

Fidelity of $\phi 29$ DNA Polymerase

COMPARISON BETWEEN PROTEIN-PRIMED INITIATION AND DNA POLYMERIZATION*

(Received for publication, May 29, 1992)

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$\phi 29$ DNA polymerase is able to catalyze two different synthetic reactions: protein-primed initiation and DNA polymerization. We have studied the fidelity of $\phi 29$ DNA polymerase when carrying out these two reactions. Global fidelity was dissected into three steps: insertion discrimination, mismatch elongation, and proofreading. The insertion discrimination of $\phi 29$ DNA polymerase in DNA polymerization ranged from 10^4 to 10^6 . The efficiency of mismatch elongation was 10^5 – 10^6 -fold lower than that of a properly paired primer terminus. These factors indicate that DNA polymerization catalyzed by $\phi 29$ DNA polymerase is a highly accurate process.

Conversely, the insertion fidelity of protein-primed initiation was quite low, the insertion discrimination factor being about 10^2 . Mismatch elongation discrimination was also rather low: mismatched terminal protein (TP)·dNMP complexes were elongated from 2- to 6-fold more slowly than the correct TP·dNMP complex. Even more, the $3' \rightarrow 5'$ exonuclease activity of $\phi 29$ DNA polymerase was unable to act on the TP·dNMP initiation complex, precluding the possibility that a wrong dNMP covalently linked to TP could be excised and corrected. Therefore, protein-primed initiation can be predicted as a quite inaccurate reaction. The problem of maintaining the sequence at the DNA ends is discussed in the context of a recently described model for protein-primed initiation.

DNA replication *in vivo* has been described as a highly accurate process, with error frequencies between 10^{-9} and 10^{-12} (1, 2). This faithful genome duplication relies on multiple sequential steps to discriminate against errors, and the critical role of DNA polymerases in ensuring a precise DNA synthesis has been clearly established. In this sense, the number of purified DNA polymerases and *in vitro* studies examining their ability for base selection are increasing (3–12). Despite the fact that *in vitro* studies probably cannot mimic precisely the actual *in vivo* conditions in which DNA synthesis occurs, it has proven to be a powerful method to

elucidate the mechanisms responsible for the fine nucleotide selection exhibited by most DNA polymerases.

Two fidelity mechanisms have been shown to operate during DNA polymerization (for a review, see 13): (a) nucleotide insertion discrimination (DNA polymerases preferentially select the correct dNTP to catalyze phosphodiester bond formation); (b) exonucleolytic proofreading (the $3' \rightarrow 5'$ exonuclease activity of DNA polymerases excises noncomplementary nucleotides at the $3'$ -OH primer terminus more rapidly than correctly paired nucleotides). The insertion discrimination step can operate at two levels. First, wrong dNTPs might be bound with lower affinity than right ones, probably because of a faster dissociation rate of mispaired dNTPs (3, 4, 7, 11). Second, the rate of phosphodiester bond catalysis can be slower for incorrect dNTPs than for correct ones (5, 6, 11). In addition, intermediate conformational changes of DNA polymerase can be especially disfavored when a mispaired dNTP is bound (5, 11). The exonucleolytic proofreading is probably based on two mechanisms. First, a mispaired $3'$ -terminus is intrinsically easier to melt and is, therefore, a better substrate for exonuclease activity, according to the "melt and slide" model for exonucleolysis (14). Second, it has been proven that the addition of the next nucleotide onto a mismatched $3'$ -terminus is an inefficient step, increasing the exposure time of the mispaired end to the exonuclease activity (5, 11, 15). All of these steps have been analyzed in different DNA polymerases, and the relative importance of each one is dependent on the specific DNA polymerase.

In this paper we present the characterization of the fidelity mechanisms exhibited by bacteriophage $\phi 29$ DNA polymerase. Two main points justify the relevance of studying the error discrimination ability of another DNA polymerase. First, $\phi 29$ DNA polymerase is able to use two different molecules as primers to catalyze nucleotide incorporation. During DNA polymerization, $\phi 29$ DNA polymerase uses a DNA primer as donor of free $3'$ -OH groups, but for the initiation of replication of the $\phi 29$ linear genome, the $-OH$ group is provided by a serine residue of the $\phi 29$ terminal protein (for review, see 16). These two reactions are thought to share the same active center (17, 18), but they show clear biochemical differences (19, 20). Therefore, it is important to elucidate and compare the fidelity mechanisms exhibited in these two different reactions, trying to correlate these mechanisms with the specific characteristics of protein-primed or DNA-primed nucleotide incorporation. For these correlations it is also important to bear in mind the possibly different physiological relevance of error discrimination at the two steps of $\phi 29$ DNA replication (initiation of DNA replication and subsequent DNA polymerization). On the other hand, these studies represent the first kinetic characterization of a protein-primed initiation reaction. Therefore, it has been possible to compare not only the fidelity mechanisms operating in protein-primed initiation and DNA polymerization but also the kinetic pa-

* This investigation has been aided by Research Grant 5R01 GM27242-13 from the National Institutes of Health, by Grant PB90-0091 from Dirección General de Investigación Científica y Técnica, by Grant BIOT CT91-0268 from European Economic Community, and by an institutional grant from Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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rameters of correct nucleotide incorporation in these two reactions. The fidelity of the initiation reaction was studied separately in three individual steps: insertion discrimination, proofreading, and mismatch elongation. In the case of DNA polymerization, only insertion discrimination and mismatch elongation were evaluated; the proofreading ability of $\phi 29$ DNA polymerase has been the subject of a recent publication (21).

MATERIALS AND METHODS

Nucleotides and Proteins—Ultrapure unlabeled dNTPs were from Pharmacia LKB Biotechnology; [α - ^{32} P]dNTPs (400 Ci/mmol) and [γ - ^{32} P]ATP (3,000 Ci/mmol) were obtained from Amersham International Plc. T4 polynucleotide kinase was purchased from New England Biolabs. Wild-type and exonuclease-deficient (D12AD66A; 22) $\phi 29$ DNA polymerases were purified essentially as described (23), with modifications to be published elsewhere. $\phi 29$ TP¹ was purified as described (24).

DNA Templates—TP·DNA complex was obtained as described (25). Complementary single-stranded oligonucleotides SP1 (5'-GAT-CACAGTGAGTAC) and SP1c+6 (5'-TCTATTGTACTACTGT-GATC) were synthesized in a 391 DNA Synthesizer (Applied Biosystems) as described (26), purified by 20% PAGE, ethanol precipitated and, in the case of SP1, 5'-labeled with [γ - ^{32} P]ATP and T4 polynucleotide kinase. Further separation by PAGE was necessary to purify the full-size 5'-labeled oligonucleotide. The specific activity obtained was 2.5×10^5 cpm/pmol. The primer/template 5'- ^{32} P-SP1/SP1c+6 molecule was obtained by hybridization between SP1 and SP1c+6.

