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Construction and characterization of a bacteriophage T4 DNA polymerase deficient in 3′ → 5′ exonuclease activity

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ABSTRACT **Bacteriophage T4 DNA polymerase has a proofreading 3′ → 5′ exonuclease that plays an important role in maintaining the accuracy of DNA replication. We have constructed a T4 DNA polymerase deficient in this exonuclease by converting Asp-219 to Ala. The exonuclease activity of the mutant T4 DNA polymerase has been reduced by a factor of at least 10^7, but it retains a polymerase activity whose kinetic parameters, k_{cat}, K_d DNA, and K_d dATP, are very close to those of the wild-type enzyme. Bacteriophage T4 with the mutant polymerase gene has a markedly increased mutation frequency. Asp-219 in T4 DNA polymerase is within a sequence similar to those surrounding Asp residues previously shown to be essential for the exonuclease activities of the Klenow fragment of *Escherichia coli* DNA polymerase I (Asp-424), bacteriophage ϕ29 DNA polymerase (Asp-66), and *Saccharomyces cerevisiae* DNA polymerase δ (Asp-405). Thus, these studies support the proposal that there are similar sequences in the active sites for the proofreading exonucleases of these and related DNA polymerases.**

Bacteriophage T4 DNA polymerase, one of the fastest and most accurate polymerases known, is a key component of the multienzyme complex responsible for replication of the viral genome *in vivo*. T4-encoded proteins including T4 DNA polymerase (gene *43*), the genes *44/62* and *45* polymerase accessory proteins, gene *32* single-stranded DNA binding protein, and the genes *61* and *41* primase-helicase can be combined to reconstitute an *in vitro* replication system capable of leading- and lagging-strand DNA synthesis (reviewed in refs. 1–3). This T4 system exhibits properties closely resembling its *in vivo* counterpart and is thus an excellent model system for studies of T4 DNA replication. Moreover, recent reports have demonstrated distinct functional and structural similarities between the T4, *Escherichia coli*, and eukaryotic DNA replication proteins. The amino acid sequence of T4 polymerase, a single polypeptide of 898 residues (4), has several colinear regions where it shows homology with those of a large family of DNA polymerases that include human polymerase α and yeast polymerases α, ϵ, and δ (4–9). In each replication system, there is a complex of polymerase accessory proteins that increases the processivity of the polymerase. One member of each complex is a multisubunit protein with a DNA-stimulated ATPase (T4 44/62, *E. coli* γ complex, eukaryotic RF-C) that is stimulated by another accessory protein [T4 45, *E. coli* β, eukaryotic PCNA (proliferating cell nuclear antigen)] (10–16).

To fully understand the mechanism by which the replication complex carries out processive and highly accurate DNA synthesis, it is important to understand the role of each individual component upon which a complete model of the active complex can be built. T4 DNA polymerase has a 3′ → 5′ exonuclease activity that has been shown to increase the accuracy of synthesis by removing nucleotides incorrectly incorporated by the polymerase (17–20). We wished to construct a mutation in T4 DNA polymerase that would remove this exonuclease activity, without altering the polymerase activity, in order to evaluate the role of the exonuclease in maintaining the fidelity of DNA replication and to facilitate kinetic studies of the mechanism by which T4 DNA polymerase alone catalyzes DNA synthesis. Previous studies from one of our laboratories (21) to define a minimal kinetic scheme for T4 DNA polymerase (Scheme I) were complicated by the vigorous exonuclease activity of this enzyme. Exonuclease-deficient enzymes of T7 DNA polymerase (22, 23) and *E. coli* DNA polymerase I (Klenow fragment) (24) have played integral roles in the delineation of their kinetic mechanisms.

The active site for exonuclease must be toward the N terminus of T4 polymerase, since the protein made by the B22 amber mutant, missing the C-terminal 20% of the intact protein, retains an altered exonuclease but lacks the polymerase activity of the wild-type enzyme (25). Analysis of the crystal structure of the Klenow fragment of *E. coli* polymerase I and the enzymatic activities of its mutants indicate that Asp-424 is involved in coordination of a metal ion and is essential for exonucleolytic cleavage (26–29). Amino acid sequence comparisons (6) have suggested that Asp-219 in T4 DNA polymerase is in an analogous position. In this report, we describe the construction and initial characterization of a T4 DNA polymerase mutant with alanine replacing Asp-219. We show that this polymerase mutant has no measurable exonuclease, but retains a polymerase activity whose kinetic parameters are very close to those of the wild type. Bacteriophage T4 with the mutant polymerase gene have a markedly increased mutation frequency.

