase mutants were expressed in E. coli BL21(DE3) cells and further purified essentially as described for the wild-type DNA polymerase (26).

Polymerase/3′–5′-Exonuclease (pol/exo)-coupled Assay—The primer-template molecule 15/21-mer (double-stranded DNA; dsDNA) contains a 6-nucleotide 5′-protruding end and therefore can be used as substrate for the exonuclease activity (dsDNA) and also for DNA-dependent DNA polymerization. The incubation mixture contained, in 12.5 μl, 50 mM Tris-HCl, pH 7.5, either 10 mM MgCl₂ or 1 mM MnCl₂, 1 mM dithiothreitol (DTT), 4% (v/v) glycerol, 0.1 mg/ml bovine serum albumin (BSA), and 25 ng of wild-type or the indicated mutant φ29 DNA polymerase. Protein was incubated with 1.2 nm of 5′-labeled 15/21-mer substrate and the indicated increasing concentrations of the four dNTPs. After incubation for 5 min at 25 °C, 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. Polymerization or 3′–5′-exonuclease was detected as an increase or decrease, respectively, in the size (15-mer) of the 5′-labeled primer.

Measurement of DNA Polymerase Fidelity of Mutant φ29 DNA Polymerases—The measurements were performed essentially as described (30). The incubation mixture contained in a final volume of 12.5 μl, 50 mM Tris-HCl, pH 7.5, either 10 mM MgCl₂ or 1 mM MnCl₂, 1 mM DTT, 4% (v/v) glycerol, 0.1 mg/ml BSA, 1.2 nm of the 5′-labeled 15/21-mer-A substrate, and the indicated concentrations of either the correct (dTTP) or incorrect (dCTP) nucleotide. Reaction times and enzyme concentration were adjusted for each polymerase to optimize product detection while ensuring that all reactions were conducted in the steady state. Only those reactions that fell within the linear range of substrate utilization (<30% primer extension) were used for analysis. Apparent value for Michaelis-Menten constant (Kₘ) for the correct dTTP in the presence of 1 mM MnCl₂ was carried out with the following amounts of exonuclease-deficient DNA polymerase: WTExo⁻⁻⁻ (1 ng); G481FExo⁻⁻⁻ (25 ng); G481LExo⁻⁻⁻ (1 ng); W483FExo⁻⁻⁻ (1 ng); W483LExo⁻⁻⁻ (4 ng); A484FExo⁻⁻⁻ (1 ng); E486AExo⁻⁻⁻ (2 ng); and E486DExo⁻⁻⁻ (2 ng). In the presence of 10 mM MgCl₂, the amount of DNA polymerases were as follows: WTExo⁻⁻⁻ (1.25 ng); G481FExo⁻⁻⁻ (30 ng); G481LExo⁻⁻⁻ (3 ng); W483FExo⁻⁻⁻ (1 ng); W483LExo⁻⁻⁻ (1.5 ng); A484FExo⁻⁻⁻ (1.5 ng); E486AExo⁻⁻⁻ (1 ng), and E486DExo⁻⁻⁻ (1 ng). Estimation of the Kₘ value for the incorrect dCTP was carried out in the presence of 1 mM MnCl₂ and the following amounts of DNA polymerase: WTExo⁻⁻⁻ (2 ng); G481FExo⁻⁻⁻ (14 ng); G481LExo⁻⁻⁻ (6 ng); W483FExo⁻⁻⁻ (1.5 ng); W483LExo⁻⁻⁻ (5 ng); A484FExo⁻⁻⁻ (6 ng); E486AExo⁻⁻⁻ (6 ng), and E486DExo⁻⁻⁻ (4 ng). Samples were incubated for 30 s at 25 °C and quenched by adding EDTA up to 10 mM. Reactions were analyzed by electrophoresis in 8 m urea, 20% polyacrylamide gels and quantified using a PhosphorImager (GE Healthcare). Formation of the extended product was plotted against dNTP concentration. Apparent values for Kₘ and the corresponding V_max were obtained by least squares nonlinear regression to a rectangular hyperbola using Kaleidagraph 3.6.4 software. The discrimination factor during nucleotide addition was defined as the ratio of incorrect to correct insertion catalytic efficiencies (30). Data are shown as mean ± S.D. corresponding to four independent experiments.