Measurement of Nucleotide Insertion Rates Using DNA as Primer— $\phi 29$ DNA polymerase was allowed to interact with template/primer DNA (SP1/SP1c+6) at equilibrium by preincubating for 15 min at 0 °C all of the components of the final reaction except the metal activator. Then, the incubation mixture was tempered to 30 °C and the reactions started by adding MgCl₂ or MnCl₂. The final reaction mixture contained, in 12.5 μ l, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 1 nM 5'- ^{32} P-SP1/SP1c+6, 30 nM exonuclease-deficient $\phi 29$ DNA polymerase, 10 mM MgCl₂ or 1 mM MnCl₂, and various concentrations of dNTPs. After 2 s at 30 °C, reactions were stopped with EDTA up to 100 mM. The amount of elongated primers was analyzed by 20% PAGE, autoradiography, and densitometry. At the highest dATP (correct nucleotide) concentrations all the primers were elongated in less than 2 s, preventing an estimation of the nucleotide insertion rate in linear conditions. In the case of wrong dNTPs, the highest concentrations produced severe inhibition of nucleotide insertion. Therefore, these concentrations were discarded to calculate nucleotide insertion rates.

Misinsertion in the Presence of Different Metal Activators Using DNA as Primer—The reaction mixture was as described in the previous section but using 25 μ M dATP, 3 nM exonuclease-deficient $\phi 29$ DNA polymerase and, as metal activators, 10 mM MgCl₂, 1 mM MnCl₂, 2 mM ZnCl₂, 1 mM CoCl₂ or 0.5 mM FeSO₄. Incubation was for 5 min at 30 °C, and reactions were directly quenched with 3 μ l of loading buffer. DNA polymerization products were analyzed by PAGE, autoradiography, and densitometry.

Measurement of Mismatch Elongation Using DNA as Primer—Primers with 3'-terminal mismatches were formed in the same reaction mixture as described previously but using 60 nM exonuclease-deficient $\phi 29$ DNA polymerase and a 100 μ M concentration of the selected wrong nucleotide to be incorporated at the primer terminus. After 15 min at 30 °C all of the primer molecules were elongated one position, the desired wrong dNMP being at their 3'-end (not shown). Mismatch elongation rate was measured by adding various concentrations of the next correct nucleotide (dATP) and further incubating for different times (depending on the particular mismatch) at 30 °C. The reactions were stopped by the addition of EDTA to 100 mM. Mismatch elongation was analyzed by PAGE, autoradiography, and densitometry.

Measurement of Insertion Rates in Protein-primed Initiation— $\phi 29$ DNA polymerase was allowed to interact with TP and template (TP·DNA) by preincubating for 15 min at 0 °C all of the components of the final reaction except the metal activator. Then, the incubation

mixture was tempered to 30 °C, and the reactions were started by adding MgCl₂ or MnCl₂. The final reaction mixture contained, in 25 μ l, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 1.5 nM TP·DNA (two origins/molecule), 150 nM TP, 15 nM exonuclease-deficient $\phi 29$ DNA polymerase, and 10 mM MgCl₂ or 1 mM MnCl₂. The final dNTP concentration was varied keeping constant the [α - ^{32} P]dNTP concentration (0.05 μ M for dATP or 0.25 μ M for wrong dNTPs) and adding different concentrations of the corresponding unlabeled dNTP. Incubation was at 30 °C for different times to calculate the reaction velocity, and reactions were stopped by the addition of EDTA to 10 mM and SDS to 0.1%. The samples were filtered through Sephadex G-50-spun columns in the presence of 0.1% SDS, and the excluded volume was subjected to 10% SDS-PAGE. The TP·dNMP complexes were detected by autoradiography and quantified by densitometry in the presence of convenient markers. When initiation complexes were expected to be elongated giving rise to different TP·(dNMP)_n products, electrophoresis was carried out in 12% SDS-PAGE (360 $\times$ 280 $\times$ 0.5 mm). In these conditions, the different TP·(dNMP)_n complexes are resolved.

Misinsertion in the Presence of Different Metal Activators in Protein-primed Initiation—The reaction mixture was as described in the preceding section but using 0.1 μ M [α - ^{32} P]dNTP (1 μ Ci) and 1 mM MnCl₂, 1 mM FeSO₄, 2 mM ZnCl₂, 0.5 mM CoCl₂ or 10 mM MgCl₂ as the metal activator. After incubation for 2 min at 30 °C, the reactions were stopped by the addition of EDTA to 10 mM and SDS to 0.1% and analyzed as described in the previous section.

Measurement of Mismatch Elongation in Protein-primed Initiation—Mismatched TP·dNMP complexes were formed in the same reaction mixture as described in the previous section, using 0.25 μ M (2.5 μ Ci) of the wrong nucleotide to be covalently linked to TP. After 15 min at 30 °C, the elongation rates of these mismatches were measured by adding various concentrations of dATP (the next correct nucleotide) and further incubating for 5 min at 30 °C. To avoid the interference of initiation complexes synthesized in the second incubation, the labeled dNTP was diluted with its corresponding unlabeled dNTP at 100 μ M. The reactions were stopped by the addition of EDTA to 10 mM and SDS to 0.1% and analyzed as described for nucleotide insertion rate measurements.

RESULTS

Insertion Fidelity of $\phi 29$ DNA Polymerase during DNA Polymerization—The ability of $\phi 29$ DNA polymerase to discriminate between right and wrong nucleotides at the insertion step of DNA polymerization was evaluated using a highly purified exonuclease-deficient mutant (22) to avoid the interference of the 3' $\rightarrow$ 5' exonuclease activity. The DNA substrate was a 5'-labeled 15/21-mer (SP1/SP1c+6), which offers a T as first nucleotide on the template, and the rate of nucleotide insertion was measured by quantifying the elongation products of the 5'-labeled primer (4) (see "Materials and Methods"). These experiments were carried out using Mg²⁺ or Mn²⁺ as the metal activator. Mn²⁺ has been described as an efficient activator of the synthetic activities of $\phi 29$ DNA polymerase (20) although, in general, Mn²⁺-activated DNA polymerases are error-prone (6, 27–31). Therefore, it was interesting to examine the influence of the metal activator on the insertion fidelity of $\phi 29$ DNA polymerase.