MATERIALS AND METHODS

Materials. Radionucleotides, [α-^{32}P]dATP, [γ-^{32}P]ATP, and deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate, were purchased from New England Nuclear. Ultrapure, unlabeled dNTPs were obtained from Pharmacia. Oligonucleotides were purified and 5′ ^{32}P-end-labeled as described by Capson *et al.* (21).

Construction of Polymerase Mutants. A phagemid containing T4 gene *43* under control of the Plac promoter [pPST4pol (formerly pPS701)] was constructed by P. Spacciapoli and N.G.N. (unpublished data) using the wild-type polymerase gene from pTL43W, the gift of T.-C. Lin and W. Konigsberg (30). *In vitro* mutagenesis was carried out as described by Kunkel *et al.* (31) except that T4 DNA polymerase and the 44/62 and 45 polymerase accessory proteins were used for DNA synthesis. The Asp-219 to Ala (D219A) mutation was created with the oligonucleotide complementary to GAGGGGTTT**GC**CGTTCCGTATATC and the D189A plus E191A mutation with GACCGAGTAATTTATATGCCATTCGCTAATGCGCGTGATATGCTCATGG (new *Bst*UI

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site is underlined). Phagemids with the double mutant were identified by digestion with *Bst*UI. The region of the D219A phagemid including gene *43* was subcloned into pUC18 and sequenced with Sequenase (United States Biochemical).

The wild-type and D219A mutant polymerases were induced with isopropyl β-D-thiogalactopyranoside and purified to >95% by the procedure of P. Spacciapoli and N.G.N. (unpublished data). Enzymes were stored at −70°C in 50% (vol/vol) glycerol/50 mM Tris·HCl, pH 7.5/0.25 M KCl/1 mM EDTA/10 mM 2-mercaptoethanol.

Construction of Bacteriophage T4 with the D219A Mutation in Gene *43* and Analysis of Its Mutation Frequency. *E. coli* MV1190 (*supE*) containing the wild-type or D219A gene *43* phagemid was infected at a multiplicity of 5 with T4 *am4306* (obtained from J. Drake, National Institute of Environmental Health Sciences), which has an amber mutation in gene *43* located 18 bases downstream (32) from the mutation creating the D219A polymerase. Nonamber recombinant progeny were identified by plating on *E. coli* B, and total phage progeny were titered on *E. coli* CR63. Wild-type and D219A recombinant phage were produced at frequencies of 4.9 and 6.6×10^{-2}, respectively. The am4306 polymerase mutation was poorly suppressed in this host; few progeny phage (<1 $\times 10^8$ per ml) were found when the phagemids encoding polymerase were replaced by the vector. Individual plaques from the *E. coli* B plates were suspended in 0.3 ml of broth overnight at 4°C and titered on *E. coli* B at 30°C to determine the total phage and the phage resistant to acriflavine (0.5 μg/ml) in each plaque.

Kinetic Analysis. All kinetic experiments were carried out in an assay buffer consisting of 67 mM Tris·HCl, pH 8.8/10 mM 2-mercaptoethanol/60 mM NaCl at 20°C unless otherwise indicated and are described in their respective figure legends. The sequence of the 13/20-mer primer-template is shown in the legend of Fig. 1. All rapid quench studies were performed with the instrument described by Johnson (33). Except as indicated, product analysis and rate profiles were done as described (21).