Replication of Primed M13 DNA—The incubation mixture contained, in 25 μl, 50 mM Tris-HCl, pH 7.5, either 10 mM MgCl₂ or 1 mM MnCl₂, 1 mM DTT, 4% (v/v) glycerol, 20 mM ammonium sulfate, 0.1 mg/ml BSA, 40 μM each of dCTP, dGTP, dTTP, and [α-32P]dATP (1 μCi), 0.25 μg of primed M13mp18 single-stranded DNA, and 100 ng of either wild-type or mutant φ29 DNA polymerases. After incubation for the indicated times at 30 °C, the reactions were stopped by adding 10 mM EDTA and 0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns. Relative activity was calculated from Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels, as described (31). After electrophoresis, gels were dried and autoradiographed.

Replication Assay (Protein-primed Initiation Plus Elongation) Using φ29 TP-DNA as Template—The replication assay was performed as described (32). The incubation mixture contained in a final volume of 25 μl, 50 mM Tris-HCl, pH 7.5, 20 mM ammonium sulfate, 1 mM DTT, 4% (v/v) glycerol, 0.1 mg/ml BSA, 20 μM each of dNTP and [α-32P]dATP (1 μCi), 10 ng of purified TP, 20 ng of either purified wild-type or mutant DNA polymerase, and 0.5 μg of φ29 TP-DNA, in the presence of either 10 mM MgCl₂ or 1 mM MnCl₂. After incubation for the indicated times 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 NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels, as described (31). After electrophoresis, the position of unit length φ29 DNA (19285 bases) was detected by ethidium bromide staining, and then the gels were dried and autoradiographed.

Protein-primed Initiation Assay (TP-dAMP Formation)—The ability to carry out the initiation step during TP-DNA replication was analyzed as described (32). The incubation mixture contained in 25 μl, 50 mM Tris-HCl, pH 7.5, either 10 mM MgCl₂ or 1 mM MnCl₂, 20 mM ammonium sulfate, 1 mM DTT, 4% (v/v) glycerol, 0.5 μg of φ29 TP-DNA as template, 0.1 mg/ml BSA, 0.2 μM dATP (1 μCi of [α-32P]dATP) and the indicated amount of TP and DNA polymerase. Samples were incubated for 4 min (with Mg²⁺) or 2 min (with Mn²⁺) at 30 °C. Reactions were stopped by adding 10 mM EDTA, 0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns and further analyzed by SDS-PAGE in 12% polyacrylamide gels. Quantification was done by densitometric analysis of the labeled band corresponding to the TP-dAMP complex detected by autoradiography.

Pyrophosphorolysis—Pyrophosphorolytic activity was measured on the 15/21-mer substrate. The reaction mixture contained in 12.5 μl, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% (v/v) glycerol, 0.1 mg/ml BSA, 10 ng of the 3′–5′-exonuclease-deficient wild-type or mutant φ29 DNA polymerases to avoid exonuclease activity on this substrate, 1.2 nm of the 5′-labeled 15/21-mer, either 10 mM MgCl₂ or 1 mM MnCl₂ as metal acti-
vator, and the indicated concentrations of tetra-sodium pyrophosphate (Merck). After incubation for 5 min at 25 °C, the reaction was stopped by adding EDTA to 10 mM. Samples were analyzed by electrophoresis in 8 M urea, 20% polyacrylamide gels.

RESULTS

LEXE Motif of Replicative DNA Polymerases—Multiple amino acid sequence alignments of family B DNA polymerases allowed the identification in their polymerization domain of a conserved motif with the consensus sequence LE\textsubscript{X}E\textsubscript{E} (24). The second glutamate of this motif was previously proposed to interact with the γ-phosphate of the incoming nucleotide through a third noncatalytic metal ion and a water molecule at the polymerization active site of bacteriophage RB69 DNA polymerase (5, 16, 23). In the protein-primed subgroup of DNA polymerases, only the second Glu residue is conserved (in cyan in Fig. 2). Preceding the first Glu, bacterial, viral, and cellular DNA polymerases show a conserved Leu residue, whereas most members of the protein-primed subgroup have an aromatic residue (Phe/Trp) (colored in green in Fig. 2). In addition, protein-primed DNA polymerases contain a conserved Gly residue located two positions N-terminally from the aromatic one (in dark blue in Fig. 2), unlike the Leu/Ile amino acid exhibited by the polymerases belonging to the cellular, viral, and bacterial subgroups.