The kinetic studies were carried out in conditions in which each DNA polymerase molecule catalyzed a single base insertion onto a previously bound template/primer molecule. These conditions were achieved by preincubating a 30-fold molar excess of $\phi 29$ DNA polymerase over template/primer and starting the reaction by the addition of the metal-chelated dNTP (see "Materials and Methods"). This approach allows a direct estimation of the true nucleotide incorporation rate, avoiding other possible rate-limiting steps such as DNA association or dissociation.

The dNTP insertion rates were measured at different dNTP concentrations and analyzed by linear regression. The initial slope in a plot of rate versus dNTP concentration defines the apparent second-order rate constant k_{cat}/K_m (in-

¹ The abbreviations used are: TP, terminal protein; TP·DNA, terminal protein- $\phi 29$ DNA covalent complex; PAGE, polyacrylamide gel electrophoresis.

TABLE I

Insertion efficiencies and discrimination factors in Mg^{2+} - and Mn^{2+} -activated DNA polymerizationInsertion rates were measured at different dNTP concentrations within a linear range, and analyzed as described under "Materials and Methods" to obtain insertion efficiencies (k_{cat}/K_m) and discrimination factors (f).

	Mg^{2+}		Mn^{2+}	
	k_{cat}/K_m	Discrimination factors	k_{cat}/K_m	Discrimination factors
	$s^{-1} \mu M^{-1}$		$s^{-1} \mu M^{-1}$	
T:dAMP ^a	6.8 ± 1.9^b	1	15.1 ± 5.1	1
T:dCMP	$(1.5 \pm 0.3) \times 10^{-4}$	4.5×10^4	$(6.2 \pm 1.8) \times 10^{-4}$	2.5×10^4
T:dGMP	$(3.9 \pm 0.7) \times 10^{-5}$	1.8×10^5	$(1.0 \pm 0.4) \times 10^{-3}$	1.5×10^4
T:dTMP	$(2.8 \pm 0.4) \times 10^{-6}$	2.5×10^6	$(3.8 \pm 0.3) \times 10^{-4}$	4.0×10^4

^a T:dNMP stands for the insertion of a dNMP residue opposite T on the template, using SP1/SP1c + 6 as primer/template.^b The errors of the mean were calculated by standard statistical methods with a confidence interval of 90%. Only the errors of the insertion efficiencies are indicated, for simplicity.

sion efficiency). The results obtained with the right (dATP) and wrong (dCTP, dGTP, and dTTP) nucleotides using Mg^{2+} or Mn^{2+} as the metal activator are shown in Table I. Individual estimations of k_{cat} and K_m for the correct nucleotide were not possible because of its fast insertion rate (the minimal reaction time, 2 s, was too long to measure the maximal velocity of correct nucleotide insertion accurately). In the case of wrong nucleotides, the hyperbolic behavior of insertion rate as a function of dNTP concentration overlapped with the inhibition observed at high dNTP concentrations, precluding an accurate estimation of k_{cat} and K_m . Nevertheless, it is important to point out that fidelity values can be obtained from the ratio of the insertion efficiencies for right versus wrong nucleotides ($f = (k_{cat}/K_m)_R / (k_{cat}/K_m)_W$). As shown in Table I, the discrimination values obtained with Mg^{2+} as the metal activator ranged from 4.5×10^4 (T:dCMP mispair)² to 2.5×10^6 (T:dTMP mispair). Mn^{2+} , in general, reduced the insertion fidelity of $\phi 29$ DNA polymerase, but the extent of this reduction was dependent on the individual mispair; the formation efficiency of T:dGMP and T:dTMP mispairs was increased 10- and 100-fold, respectively, whereas the misinsertion efficiency of the T:dCMP mispair was not altered significantly.

The insertion fidelity of $\phi 29$ DNA polymerase was qualitatively evaluated in the presence of other metal ions known to support its polymerization activity (20). In this assay we examined the elongation of the 15/21-mer (SP1/SP1c+6) with dATP (see "Materials and Methods"). The correct incorporation of two dAMP residues is expected, and further elongation of the primer would imply the formation of an A:dAMP mispair (Fig. 1A). This approach allows a direct comparison between the efficiency of correct versus wrong nucleotide insertion. As shown in Fig. 1B, all of the metal activators tested, except Zn^{2+} , reduced the fidelity of $\phi 29$ DNA polymerase. This result, together with the previous kinetic study (Table I), shows that the insertion fidelity of $\phi 29$ DNA polymerase is markedly affected by the metal ion used as activator.

Elongation Efficiency of Mismatched DNA Primer Termini—Another important fidelity mechanism for $3' \rightarrow 5'$ exonuclease-containing DNA polymerases is the competition between mismatch extension and exonucleolytic excision. A slow rate of mismatch elongation will stall DNA polymerase after misincorporation, increasing the time for proofreading to act. In the absence of proofreading, an inefficient extension of mismatched primers would eventually lead to DNA dissociation and abortion of DNA synthesis after misinsertion. In

the case of $\phi 29$ DNA polymerase, its strong $3' \rightarrow 5'$ exonuclease activity has been shown to act as a proofreading activity (21).

The 15/21-mer primer/template was elongated by the addition of one nucleotide forming the three possible mismatches (T:dCMP, T:dGMP, and T:dTMP). Then, the mismatch extension efficiency (k_{cat}/K_m) of these three mismatches was estimated measuring the extension rate at different dATP concentrations (dATP is the next correct nucleotide), using Mg^{2+} or Mn^{2+} as the metal activator (Fig. 2a) (see "Materials and Methods"). These experiments were carried out with a 60-fold molar excess of the exonuclease-deficient $\phi 29$ DNA polymerase mutant over mismatched primers. In these conditions DNA binding is saturated, and the elongation rate reflects the nucleotide insertion rate. On the other hand, it has been observed in other systems that inefficient mismatch extension is mainly because of a slow nucleotide insertion rather than a low affinity for mismatched primers (32).