RESULTS

Preliminary Screening of Mutant T4 DNA Polymerases. Our construction of mutants in T4 DNA polymerase likely to be defective in exonuclease activity was guided by the sequence analysis of Bernad *et al.* (6), who suggested that T4 polymerase residues Asp-189, Glu-191, and Asp-219 were analogous, respectively, to Asp-355, Glu-357, and Asp-424 in the Klenow fragment of *E. coli* polymerase I (see *Discussion*). The phagemid in which T4 DNA polymerase Asp-219 was replaced by alanine expressed polymerase at a high level comparable to the wild type. Preliminary analysis (data not shown) suggested that this mutant polymerase was exonuclease deficient. Phagemids in which both Asp-189 and Glu-191 were replaced by alanine made little or no polymerase, judged by SDS/polyacrylamide gel electrophoresis and by polymerase assays of the extracts. The entire polymerase gene in the phagemid encoding the D219A mutation was sequenced as described. This sequence was identical to that of the wild type (4) except for the D219A (GAC → GCC) mutation.

Polymerase Activity of the D219A Mutant Polymerase. If the exonuclease-deficient mutant is to facilitate kinetic studies, it should exhibit properties similar to the wild-type T4 polymerase in all but its exonuclease activity. A preliminary experiment was performed to evaluate the pre-steady-state and steady-state kinetics of single nucleotide incorporation by the D219A mutant. In these experiments, the enzyme is provided with only the next correct nucleotide limiting the reaction to a single base incorporation per substrate molecule. For the 13/20-mer DNA substrate shown in the legend of Fig. 1, dATP is the next correct nucleotide. Upon its incorporation, the 13/20-mer is converted to 14/20-mer product. The resulting time course revealed an initial burst phase followed by a second slower phase (Fig. 1). The burst phase corresponds to the turnover of all initial enzyme-bound species. The slower steady-state phase is characteristic of a slow release of product DNA and rebinding of substrate (multiple turnovers) (21). Extrapolation of the steady-state data to the product intercept (of the ordinate) is representative of the DNA·enzyme complexes formed during preincubation and trapped by dAMP incorporation. The burst amplitude and both the pre- and steady-state rate constants were comparable to those observed for the wild-type polymerase (Table 1).

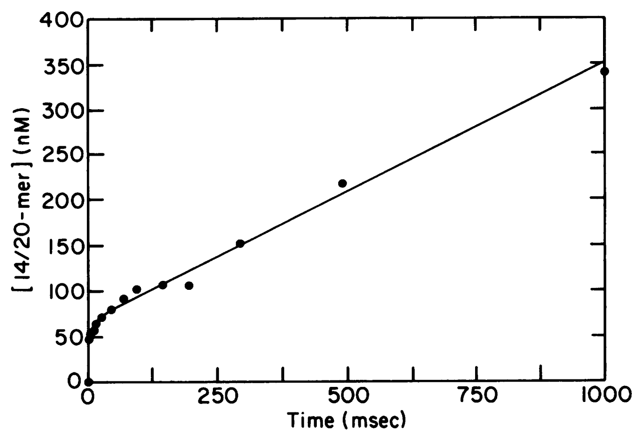
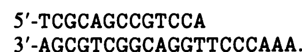


FIG. 1. Biphasic kinetic behavior of single nucleotide incorporation by the D219A mutant T4 DNA polymerase. The rapid quench experiment was performed by mixing a solution containing the T4 D219A polymerase (150 nM), 5′-^{32}P-labeled 13/20-mer (1 μM), and EDTA (0.1 mM) in assay buffer (pH 8.8) with an equal volume of a solution containing $MgCl_2$ (20 mM) and dATP (200 μM) in assay buffer. The reaction was terminated at various times by addition of EDTA (0.25 M). The data were fit to the equation: $y = Ae^{(-kt)} + mx + b$. The burst amplitude is representative of all active enzyme·DNA complexes formed during preequilibration. The sequence for the 13/20-mer substrate is


5′-TCGCAGCCGTCCA
3′-AGCGTCGGCAGGTTCCCAAA.

Comparison of the Exonuclease Activities of the Wild-type and D219A Mutant T4 DNA Polymerases. To determine the magnitude of the loss of exonuclease activity of the mutant D219A T4 DNA polymerase, a rapid quench experiment was performed to compare the activities of the purified wild-type and exonuclease-deficient enzymes on single-stranded DNA substrate. The data in Fig. 2 indicate that the mutant displays no detectable exonuclease activity, placing the rate constant at $<1 \times 10^{-5}$ sec^{-1}, compared to 100 sec^{-1} for the wild-type T4 DNA polymerase. Therefore, the exonuclease activity for the D219A polymerase has been lowered by a factor of at least 10^7 compared to the wild-type enzyme.