Crystallographic resolution of Φ29 DNA polymerase ternary complex structure showed that the corresponding residues Gly-481 and Trp-483 are forming a structural cavity with residue Leu-253 (from motif A) (see Fig. 1B) (3). Such hydrophobic pocket would allocate the side chain of Φ29 DNA polymerase Val-250, a residue that participates in the coordination of metal B (3, 33).

To analyze the effect of mutations in each of the amino acids described above, single changes were introduced at residues Gly-481, Trp-483, Ala-484, and Glu-486 of Φ29 DNA polymerase. Gly-481 was mutated to Leu (G481L), as this residue is the most abundant in the rest of DNA polymerases, and to Phe (G481F), because several bacterial/viral DNA polymerases contain this residue at the homologous position. Trp-483 was substituted for Phe (W483F), as in other protein-primed members, and for Leu (W483L), because this residue is conserved in the rest of DNA polymerases. Ala-484 was changed into Glu (A484E) as in the case of the cellular, viral, and bacterial DNA polymerases. Finally, residue Glu-486 was replaced by Ala (E486A), to eliminate the electronegative charge, and by Asp (E486D), to conserve it. Site-directed mutagenesis and protein

FIGURE 2. Multiple alignment of the LEXE motif of family B DNA polymerases. The sequences of DNA polymerases aligned are subgrouped in bacterial/viral, cellular, and those able to use a protein as primer (protein-primed subgroup). DNA polymerase nomenclature and sequence references are compiled in Ref. 24, with the exception of linear mitochondrial plasmid DNA polymerase from the following: Enterobacteria phage RB69 (RB69; GenBank\textsuperscript{TM} accession number NP_861746); Pyrodictionium occultum DNA polymerase II (pol II; GenBank\textsuperscript{TM} accession number BAA07579); Schizosaccharomyces pombe (S.p.; GenBank\textsuperscript{TM} accession number AAA3503); linear mitochondrial plasmid DNA polymerases from Gelasinopora sp. (GenBank\textsuperscript{TM} accession number S62752); Brassica napus (GenBank\textsuperscript{TM} accession number NP862323); Flammulina velutipes (GenBank\textsuperscript{TM} accession number BAB13496); Pichia klyveri (GenBank\textsuperscript{TM} accession number CAA72340); Porphyra purpurea (GenBank\textsuperscript{TM} accession number NP_049297); Morchella conica (pMC3-2; GenBank\textsuperscript{TM} accession number CAAT4364); linear plasmid pAL2-1 DNA polymerase from Podospora anserina (pAL2-1; GenBank\textsuperscript{TM} accession number X60707); Streptococcus pneumoniae phage Cp-1 DNA polymerase (GenBank\textsuperscript{TM} accession number Q37989); and DNA polymerase from the Bacillus phage GA-1 (GA-1; GenBank\textsuperscript{TM} accession number NP_073685). Numbers between slashes indicate the position of the first amino acid residue aligned relative to the N terminus of each DNA polymerase sequence. The first and second Glu residues are in white and cyan letters, respectively. Residues Gly and Trp/Phe, conserved in the protein-primed subgroup of DNA polymerases, are indicated in blue and green letters, respectively. Other moderately conserved residues are in black letters over a gray background. Φ29 DNA polymerase residues Gly-481, Trp-483, Ala-484, and Glu-486, studied here, are indicated with black dots.
purification were performed as described under “Experimental Procedures.”

DNA-primed Polymerization Activity of Mutant φ29 DNA Polymerases—As in most replicases, the exonuclease and polymerization activities of φ29 DNA polymerase reside in the structurally independent N- and C-terminal domains, respectively (34, 35). Despite their structural separation, both active sites must work in concert to ensure a productive and faithful DNA synthesis, preventing the accumulation of errors in the newly synthesized strand while allowing a proper elongation rate. The decision to synthesize versus to degrade the primer-terminus depends on several factors, the relative velocity of both activities and the comparative stabilization of the primer-terminus at such active sites (36).