The extension efficiency of the three mismatches, as well as the elongation discrimination factors, defined by the ratio of these mismatch extension efficiencies versus the extension efficiency of a properly paired DNA primer (unextended 15/21 primer/template), are shown in Table II. Using Mg^{2+} as the metal activator, the discrimination factor for elongating T:dGMP and T:dTMP mismatches was 2.7×10^5 and 4.2×10^6 , respectively. These factors are comparable to other values reported in different systems (5, 11, 15). However, the elongation efficiency of the T:dCMP mispair was surprisingly high (only 8-fold lower than the extension efficiency of the correctly paired primer terminus). This efficiency was so high that it does not seem to reflect the actual mismatch elongation. In fact, taking into account the nucleotide composition of the template strand, we think that the most plausible explanation for this result would be a misalignment event, as depicted in Fig. 2b. The mismatched $3'$ -terminal dCMP residue might pair with the previous G in the template, dislocating the primer strand. In this conformation, the $3'$ -primer terminus would be properly paired and could be efficiently elongated. Misalignment-mediated mutagenesis has been described in other systems (for a review, see 33). Using Mn^{2+} as the metal activator, the elongation efficiencies of T:dGMP and T:dTMP mismatches increased 100- and 10-fold, respectively (see Table II). However, the T:dCMP mismatch was elongated much more inefficiently than in the presence of Mg^{2+} . According to the previous interpretation, this slow mismatch elongation would indicate that in the presence of Mn^{2+} the misaligned conformation is disfavored with respect to the extended mismatched conformation (in fact, Mn^{2+} has been described as stabilizing mismatched duplexes (34)). In

² The first symbol (N) stands for the nucleotide present on the template; the second symbol (dNMP) represents the nucleotide inserted at the primer terminus, opposite N.

this sense, the measured efficiency would reflect the actual mismatch elongation efficiency.

Insertion Fidelity of $\phi 29$ DNA Polymerase during Protein-primed Initiation—The fact that protein-primed initiation is a DNA-instructed reaction, as has been demonstrated recently (35), offers the possibility of quantifying the fidelity of this reaction and of elucidating the mechanisms responsible for nucleotide selection when a protein is used as primer.

The kinetic characterization of the insertion fidelity during protein-primed initiation was carried out using the natural TP·DNA as template (which offers a T as first template nucleotide), TP as primer, and an exonuclease-deficient $\phi 29$ DNA polymerase mutant (see “Materials and Methods”). In this reaction, it was especially relevant to compare the insertion discrimination obtained with Mg^{2+} and with Mn^{2+} because Mn^{2+} is a much better activator of the initiation reaction than Mg^{2+} (20). The interference of other possible rate-limiting steps as TP·DNA polymerase complex formation or

origin positioning was avoided by preincubating a 10-fold molar excess of TP over $\phi 29$ DNA polymerase; this TP·DNA polymerase complex was incubated in a 5-fold molar excess over template origins, and the reaction was started by the addition of the metal-chelated dNTP. The reaction time was short enough to ensure that most of the origins were not used (see “Materials and Methods”). Under these conditions, measuring the insertion rate at different dNTP concentrations and by standard double-reciprocal plot analysis, it was possible to estimate the K_m and k_{cat} for correct (dATP) and incorrect (dCTP, dGTP, and dTTP) nucleotides (Table III). In the presence of Mn^{2+} , the discrimination factors obtained ranged from 1.1×10^2 (T:dGMP) to 1.3×10^3 (T:dCMP). It can be also observed that the kinetic parameter mainly responsible for this insertion fidelity is K_m . The K_m ratio of wrong nucleotides versus dATP varied between 82 (dGTP) and 235 (dCTP), whereas the k_{cat} for dATP was only 1.4 or 5.4 times greater than the k_{cat} for dGTP or dCTP, respectively. The misinsertion reactions in the presence of Mg^{2+} were so weak that only the misinsertion efficiency (k_{cat}/K_m) is indicated. In fact, the reaction obtained with dCTP was so low that this parameter could not be accurately estimated. The fidelity factors that could be determined in the presence of Mg^{2+} were quite similar to those obtained in the presence of Mn^{2+} .

The insertion discrimination of $\phi 29$ DNA polymerase in protein-primed initiation was also examined qualitatively in the presence of other metal ions known to activate the initiation reaction (20). As shown in Fig. 3, the initiation reaction obtained with dATP was always higher than the one obtained with the other (wrong) dNTPs, and there was no apparent stimulation of misincorporation using different metal activators. Therefore, the insertion fidelity of the initiation reaction does not seem to be severely affected by the metal ion used as activator.

Editing on the TP·dNMP Complex—The ability of $\phi 29$ DNA polymerase to catalyze DNA polymerization as well as protein-primed initiation using the same active site (17, 18) led to the proposal that TP is structurally equivalent to the double-stranded portion of a template/primer DNA molecule (36). This model poses the question of whether the TP·dNMP complex could be a substrate for the $3' \rightarrow 5'$ exonuclease activity of $\phi 29$ DNA polymerase. First, the sensitivity to the exonucleolytic activity of the phosphodiester bond between Ser²³² in TP and dAMP (the first 5'-nucleotide of the TP·DNA) could be different from the standard internucleotidic phosphodiester bond. Second, although perhaps formally analogous, TP and a template/primer DNA molecule could exhibit structural differences, especially concerning the melt

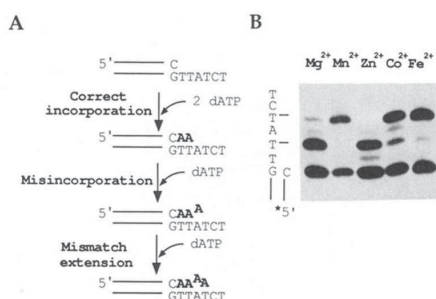


FIG. 1. Misinsertion in the presence of different metal ions. Panel A, diagram showing successive correct incorporation, misincorporation, and mismatch elongation using dATP as the only nucleotide. Panel B, electrophoretic analysis of SP1/SP1c+6 primer extension using 25 μM dATP and different metal ions as activators (see “Materials and Methods”). The asterisk at the 5'-end of the primer strand stands for ³²P labeling.

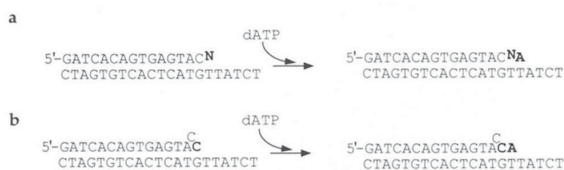


FIG. 2. Diagram of the strategy used to measure the rate of mismatch elongation. Panel a, true mismatch elongation. Panel b, primer misalignment leading to elongation of a correctly paired (but dislocated) primer terminus.

TABLE II

Elongation efficiencies and discrimination factors in Mg^{2+} - and Mn^{2+} -activated DNA polymerization

Elongation rates were measured at different dATP concentrations and analyzed as described for Table I. Elongation efficiencies (k_{cat}/K_m) and discrimination factors are shown.