Equilibrium Dissociation Constant, K_d, of the 13/20-mer and T4 D219A Exonuclease-Deficient DNA Polymerase. As described above, the polymerization time course for the wild-type and mutant T4 polymerases is biphasic in nature.

Table 1. Comparison of wild-type and D219A exonuclease-deficient T4 DNA polymerases

	Wild type	D219A
K_d DNA	70 ± 7 nM	40 ± 4 nM
K_d dATP	20 ± 3 μM	10 ± 2 μM
k_{cat}	1–3 sec^{-1}	3–4 sec^{-1}
k_{ps}	400 sec^{-1}	400 sec^{-1}

Data for wild-type enzyme are from ref. 21.

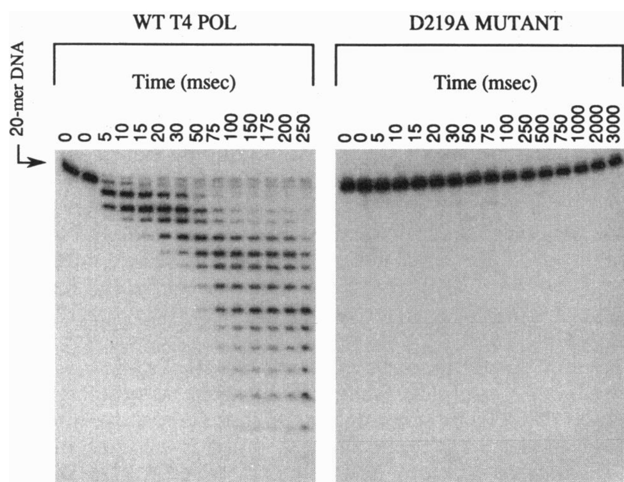
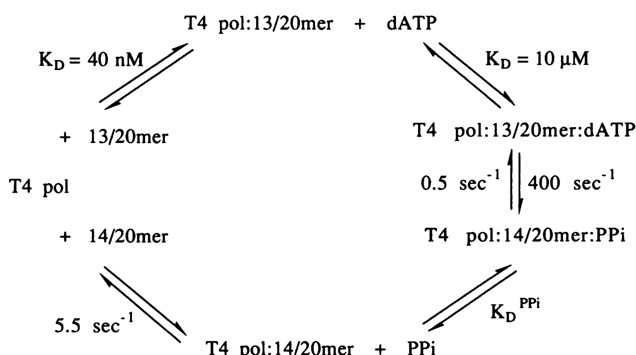


FIG. 2. Comparison of exonuclease activities of wild-type T4 DNA polymerase and the D219A exonuclease-deficient mutant. Assays were performed by incubating excess polymerase enzyme (1.2 μM) with the single-stranded 20-mer DNA template (200 nM) shown in the legend of Fig. 1. Reactions were initiated by addition of an equal volume of a solution of $MgCl_2$ (20 mM) in assay buffer, and the reaction was terminated at various times by addition of EDTA (0.25 M). Autoradiograph of the 20% polyacrylamide gel of products generated by the Molecular Dynamics Phosphorimager is shown. Lanes 0–250 msec, the time course for wild-type (WT) T4 polymerase; lanes 0–3000 msec, the time course for D219A mutant enzyme. A rate constant of 100 sec^{-1} (21) describes the stepwise product formation for the native enzyme, whereas no exonuclease activity (<1×10^{-5} sec^{-1}) was detected with the D219A mutant.

Measurement of the burst amplitude as a function of DNA levels allowed determination of the T4 polymerase·13/20-mer DNA (E·D) dissociation constant. The K_d for the 13/20-mer DNA and D219A T4 polymerase was measured by incubating the T4 D219A exonuclease-deficient polymerase with various amounts of 13/20-mer DNA (Fig. 3). The K_d was comparable with that of the wild-type polymerase (Table 1).