To evaluate how the amino acid substitutions introduced affected the dynamic equilibrium between the 3′→5′-exonuclease and polymerization activities of the DNA polymerase, we studied the functional coupling between synthesis and degradation on a primer-template hybrid molecule (15/21-mer) as a function of dNTP concentration (see Fig. 3A and see under “Experimental Procedures”). Without nucleotides, the only bands detected correspond to primer degradation products by the 3′→5′-exonuclease activity. Under these conditions, the extent of degradation reflects the level of the 3′→5′-exonuclease activity of the different mutants. As expected, based on the positioning of the LEXE motif at the polymerization domain, except mutant G481F, none of the mutant derivatives showed significant defects to degrade the primer strand. As the concentration of the unlabeled dNTP increases, exonuclease activity is progressively competed by the 5′→3′ polymerization one. Net dNMP incorporation is observed as an increase in the length of the labeled primer, allowing us to define the dNTP concentration needed to obtain an efficient elongation for each mutant derivative (pol/exo ratio). When Mg²⁺ was used as metal activator, and in the presence of 50 nM dNTPs, the wild-type φ29 DNA polymerase competed out its 3′→5′-exonuclease activity, a phenotype only shared by mutant A484E (Fig. 3A). In contrast, the rest of the mutants were unable to overcome their exonuclease activity at 100 nM dNTPs. Thus, although mutants W483L and G481F did not give rise to any elongation product, mutants G481L, W483F, E486A, and E486D only yielded a 1 elongation product at the highest dNTPs concentration assayed. Remarkably, the exonuclease activity of mutants E486A and E486D was higher than that of the wild-type enzyme.

Any strong impairment in nucleotide incorporation, such as a mutation at the polymerization active site, would result in the exonuclease activity overcoming polymerization, precluding a
specific analysis of the synthetic features of the enzyme. Therefore, \( \phi 29 \) DNA polymerase mutants were engineered to include additional mutations at two conserved aspartic residues (D12A/D66A (29)) responsible for the exonuclease activity, eliminating their proofreading proficiency (see “Experimental Procedures”). The \( \phi 29 \) DNA polymerase mutant D12A/D66A was used as a control for the wild-type polymerization active site. When Mg\(^{2+}\) was used as the metal activator, both the wild-type DNA polymerase and mutant A484E required \( 5 \) nM dNTPs concentration to elongate the primer molecule up to the 20-mer position (Fig. 3B). However, none of the other mutants added more than one nucleotide. This result led us to conclude that the defects observed in the polymerization activity, especially in mutants E486A and E486D, were not attributable to a higher exonuclease proficiency but to a specific impairment of the polymerization catalysis. Interestingly, when Mn\(^{2+}\) was used as metal activator, with the exception of mutant G481F, the polymerization activity of all mutant derivatives improved notably as they reached the 20-mer position at 5 nM dNTPs, as in the wild-type enzyme (Fig. 3C).

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**Table 1**

| \( \phi 29 \) DNA polymerase mutant | WT | G481F | G481L | W483F | W483L | A484E | E486A | E486D |
|-------------------------------------|----|-------|-------|-------|-------|-------|-------|-------|
| \( K_m \) (nM)                  | 3 ± 0.3 | 2634 ± 93 | 12 ± 4 | 23 ± 4 | 113 ± 10 | 8 ± 2 | 74 ± 1 | 54 ± 6 |
| \( k_{cat} \) (s\(^{-1}\))     | 0.77 | 0.05 | 0.57 | 1.1 | 0.86 | 0.81 | 0.99 | 1.02 |
| \( f_{dis} \) \( K_m \) (nM\(^{-1}\)s\(^{-1}\)) | 0.255 | 1.7 \( \times \) 10\(^{-5}\) | 3.1 \( \times \) 10\(^{-2}\) | 4.7 \( \times \) 10\(^{-2}\) | 7.5 \( \times \) 10\(^{-3}\) | 0.1 | 1.3 \( \times \) 10\(^{-2}\) | 1.9 \( \times \) 10\(^{-2}\) |