	Mg^{2+}		Mn^{2+}	
	k_{cat}/K_m	Discrimination factors	k_{cat}/K_m	Discrimination factors
	$s^{-1} \mu M^{-1}$		$s^{-1} \mu M^{-1}$	
G:dCMP ^a	6.8 ± 1.9^b	1	15.1 ± 5.1	1
T:dCMP ^c	0.8 ± 0.2	8.1	$(5.4 \pm 1.1) \times 10^{-4}$	2.8×10^4
T:dGMP	$(2.5 \pm 0.6) \times 10^{-5}$	2.7×10^5	$(7.3 \pm 0.4) \times 10^{-3}$	2.1×10^3
T:dTMP	$(1.6 \pm 1.1) \times 10^{-6}$	4.2×10^6	$(7.3 \pm 5.7) \times 10^{-5}$	2.1×10^5

^a G:dCMP stands for the elongation of the SP1/SP1c + 6 primer/template with dATP. This DNA molecule has a G:C pair at its primer terminus, C being on the primer strand.

^b The errors of the mean were calculated as in Table I.

^c Mismatches were preformed by adding one wrong nucleotide onto the SP1/SP1c + 6 primer terminus, opposite T on the template. Hence, T:dNMP stands for the elongation of these preformed primer termini (N being on the primer strand) with dATP (see Fig. 2a).

TABLE III

Kinetic constants and discrimination factors in Mg^{2+} - and Mn^{2+} -activated protein-primed insertion

Insertion rates were measured at different dNTP concentrations and analyzed as described under "Materials and Methods" to obtain kinetic constants (K_m and k_{cat}), insertion efficiencies (k_{cat}/K_m), and discrimination factors.

	Mg^{2+}				Mn^{2+}			
	K_m	k_{cat}	k_{cat}/K_m	Discrimination factors	K_m	k_{cat}	k_{cat}/K_m	Discrimination factors
	μM	s^{-1}	$s^{-1} \mu M^{-1}$		μM	s^{-1}	$s^{-1} \mu M^{-1}$	
TP·dAMP:T ^a	5.3	3.9×10^{-4}	$(7.4 \pm 0.3) \times 10^{-5}$	1	0.2	1.5×10^{-3}	$(7.5 \pm 1.5) \times 10^{-3}$	1
TP·dCMP:T	ND ^c	ND	ND	ND	47.1	2.8×10^{-4}	$(5.9 \pm 0.4) \times 10^{-6}$	1.3×10^3
TP·dGMP:T	ND	ND	$(2.1 \pm 0.3) \times 10^{-7}$	3.5×10^2	16.5	1.1×10^{-3}	$(6.7 \pm 0.4) \times 10^{-5}$	1.1×10^2
TP·dTTP:T	ND	ND	$(2.5 \pm 0.5) \times 10^{-7}$	3.0×10^2	20.1	6.2×10^{-4}	$(3.1 \pm 0.6) \times 10^{-5}$	2.4×10^2

^a TP·dNMP:T stands for the covalent linking of a dNMP residue onto the terminal protein primer, opposite T on the $\phi 29$ DNA template.

^b The errors of the mean were calculated as in Table I.

^c ND, not determined.

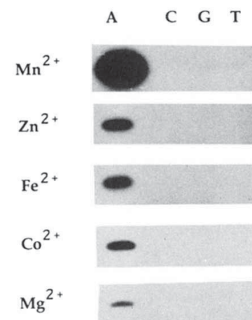


FIG. 3. Protein-primed misinsertion in the presence of different metal ions. Electrophoretic analysis of protein-primed initiation showing the TP·[³²P]dNMP band obtained with each of the four [³²P]dNTPs and different metal ions as activators (see "Materials and Methods"). The intensity of the TP·[³²P]dAMP band reflects the activation efficiency of each metal at its optimal concentration (20).

and slide model for exonucleolytic editing (14).

The ability of $\phi 29$ DNA polymerase to excise the first nucleotide covalently linked to TP was examined comparing the initiation reaction at low dATP concentration (0.25 μM) using the wild-type $\phi 29$ DNA polymerase and the exonuclease-deficient mutant. If the TP·dAMP complex is susceptible to exonucleolytic degradation, wild-type $\phi 29$ DNA polymerase is expected to excise the dAMP residue covalently linked to TP, yielding a lower amount of TP·dAMP complexes than the exonuclease-deficient mutant, and releasing dAMP. This experiment was carried out in the absence of template (36) to ensure that only one dAMP residue is inserted on TP. This caution is necessary because TP·DNA has three consecutive Ts at its 3' ends, allowing potential incorporation of several dAMP residues on TP. As shown in Fig. 4A, the amount of TP·dAMP complexes formed by wild-type polymerase or the exonuclease-deficient mutant was quite similar. In addition, no [³²P]dAMP was released by the wild-type enzyme (Fig. 4B). This result strongly suggests that there is no exonucleolytic activity on the TP·dAMP complex. As controls, the results obtained with TP·DNA as template are shown in Fig. 4, C and D. The incorporation of dAMP on TP catalyzed by the wild-type enzyme was much lower than with the exonuclease-deficient mutant (Fig. 4C), and most of the input [³²P]dATP was turned over to [³²P]dAMP after 1 h (Fig. 4D). Therefore, in the presence of TP·DNA as template, dAMP incorporation proceeds further from initiation, producing elongated products susceptible to exonucleolytic degradation (in fact, the TP·dAMP band obtained with the exonuclease-deficient mutant (Fig. 4C) appeared slightly shifted up in

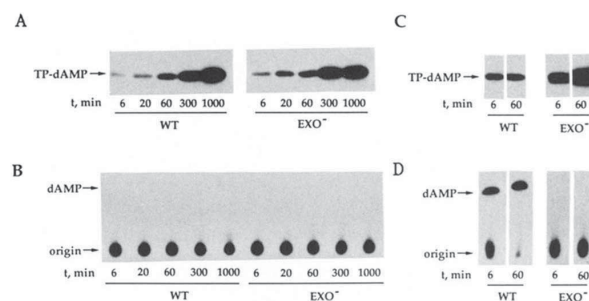


FIG. 4. Comparison between protein-primed initiation catalyzed by wild-type (WT) and exonuclease-deficient (EXO⁻) mutant $\phi 29$ DNA polymerases. **Panel A**, time course of TP·dAMP formation. Reactions were carried out and analyzed as described for Fig. 3 but omitting template, using 65 nM either exonuclease-deficient or wild-type $\phi 29$ DNA polymerase (as indicated) and 1 mM Mn^{2+} as the metal activator. Reaction times are indicated. The position of the TP·[³²P]dAMP band is indicated. **Panel B**, samples of the assay shown in panel A were subjected to polyethyleneimine-cellulose thin-layer chromatography for dAMP turnover analysis. The position expected for [³²P]dAMP as well as the chromatographic origin are indicated. **Panel C**, TP·dAMP formation in the presence of TP·DNA as template. Reactions were carried out for the indicated times using Mn^{2+} as the metal activator; analysis was as described for Fig. 3. **Panel D**, dAMP turnover analysis of the samples shown in panel C.