Scheme I

Equilibrium Dissociation Constant, K_d, for dATP Binding to the T4 D219A Polymerase·13/20-mer Complex. The K_d for dATP binding to the T4 polymerase·DNA complex was derived from the computer simulation of each time course after addition of dATP to all initial E·D complexes (Fig. 4). The value for the K_d of dATP binding (Table 1) for the D219A mutant is very similar to that of wild type.

Increased Mutation Frequency of T4 Bacteriophage with the D219A Exonuclease-Deficient DNA Polymerase. We have compared the mutation frequencies of T4 phage with the wild-type and D219A polymerases by using a modification of the acriflavine-resistance assay used by Reha-Krantz and Lambert (34). The absence of the proofreading exonuclease greatly increases the frequency of acriflavine-resistant mutants (Table 2). The number of phage recovered from each D219A plaque was significantly reduced, presumably due to the increased frequency of lethal mutations, and the plaque morphology was extremely heterogeneous, as expected for a strong mutator polymerase.

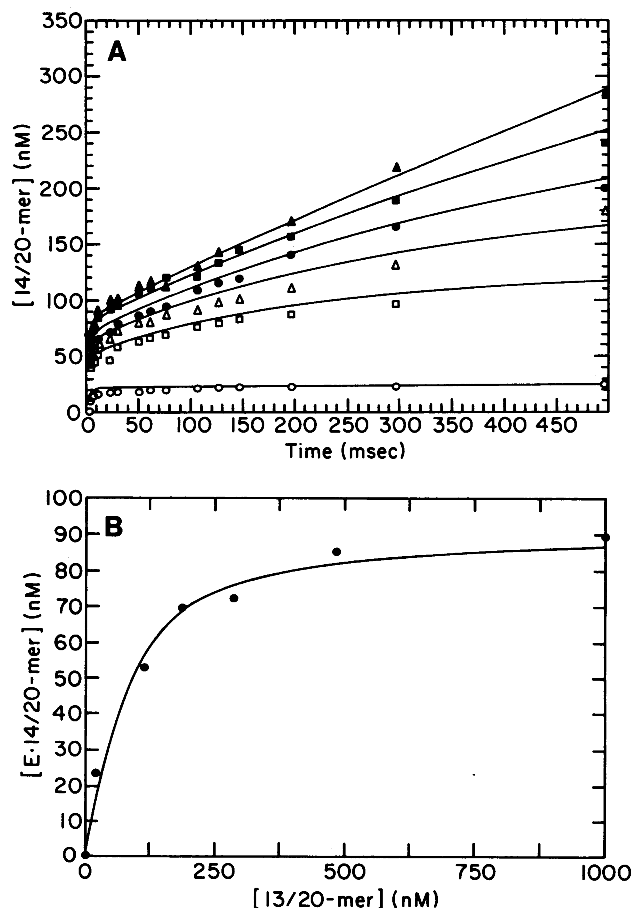


FIG. 3. (*A*) Determination of equilibrium K_d of the 13/20-mer and T4 D219A DNA polymerase. Variable concentrations of 5′ ^{32}P-labeled 13/20-mer (○, 50 nM; □, 250 nM; △, 400 nM; ●, 600 nM; ■, 1 μM; ▲, 2 μM) were incubated with the T4 D219A DNA polymerase (184 nM)/0.1 mM EDTA in one syringe and the reaction was initiated by mixing with an equal volume of a solution containing $MgCl_2$ (20 mM) and dATP (40 μM). The reaction was terminated at various times by the addition of EDTA (0.25 M). Solid curves were ultimately generated by computer simulation as described (21) with the kinetic sequence shown in Scheme I. (*B*) Plot of the burst amplitude as a function of 13/20-mer concentration. The resulting curve was fit to the function [T4 polymerase·13/20-mer] = 0.5 (K_d + [T4 polymerase] + [13/20-mer]) − {0.25 (K_d + [T4 polymerase] + [13/20-mer])^2 − ([T4 polymerase] + [13/20-mer])}$^{1/2}$. K_d was calculated to be 40 ± 4 nM.