**Table 2**

**Nucleotide insertion fidelity of \( \phi 29 \) DNA polymerase mutants**

| Mutant  | \( K_m \) (nM) | \( k_{cat} \) (s\(^{-1}\)) | \( f_{rel} \) | \( f_{dis} \) |
|---------|----------------|-----------------|---------------|---------------|
| Wild type | 1 ± 2 | 0.8 | 8 | \( 24 ± 2 \) | 4 \( \times \) 10\(^{-2}\) | 1.6 \( \times \) 10\(^{-3}\) | 480 | 1 |
| G481F | 26 ± 2 | 5.1 \( \times \) 10\(^{-2}\) | 1.9 \( \times \) 10\(^{-3}\) | 89 ± 1 | 1.5 \( \times \) 10\(^{-2}\) | 1.6 \( \times \) 10\(^{-4}\) | 12 | 41 |
| G481L | 7 ± 2 | 0.16 | 2.3 \( \times \) 10\(^{-2}\) | 83 ± 4 | 1.2 \( \times \) 10\(^{-2}\) | 1.4 \( \times \) 10\(^{-4}\) | 164 | 3 |
| W483F | 7 ± 2 | 0.17 | 2.4 \( \times \) 10\(^{-2}\) | 29 ± 4 | 3.2 \( \times \) 10\(^{-2}\) | 1.1 \( \times \) 10\(^{-3}\) | 22 | 22 |
| W483L | 4 ± 0.5 | 0.29 | 7.2 \( \times \) 10\(^{-2}\) | 132 ± 12 | 7 \( \times \) 10\(^{-2}\) | 5.3 \( \times \) 10\(^{-5}\) | 1373 | 0.3 |
| A484E | 3 ± 0.2 | 0.27 | 8.8 \( \times \) 10\(^{-2}\) | 27 ± 6 | 3.6 \( \times \) 10\(^{-2}\) | 1.3 \( \times \) 10\(^{-3}\) | 68 | 7 |
| E486A | 8 ± 1 | 0.16 | 1.9 \( \times \) 10\(^{-3}\) | 92 ± 2 | 1.8 \( \times \) 10\(^{-2}\) | 1.9 \( \times \) 10\(^{-4}\) | 98 | 5 |
| E486D | 7 ± 1 | 0.2 | 2.8 \( \times \) 10\(^{-3}\) | 100 ± 9 | 1.8 \( \times \) 10\(^{-2}\) | 1.8 \( \times \) 10\(^{-4}\) | 159 | 3 |

**Procesive DNA Synthesis Coupled to Strand Displacement by Mutant \( \phi 29 \) DNA Polymerases—\( \phi 29 \) DNA polymerase is characterized by its ability to couple procesive DNA polymerization to strand displacement (22). These two features (procesivity and strand displacement) allow the DNA polymerase to account for \( \phi 29 \) DNA replication without the assistance of processivity factors and helicase-like DNA-unwinding proteins. To analyze the ability of mutant derivatives to perform procesive DNA synthesis coupled to strand displacement, we carried out singly primed M13 rolling circle replication assays in which the polymerase starts replication from the 3’-OH group of a hybridized primer. The first replication round does not require any unwinding activity, but once it is completed and the polymerase encounters the 5’-end of the primer, the next rounds of replication will entail an active strand displacement. As shown in Fig. 4A and Table 3, in the presence of Mg\(^{2+}\), mutants G481F, W483L, and E486A showed a negligible activity on this substrate. The activity exhibited by mutants G481L, W483F, and A484E was 1.5-, 4-, and 7-fold lower, respectively, than that of the wild-type enzyme, and the length of the replication products yielded by the two former mutants was shorter (Fig. 4A, compare the 5-min lanes), suggesting a reduced replication efficiency. **
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velocity. Interestingly, despite the fact that mutant E486D synthesized more product than the wild-type polymerase (160%), it also exhibited a reduced velocity. As described in the exo/pol assays, the presence of Mn\(^{2+}\) ions promoted partial (mutant W483L) or total (mutants G481L and W483F) recuperation of the polymerization activity, unlike mutants G481F and A484E whose polymerization efficiency was not improved (Fig. 4B). Notably, the presence of Mn\(^{2+}\) ions not only enhanced the amount of product yielded by mutants E486A and E486D, but the replication velocity was even faster than that exhibited by the wild-type enzyme.