lower exposures, suggesting that several dAMP residues were covalently linked to TP). To evaluate which is the first elongated product that can be substrate for the exonuclease activity of $\phi 29$ DNA polymerase, dAMP incorporation on TP catalyzed by wild-type polymerase and the exonuclease-deficient mutant was compared at different dATP concentrations (Fig. 5A). This experiment was also carried out with an incorrect nucleotide (dTTP) to test the validity of these results when a wrong TP·dNMP complex is formed (Fig. 5B). Reactions were analyzed by SDS-PAGE allowing the separation of the different TP·(dNMP)_n complexes (see "Materials and Methods"). In these conditions, if the 3' → 5' exonuclease activity of $\phi 29$ DNA polymerase is able to excise the dNMP residue at the 3'-end of a TP·(dNMP)_n complex, the band corresponding to the TP·(dNMP)_{n-1} complex is expected to accumulate, taking the exonuclease-deficient mutant as reference. As shown in Fig. 5A, the reaction obtained with the wild-type $\phi 29$ DNA polymerase mostly corresponds to the TP·dAMP complex, even at the highest dATP concentration tested (1 μM). On the other hand, the appearance of the subsequent elongated products (TP·(dAMP)₂, TP·(dAMP)₃, ...) is fairly evident with the exonuclease-deficient mutant from the lowest dATP concentration tested (0.05 μM) (Fig. 5A). These results strongly suggest that the first elongated

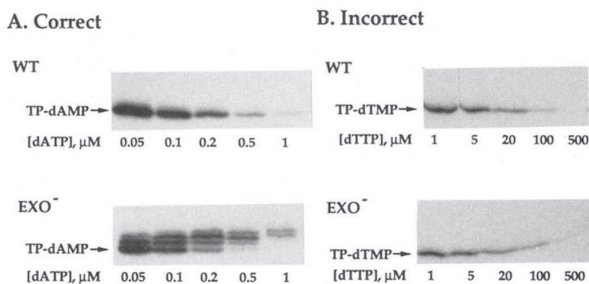


FIG. 5. Nucleotide requirements for the formation of the TP·dNMP initiation complex and the first elongated products. Panel A, comparison between protein-primed initiation and elongation catalyzed by wild-type (WT) $\phi 29$ DNA polymerase or the exonuclease-deficient (EXO⁻) mutant. Reactions were carried out and analyzed as described for Fig. 3, using Mn^{2+} as the metal activator and different concentrations of dATP, as indicated. The position of the TP·[³²P]dAMP band is shown with an arrow. Panel B, comparison between protein-primed misinsertion catalyzed by wild-type or exonuclease-deficient $\phi 29$ DNA polymerase. Reactions were carried out and analyzed as described for Fig. 3, using Mn^{2+} as the metal activator and different concentrations of dTTP, as indicated. The position of the TP·[³²P]dTMP band is shown with an arrow.

product susceptible of exonucleolytic degradation is just the TP·(dAMP)₂ complex. The TP·(dAMP)₄ complex obtained with the exonuclease-deficient mutant (Fig. 5A) is probably mediated by primer slippage on the 3'-TTT sequence of the template. As shown in Fig. 5B, there was no elongated product when the initiation reaction was carried out with a wrong nucleotide (its appearance would imply the elongation of the initial mismatch inserting again a wrong nucleotide), but the similar amount of initiation reaction obtained at different dTTP concentrations, using either the wild-type protein or the exonuclease-deficient mutant, indicates that there is no exonuclease activity on the TP·dNMP initiation complex, not even when it does not match with the 3'-end of the template.

Elongation Efficiency of Mismatched Initiation Complexes—After verifying that the initiation complex cannot be proof-read and that the insertion fidelity of the initiation reaction is rather low (10^2 – 10^3 , see Table III), mismatch elongation appeared as a crucial step for error avoidance in the protein-primed initiation reaction.

The mismatch elongation efficiency of $\phi 29$ DNA polymerase when the mismatched nucleotide is directly bound to TP was measured in a pulse-chase experiment (see "Materials and Methods"). During the pulse, separate initiation reactions were carried out with each of the three different wrong nucleotides (α -³²P-labeled) to obtain the three possible mismatched TP·dNMP initiation complexes. Then, the elongation rate of these complexes was followed at different dATP concentrations (the next correct nucleotide). These studies were carried out using Mn^{2+} as the metal activator because of the low reaction that can be obtained with Mg^{2+} . As shown in Table IV, no elongation of the TP·dCMP:T mismatch was observed at the highest dATP concentration tested. This fact can be a result of a very low elongation rate of this mismatch and/or dissociation of the complex from the replication origin. In the other two cases, a high rate of mismatch elongation was observed, and most of the complexes were elongated, indicating that elongation was faster than dissociation. Mismatch elongation efficiencies were compared with the elongation efficiency of the correctly paired TP·dAMP:T complex (calculated from a continuous initiation/elongation reaction), and the corresponding discrimination factors were calculated (Table IV). These factors (2.3 and 6.3 for TP·dGMP:T and

TABLE IV
Elongation efficiencies and discrimination factors of TP·dNMP complexes

Elongation rates were measured at different dATP concentrations as described under "Materials and Methods" and analyzed as described for Tables I and II. Elongation efficiencies (k_{cat}/K_m) and discrimination factors are shown.

	k_{cat}/K_m $\text{s}^{-1} \mu\text{M}^{-1}$	Discrimination factors
TP·dAMP:T ^a	$(8.9 \pm 1.9) \times 10^{-3b}$	1
TP·dCMP:T	ND ^c	ND
TP·dGMP:T	$(3.8 \pm 2.2) \times 10^{-3}$	2.3
TP·dTMP:T	$(1.4 \pm 0.9) \times 10^{-3}$	6.3

^a TP·dNMP:T stands for the elongation of a preformed initiation complex (dNMP being covalently bound to the terminal protein) opposite T on the template. These elongations were carried out with dATP.

^b The errors of the mean were calculated as in Table I.

^c ND, no elongation was observed at the highest dATP concentration tested (400 μM).