DISCUSSION

In this paper, we describe the design, isolation, and partial characterization of a mutant T4 DNA polymerase that is deficient in exonuclease activity. Initial kinetic studies of the mutant polymerase indicate that full polymerase activity is retained while virtually all of the exonuclease activity has been removed.

T4 DNA polymerase has discrete regions with strong amino acid sequence similarity to a large number of other polymerases (polymerase B family), including many eukaryotic cellular and viral polymerases, and more limited sequence similarity to the polymerase A family that includes *E.*

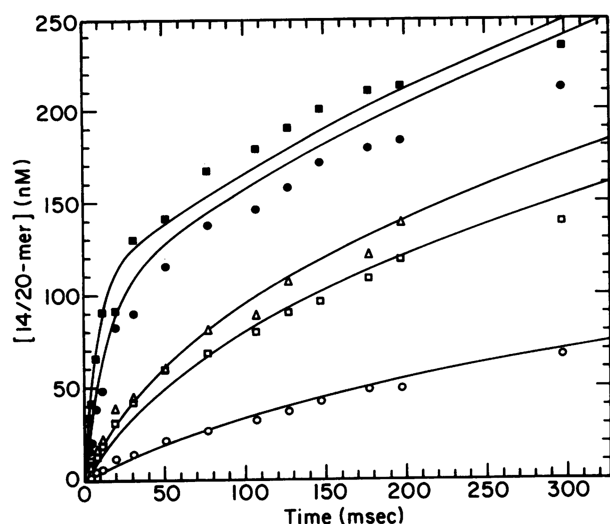


FIG. 4. Determination of equilibrium K_d for dATP binding to the D219A T4 polymerase·13/20-mer complex. D219A T4 polymerase (244 nM) was preincubated with 5′ ^{32}P-end-labeled 13/20-mer (800 nM)/0.1 mM EDTA in one syringe and mixed with an equal volume of a solution containing $MgCl_2$ (20 mM) and various concentrations of dATP (○, 0.250 μM; □, 0.75 μM; △, 1 μM; ●, 5 μM; ■, 10 μM; data not shown for 20, 40, 80, and 100 μM). At various times, the reaction was terminated by addition of EDTA (0.25 M). Products were analyzed as described. Solid curves were ultimately generated by computer simulation using the KINSIM program and the kinetic sequence in Scheme I.

coli DNA polymerase I and phage T7 polymerase (4–9, 35, 36). The crystal structure of the Klenow fragment of *E. coli* polymerase I suggests that its exonuclease active site is in the N-terminal portion of the polypeptide in a separate domain from the polymerase (26–29). Mutagenesis of any of three carboxylic acid amino acids (Asp-355, Asp-424, and Asp-501) greatly decreased the exonuclease, but not the polymerase, activity of the Klenow fragment (28). Each of these residues has been shown to interact directly with one or both of the two metal ions that are essential for exonucleolytic cleavage by this enzyme (26–29). Mutations of Glu-357 decrease the exonuclease activity less severely, and this residue is now thought to play a role in the hydrolytic reaction other than metal binding (28, 29).

There is now strong evidence that polymerase B family members have amino acids essential for their exonuclease activity that are located within sequences homologous to the sequences containing the essential residues in the Klenow fragment. T4 DNA polymerase Asp-219 is within a sequence similar to that of Klenow Asp-424. We have shown that conversion of Asp-219 to alanine yields a mutant enzyme lacking any measurable exonuclease, whose polymerase is comparable to the wild type. Bacteriophage T4 with this D219A polymerase have a very strong mutator phenotype (Table 2). The corresponding mutation in phage φ29 (D66A) (6) and in *Saccharomyces cerevisiae* DNA polymerase δ (D405A) (35) also gives a polymerase with decreased exonuclease activity.

Table 2. Increased mutation frequency with the T4 D219A DNA polymerase

Phage	Phage per plaque	Acriflavine-resistant phage/total phage	Relative mutation frequency
Wild type	4.5×10^7	4.2×10^{-6}	1
D219A	3.3×10^6	3.2×10^{-3}	760

Data shown are averages for 10 plaques of each type.