\(\phi 29\) DNA replication involves the recognition of the two terminal replication origins by the heterodimer formed by the \(\phi 29\) DNA polymerase and a free TP molecule with the further formation of a template-directed TP-dAMP complex (initiation reaction), catalyzed by the DNA polymerase. Such an initiation complex is subsequently elongated via strand displacement to produce full-length DNA (37, 38). To ascertain to what extent the mutations introduced in the DNA polymerase affected the protein-primed replication process, replication assays were set up, using a minimal replication system based on \(\phi 29\) TP-DNA, DNA polymerase, and TP (22). As shown in the upper panel of Fig. 5A and in Table 3, in the presence of Mg\(^{2+}\) the defects displayed by the mutant DNA polymerases to replicate TP-DNA were even more severe than those observed when M13 was used as template (see above). This fact led us to analyze the first step of TP-DNA replication.

To study the initiation activity of mutant polymerases, the formation of the TP-dAMP complex (initiation reaction) was evaluated using as template \(\phi 29\) TP-DNA. As shown in the lower panel of Fig. 5A (see also Table 3), mutant polymerases were grievously (G481F, W483L, and E486A) or moderately (G481L, W483F, A484E, and E486D) affected in performing the initiation reaction. Therefore, the defective replication activity of mutant derivatives during DNA-primed replication (M13 DNA assay) would be aggravated by their additional deficiency in the TP-primed initiation reaction during TP-DNA replication. The presence of Mn\(^{2+}\) ions improved the TP-DNA replication efficiency of mutants, in a similar way to that described in the M13 DNA replication assay, with a near full recovery for mutants G481L, W483F, E486A, and E486D (see upper panel of Fig. 5B and Table 3). Again, the presence of Mn\(^{2+}\) ions increased the replication velocity of mutants E486A and E486D above the wild-type levels Analogous Mn\(^{2+}\) stimulation was observed in the initiation activity of the mutant polymerases (see lower panel of Fig. 5B).

Pyrophosphorolytic Activity of \(\phi 29\) DNA Polymerase Mutants—Besides its 3’–5’-exonuclease activity, \(\phi 29\) DNA polymerase possesses an additional inorganic pyrophosphate-dependent degradative activity, pyrophosphorolysis (39). This activity, whose optimal substrate is a duplex DNA with a protruding 5’ single strand, can be considered as the reversal of polymerization as it acts in the 3’–5’ direction releasing free dNTPs. Therefore, we analyzed the effect of the mutations on the pyrophosphorolytic activity of the enzyme, using as substrate the hybrid molecule 15/21-mer (see “Experimental Procedures”). To diminish the potential interference of the exonuclease activity in the interpretation of the results, the \(\phi 29\) DNA polymerase exonuclease-deficient mutants were assayed. As shown in Fig. 6, addition of increasing concentrations of inorganic pyrophosphate (PP\(_i\)) results in pyrophosphorolysis as indicated by the appearance of shorter products. When Mg\(^{2+}\) was used as the

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**TABLE 3**

Processive DNA synthesis of wild-type and mutant derivatives of \(\phi 29\) DNA polymerase

| Activity assay                | Metal ion | WT   | G481F | G481L | W483F | W483L | A484E | E486A | E486D |
|-----------------------------|-----------|------|-------|-------|-------|-------|-------|-------|-------|
| M13 replication             | Mg\(^{2+}\)| 100  | 7     | 66    | 24    | 4     | 15    | 16    | 159   |
|                             | Mn\(^{2+}\)| 100  | 15    | 150   | 86    | 44    | 21    | 125   | 179   |
| \(\phi 29\) TP-DNA replication | Mg\(^{2+}\)| 100  | 0.3   | 4     | 3     | 0.2   | 19    | 0.5   | 7     |
|                             | Mn\(^{2+}\)| 100  | 7     | 87    | 50    | 5     | 24    | 69    | 100   |
| \(\phi 29\) TP-DNA initiation | Mg\(^{2+}\)| 100  | 10    | 20    | 17    | 2     | 25    | 8     | 26    |
|                             | Mn\(^{2+}\)| 100  | 8     | 53    | 43    | 5     | 21    | 21    | 47    |