TP·dTMP:T, respectively) show that the elongation efficiency of a correctly matched initiation complex is not much higher than the efficiency of mismatched ones.

DISCUSSION

In this work we have evaluated the fidelity parameters governing the error discrimination ability exhibited by $\phi 29$ DNA polymerase during both DNA polymerization and protein-primed initiation.

Insertion Discrimination—The first direct conclusion that can be drawn from these studies is that $\phi 29$ DNA polymerase is able to catalyze DNA polymerization with high accuracy, comparable to that of other reported DNA polymerases (4–6, 11). With Mg^{2+} , the error frequency (the inverse of the insertion discrimination factor; see (4)) of $\phi 29$ DNA polymerase ranged from 2.2×10^{-5} (T:dCMP misinsertion) to 4×10^{-6} (T:dTMP misinsertion); the T:dGMP misinsertion showed an intermediate frequency (5×10^{-6}). Using Mn^{2+} as the metal activator, $\phi 29$ DNA polymerase showed a slight increase in the insertion efficiency of the correct nucleotide (from 6.8 to 15.1 $\text{s}^{-1} \mu\text{M}^{-1}$), whereas, in general, there was a strong increase of misinsertion efficiencies. The ability of Mn^{2+} to increase misinsertion efficiency without altering significantly the insertion efficiency of correct nucleotides is obviously one of the reasons for the error proneness of Mn^{2+} -activated DNA polymerases. In this study it was not possible to know if Mn^{2+} increased the affinity for wrong nucleotides or accelerated the mismatch catalytic rate.

Similar kinetic studies were also carried out for the protein-primed initiation reaction, obtaining independent K_m and k_{cat} estimations for correct and incorrect dNTPs, using Mn^{2+} as the metal activator. When comparing the insertion discrimination values of DNA polymerization and protein-primed initiation, at least three conclusions can be drawn. First, the insertion of the correct nucleotide (dATP) is much more efficient in DNA polymerization than in the initiation reaction. Protein-primed initiation was 10^5 or 2×10^3 times less efficient than DNA polymerization when the metal activator was Mg^{2+} or Mn^{2+} , respectively. Specific steps of the protein-primed reaction such as TP·DNA polymerase complex formation or origin recognition cannot be invoked to justify this difference, as these steps were not rate-limiting in the assay conditions. Therefore, the poor efficiency of the initiation reaction is reflecting the actual low efficiency of nucleotide insertion. Second, the insertion fidelity of protein-primed initiation is quite low, with a discrimination factor of 10^2 ,

whereas it varied from 10^4 to 10^6 in DNA polymerization. The insertion discrimination of the Mn^{2+} -activated protein-primed initiation by $\phi 29$ DNA polymerase is mainly a " K_m discrimination," K_m being about 140-fold lower for the correct dNTP than for the incorrect ones. On the other hand, the catalytic rate of the four dNTPs was rather similar (twice faster for dATP than for the other dNTPs, on average). Third, Mn^{2+} did not decrease the insertion fidelity of the initiation reaction, in contrast with the result obtained for DNA polymerization. Mn^{2+} produced a 100-fold increase of misinsertion efficiency in protein-primed initiation (quite comparable to the result obtained in DNA polymerization). However, Mn^{2+} stimulated the insertion of the correct nucleotide to a similar extent, keeping the error frequency at the same level (about 4×10^{-3}). The increased efficiency of dATP insertion in the presence of Mn^{2+} with respect to Mg^{2+} is mainly a result of an increase in the affinity for this nucleotide (a 25-fold increase in affinity versus a 4-fold increase in velocity; see also Ref. 20).

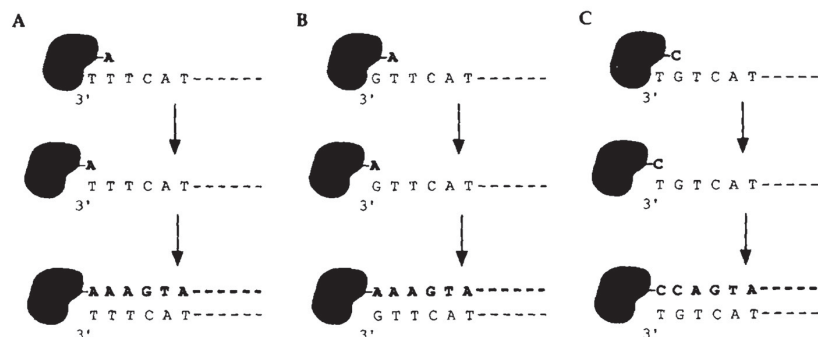
Therefore, the three main characteristics of protein-primed initiation which differentiate it from DNA polymerization at the insertion level are: poor efficiency of nucleotide insertion, low discrimination factor, and independence of this value from the metal used as activator. As discussed below, these properties are self-consistent and induce us to think of protein-primed initiation as a mechanistically unfavored reaction.

$\phi 29$ DNA polymerase has been shown to share significant amino acid homology with the Klenow fragment of *Escherichia coli* DNA polymerase I (37). Based on this homology and on site-directed mutagenesis studies (22, 37), $\phi 29$ DNA polymerase has been proposed to show a structural arrangement similar to the one observed for Klenow (38). In this model, the polymerase active site is located within a binding cleft for duplex DNA, in which the template/primer structure would be held. The two synthetic reactions carried out by $\phi 29$ DNA polymerase (DNA polymerization and protein-primed initiation) are proposed to share the same active site, and therefore, the two different primer molecules used in these reactions (DNA template/primer and TP) would have to be accommodated in the proposed binding cleft of the $\phi 29$ DNA polymerase. In this sense, the $\phi 29$ TP has been proposed to be structurally analogous to the double-stranded portion of a template/primer molecule (36). However, the interaction between TP and $\phi 29$ DNA polymerase might distort to some extent its polymerization active site. In addition, in the initiation reaction, primer (TP) and template are not covalently linked, as in DNA polymerization (considering the double-stranded region of template/primer as the real primer). These different properties of TP compared with DNA template/primer are likely diminishing the stabilization of the incoming

nucleotide, reducing its insertion efficiency. This distortion would also explain the low fidelity exhibited in this reaction. A less precise interaction with template will mask, to some extent, the differences between correct and incorrect pairing. In other systems, Mn^{2+} has been shown to increase the DNA polymerase affinity for improperly paired molecules, as wrong dNTPs or dNTP analogs (28, 39). The structural reason for this behavior is not yet clearly understood, but in some way Mn^{2+} improves the matching of such molecules with the DNA polymerase-DNA complex. In protein-primed initiation, even the right dNTP is probably not efficiently paired with template, allowing Mn^{2+} to increase the affinity for this nucleotide. The same effect would occur for wrong nucleotides, and therefore, the error frequency of this reaction is not increased in the presence of Mn^{2+} .