Changing the residues corresponding to Klenow Asp-355 and Glu-357 to alanine has been shown to give decreased exonuclease in T7 DNA polymerase (polymerase A family) (23) and phage φ29 (6), decreased exonuclease and a mutator phenotype in yeast pol ε (36), and a mutator phenotype in yeast pol δ (35). Bernad *et al.* (6) initially proposed that the corresponding T4 polymerase residues were Asp-189 and Glu-191. However, our phagemid with alanine replacing these residues did not produce stable polymerase, and Reha-Krantz *et al.* (20) reported only a slightly increased mutation frequency with T4 phage with the same mutation. Recent analyses indicate a better alignment with T4 Asp-112 and Glu-114 (9, 36).

Klenow Asp-501 can be aligned with T4 Asp-324 (9, 36). Reha-Krantz *et al.* (20) found a T4 polymerase mutation at this site (D324G) by screening for an intragenic mutation that would increase the very modest mutator activity of the E191A mutant. The mutation frequency of the E191A/D324G double mutant is similar to that found with the D219A mutant and much higher than that of the D324G single mutant. The T4 DNA polymerase of this double mutant is exonuclease deficient and inaccurate *in vitro* (20).

Together these mutation studies of DNA polymerases suggest significant similarity in the regions of these enzymes required for DNA hydrolysis. Thus, the insight gained from the powerful structural and mechanistic studies of *E. coli* polymerase I should be relevant to these other enzymes. It is not yet clear whether these other polymerases will have exonuclease and polymerase domains that are as separate as those in the Klenow fragment. All of the T4 polymerase mutants with decreased exonuclease are in the N-terminal half of the protein. The B22 amber fragment, which contains the N-terminal 80%, retains the exonuclease activity, but its binding to DNA is much weaker than that of the intact enzyme (25). This could implicate a need for the C-terminal region, or it could simply reflect aberrant folding of the truncated protein. Recent studies of the T4 tsL141 polymerase show that this C-terminal mutation (A737V) increases the exonuclease activity on duplex DNA without changing the hydrolysis of single-stranded DNA (P. Spacciapoli and N.G.N., unpublished data). One possibility is that it alters the movement of DNA between the polymerase and exonuclease active sites.

For the exonuclease-deficient mutant to be useful for detailed kinetic studies of the T4 DNA polymerase mechanism, it should exhibit properties similar to those of the wild-type enzyme, differing only in terms of its exonuclease activity. The pre-steady-state and steady-state kinetics of single nucleotide incorporation for both the wild-type and D219A mutant were evaluated under conditions of excess DNA substrate over enzyme. As described (21), the preequilibration of T4 polymerase and DNA results in a biphasic reaction profile characterized by a rapid initial phase followed by a second, slower phase. This burst represents the conversion of all productive enzyme·DNA complexes formed during preequilibration to product, while the steady-state phase represents a slow step following the chemistry step involving product release and subsequent turnover of remaining substrate. Under identical experimental conditions, both the wild-type and D219A mutant enzymes exhibited rate constants within experimental error of each other: steady-state rate constants of 1–3 sec^{-1} for the wild-type polymerase and 3–4 sec^{-1} for mutant D219A, and a burst rate constant on the order of 400 sec^{-1} for both enzymes (Table 1).

Given that the polymerase activity and the dissociation constants for dNTP and DNA were virtually unchanged compared to wild type, we examined the magnitude of the reduction of exonuclease activity for the D219A mutant. Exonuclease activity for both the wild-type and D219A enzymes was measured on the same single-stranded DNA

substrate. As shown in Fig. 2, no measurable exonuclease activity was detected by our methods for D219A. Thus, the exonuclease activity of this mutant has been decreased by a factor of at least 10^7. This loss of activity is of the same order of magnitude as those for exonuclease-deficient mutants of T7 DNA polymerase (23) and the Klenow fragment (24).

These studies demonstrate that a single amino acid change, D219A, decreases the exonuclease activity of T4 DNA polymerase by a factor of at least 10^7 without significantly changing its polymerase activity or substrate binding constants. This mutant polymerase is an excellent candidate for replacement of the wild-type protein in kinetic studies and will be useful for determination of kinetic characteristics of DNA replication carried out by bacteriophage T4 DNA polymerase that are not readily accessible with the wild-type enzyme.

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