*Numbers indicate the average percentage of activity relative to the wild-type enzyme obtained from at least three experiments.*
metal activator (Fig. 6A), both the wild-type DNA polymerase and mutant A484E showed a similar pyrophosphorolytic activity, being able to shorten the length of the 15-mer oligonucleotide to 9-mer. In contrast, the rest of the mutants were very inefficient in performing such a reaction. Thus, although mutants W483F, E486A, and E486D gave rise only to the −1 product (14-mer), no activity was detected in the case of mutants G481F and G481L. These results parallel those of the synthesized DNA was analyzed by alkaline 0.7% agarose gel electrophoresis. The migration position of unit length -d29 DNA is indicated. Lower panel, TP-primed initiation activity in the presence of Mg²⁺. The initiation assay was carried out as described under “Experimental Procedures,” in the presence of 30 ng of either wild-type or mutant DNA polymerase, 15 ng of purified TP, and 10 mM of MgCl₂ as metal activator. After incubation for 4 min at 30 °C, the samples were analyzed by SDS-PAGE and autoradiography. The electrophoretic mobility of the TP-dAMP initiation complex is indicated.

DISCUSSION

Previous studies on sequence comparisons of DNA polymerases showed three conserved carboxylic residues in the palm subdomain as the ones responsible for the catalysis of the phosphodiester bond formation (36, 40, 41). These acidic residues, identified in almost all known nucleic acid polymerases, are part of two different motifs as follows: motif A, which contains one aspartate, and motif C, containing the other two acidic residues (42–45). In the overall three-dimensional arrangement of the known enzymes, the three acidic residues of motifs A and C are juxtaposed to form the so-called catalytic triad, as the three were initially implicated in divalent metal binding at the polymerization active site. Further resolution of the structure of ternary complexes of several DNA polymerases demonstrated that the catalytic metal ions A and B were coordinated exclusively by the aspartate of motif A and one of the aspartates of motif C (46).

Biochemical and structural studies carried out with d29 DNA polymerase showed that besides the above acidic residues, the carbonyl group of Val-250, belonging to motif A (DYNLSLYP motif), also participates in the interaction with the γ-phosphate of the incoming nucleotide through its coordination with metal B (3, 33). Similarly, structural studies carried out in the DNA polymerases from Thermococcus gorgonarius and from bacteriophage RB69 showed that the carbonyl group of the corresponding residues Phe-405 and Leu-412 was also coordinating metal ion B (5, 16, 47). Thus, independently of the side chain, the presence of a carbonyl group interacting with the metal B-dNTP complex is conserved in the polymerization active site of family B DNA polymerases. The side chain of d29 DNA polymerase residue Val-250 fits into a structural cavity formed by residues Leu-253 (from the DYNLSLYP motif) and Gly-481 and Trp-483 (belonging to the LEXE motif studied here, see Figs. 1B and 7). In the rest of family B DNA polymerases, the correct orientation of the carbonyl group of the Phe/Tyr residue from the D(F/Y)XSLYP motif (homologous to the Val residue in the protein-primed DNA polymerases) is guaranteed by its packaging with the Ile or Leu residues from the LEXE motif (in black letters over a gray background in the alignment shown in Fig. 2; see also Fig. 7). Thus, whereas the shortening of the side chain in mutant W483L could provoke an altered positioning of the carbonyl group of Val-250, the change of d29 DNA polymerase residue Gly-481 into Phe could impose sterical clashes with residue Val-250, although in this case the
reduced activity does not allow us to rule out a misfolding of the protein. Those sterical clashes could lead most probably to a distortion of the spatial arrangement of the Val-250 carbonyl group that could be responsible for the low catalytic efficiency for the insertion of the incoming nucleotide, the reduced selectivity against noncorrect nucleotides, and the negligible pyrophosphorolytic activity observed with mutants at residues Gly-481 and Trp-483. The fact that Mn\(^{2+}\) partially recovers the activity of mutants at residues Gly-481 and Trp-483 could be due to the longer ionic radius of Mn\(^{2+}\) (0.8 Å) with respect to that of Mg\(^{2+}\) (0.66 Å) that could partially restore the interactions between Val-250 and the incoming nucleotide. Altogether, the results led us to conclude that the main role of the network formed by Val-250, Leu-253, Gly-481, and Trp-483 would be to maintain the correct orientation of residue Val-250 with respect to metal B that stabilizes the reactive state of the β- and γ-phosphates of the incoming dNTP (10).