Exonucleolytic Proofreading—Following misinsertion, a DNA polymerase can prevent fixation of the error by excising the mismatched nucleotide, and obviously, the longer the mismatched 3'-terminus remains unextended the more likely it will be eliminated. We have clearly shown that $\phi 29$ DNA polymerase extends mismatched primer termini much slower than correctly paired primers (2×10^6 times in the presence of Mg^{2+} , on average, excluding the special T:dCMP mismatch). The strong 3' $\rightarrow$ 5' exonuclease activity of $\phi 29$ DNA polymerase is likely able to take advantage of this mismatch elongation discrimination to increase the global fidelity of DNA polymerization. Mn^{2+} , in general, decreased these discrimination factors, and again, this fact is presumably reflecting its ability to improve the fitness of a mismatched primer terminus into the primer binding site of $\phi 29$ DNA polymerase. It was not possible to measure the mismatch elongation rates of Mg^{2+} -activated protein-primed initiation; but, at least with Mn^{2+} , mismatch elongation efficiencies were only moderately lower than the elongation efficiency of a properly paired initiation complex. This poor discrimination factor is consistent with the interpretation discussed previously. The same distortion that would mask the differences between the incoming correct or incorrect nucleotides probably persists after covalently linking TP and dNMP. If this is true, the differences between matched or mismatched TP-dNMP complexes would be also diminished. The fact that this distortion is still present after catalyzing the initiation reaction is suggested by the low elongation efficiency of a correctly paired initiation complex ($8.9 \times 10^{-3} \text{ s}^{-1} \mu\text{M}^{-1}$). This efficiency is about 2,000-fold lower than the elongation efficiency of a correctly paired DNA primer terminus, despite the fact that the elongation of an initiation complex implies the formation of a standard internucleotidic bond. Another factor that would justify the poor elongation of initiation complexes is the absence of a stabilizing DNA duplex region upstream from the primer terminus, which could not be perfectly mimicked by TP.

FIG. 6. Comparative model for protein-primed initiation and elongation on both wild-type and mutated templates. Panel A, initiation and elongation on a normal template (adapted from Ref. 35). Panel B, initiation and elongation on a template mutated at its 3'-terminal nucleotide. Panel C, initiation and elongation on a template mutated at its second position.



On the other hand, we have shown that the $3' \rightarrow 5'$ exonuclease activity of $\phi 29$ DNA polymerase cannot take advantage of this poor mismatch elongation discrimination because of the insensitivity of initiation complexes (mispair or not) to this exonuclease activity. Assuming that the exonucleolytic proofreading model proposed for Klenow (14) is also valid for $\phi 29$ DNA polymerase, the inability of its exonuclease activity to excise the nucleotide covalently bound to TP can simply reflect the impossibility of this nucleotide to reach the exonuclease active site because of steric hindrance imposed by TP. It is also possible that the phosphodiester bond linking TP and the first dNMP would be chemically resistant to the exonuclease activity of $\phi 29$ DNA polymerase. On the other hand, the bond linking the first and second nucleotides was susceptible to the exonuclease action. In the absence of structural information, we do not know if this result indicates a close vicinity between the exonuclease and the polymerization active sites or the ability of TP to place this second nucleotide into the exonuclease active site, mimicking DNA primer melting and sliding.

Physiological Implications of Protein-primed Initiation Inaccuracy—After examining the three discrimination steps presented in this work (insertion, mismatch elongation, and exonucleolytic proofreading), protein-primed initiation can be predicted as a quite inaccurate DNA-instructed reaction: wrong nucleotides are easily inserted, they cannot be proofread, and their elongation is rather efficient. From these results, a high mutational rate on the first position of $\phi 29$ DNA is likely to be expected. However, $\phi 29$ and other related phages, as $\phi 15$, PZA, PZE, Nf, M2, B103, or GA-1, have inverted terminal repeats which, in addition, are homologous between them (16). Therefore, the terminal sequences of these related phages are especially conserved. It can be argued that in the *in vitro* system used (TP, $\phi 29$ DNA polymerase, and TP·DNA) some factor(s) enhancing the fidelity of the initiation reaction has/have been lost. Although this possibility cannot be ruled out, we favor the possibility that the recently described special mechanism for $\phi 29$ protein-primed initiation and elongation (35) can be responsible for maintaining the genetic information of the very terminal nucleotide. It has been reported that the initiation reaction actually takes place opposite the second position from the $3'$ -end of the TP·DNA template (Fig. 6A). After initiation, transition to elongation would be mediated by a "sliding-back" mechanism, producing the translocation of the TP·dAMP complex to pair with the terminal T residue on the template. Then, the elongation of the initiation complex would be produced using again the second $3'$ -T residue as template (Fig. 6A). Misincorporation during the initiation reaction would produce templates whose first and second terminal nucleotides are different. These molecules will serve as normal templates for protein-primed initiation, but after the sliding back of the TP·dAMP complex to allow elongation, a mismatch will be produced with the terminal $3'$ -nucleotide on the template (Fig. 6B). Part of these TP·dAMP complexes could dissociate from template before elongation, diminishing the efficiency of these molecules as templates and therefore acting as a nonexonucleolytic proofreading. If the initiation complex is finally elongated, the second $3'$ -nucleotide of the template would be used again, restoring the two-nucleotides terminal repetition at the end of the molecule (Fig. 6B). Therefore, despite the fact that the first replication event is especially inaccurate, these errors would not be fixed. According to this model, errors will be counterselected because of a less efficient elongation after sliding back, or forgotten if elongation succeeds, because the

$3'$ -terminal nucleotide is never used as template. In this sense, it is also important to note that a mutation at the second position of the template would be much more dangerous. After sliding back, again a mispaired TP·dNMP will be produced on the first position of the template (Fig. 6C), but if this complex is elongated, the mutation will be fixed and imposed to the first position. Even more, these double mutants will behave as efficient templates both for initiation and elongation because the first and second nucleotides would be again identical (35). In this sense, the importance of having exonucleolytic proofreading on the second insertion event is clearly reinforced, to avoid the formation of templates with the second position mutated.

Thus, it could be considered that the strategy proposed for the replication of TP-containing linear genomes has minimized the potentially deleterious effect of the unfaithful initiation event. The low accuracy of protein-primed initiation would be because of special mechanistic and structural properties of the initiation reaction which differentiate it from DNA polymerization.

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