As mentioned above, the LEXE motif is characterized by a Glu residue almost invariant in all family B members (24). Crystallographic studies of bacteriophage RB69 DNA polymerase have shown that concomitantly to the fingers rotation, required to lock the dNTP in the active site, a reorientation of the side chain of Glu-686 (homolog to \(\phi 29\) DNA polymerase Glu-486) takes place. This motion allows residue Glu-686 to interact with a noncatalytical metal ion C, which indirectly contacts the γ-phosphate of dNTP through an ordered water molecule (5, 16, 23). This interaction was predicted to assist the proper positioning of the dNTP in a catalytically competent manner to be attacked by the 3'-hydroxyl group of the primer during formation of the phosphodiester bond (16). The results presented here give support to that hypothesis. We have shown that the mutations introduced at the corresponding \(\phi 29\) DNA polymerase residue Glu-486 diminished the replication velocity in the presence of Mg\(^{2+}\) ions as metal activator, possibly due to a decrease in the affinity for the incoming nucleotide. As it occurred with mutants at the residues forming the structural cavity mentioned above, the presence of Mn\(^{2+}\) enhanced dramatically all the synthetic activities of mutants at residue Glu-486, supporting a metal-binding role for this residue. Such a recovery could be due also to the longer ionic radius of Mn\(^{2+}\) with respect to that of Mg\(^{2+}\) mentioned above that could promote a different set of contacts with the incoming nucleotide, as has been reported to occur in pol β, where Mg\(^{2+}\) interacts with the β- and γ-phosphates of the incoming nucleotide while Mn\(^{2+}\) contacts the α- and γ-phosphates (48). RB69 DNA polymerase E686A mutant exhibited a phenotype closely similar to that described for mutants at \(\phi 29\) DNA polymerase residue Glu-486, showing that the reduced polymerization activity...
displayed with Mg$^{2+}$ was recovered to wild-type levels in the presence of Mn$^{2+}$ (16).

The role of the LEXE motif in guaranteeing adequate contacts with the γ-phosphate of the incoming nucleotide, either through metal B (φ29 DNA polymerase residues Gly-481 and Trp-483) or through potential metal C (φ29 DNA polymerase residue Glu-486), is supported by the deficient pyrophosphorolytic activity exhibited by mutants at those positions when Mg$^{2+}$ was used as metal activator.

A relevant parameter for measuring the fidelity of a DNA polymerase is the relation between the catalytic efficiency for the insertion of correct and incorrect nucleotides (30). As mentioned under “Results,” the discrimination value obtained for most of the mutants was lower than that of the wild-type enzyme. Considering those data, it is tempting to speculate that residues of the LEXE motif contribute to discrimination against an incorrect base pairing.

The three-dimensional structure of the family B T. gorgonarius DNA polymerase also agrees to a role as a metal ligand for the second Glu of motif LEXE (47). In this case the two Glu residues (Glu-578 and Glu-580) form an unexpected metal-binding site for Mn$^{2+}$ and Zn$^{2+}$. Its proximity to the catalytic Asp-404 and the γ-phosphate of the incoming nucleotide suggested a role in nucleotide binding and/or catalysis (47). Our biochemical data, together with the crystallographic structures of the ternary complexes of these eukaryotic type DNA polymerases, support a role for the Glu-486 residue in the coordination of a third metal ion at the polymerization active site of φ29 DNA polymerase. This metal would be critical not only for nucleotide incorporation but also for further PR, release (see Fig. 1A). The high conservation of this residue suggests a similar role as a metal ligand for the homologous Glu residue in the rest of family B DNA polymerases.

An essential role for binding a third metal ion has been described recently for the X and Y family DNA polymerases, pols β and η (17, 18). This ion has been suggested to establish an active site organization that could be necessary for pyrophosphorolysis (18), as well as to stabilize the transition state facilitating product release. These results together with the structural and biochemical studies performed in family B DNA polymerases RB69 (5, 16, 23) and φ29 (this paper) support the notion of the third metal ion as a general feature of the two-metal ion mechanism.

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