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Biochemistry

DNA polymerization in the absence of exonucleolytic proofreading: *In vivo* and *in vitro* studies

(bacteriophage T4 DNA polymerase mutants/mutator phenotype/conserved residues)

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ABSTRACT **Classical genetic selection was combined with site-directed mutagenesis to study bacteriophage T4 DNA polymerase 3′ → 5′ exonuclease activity. A mutant DNA polymerase with very little (≤1%) 3′ → 5′ exonuclease activity was generated. *In vivo*, the 3′ → 5′ exonuclease-deficient DNA polymerase produced the highest level of spontaneous mutation observed in T4, 500- to 1800-fold above that of wild type. The large reduction in 3′ → 5′ exonuclease activity appears to be due to two amino acid substitutions: Glu-191 to Ala and Asp-324 to Gly. Protein sequence similarities have been observed between sequences in the *Escherichia coli* DNA polymerase I 3′ → 5′ exonuclease domain and conserved sequences in eukaryotic, viral, and phage DNA polymerases. It has been proposed that the conserved sequences contain metal ion binding ligands that are required for 3′ → 5′ exonuclease activity; however, we find that some proposed T4 DNA polymerase metal binding residues are not essential for 3′ → 5′ exonuclease activity. Thus, our T4 DNA polymerase studies do not support the hypothesis by Bernad *et al.* [Bernad, A., Blanco, L., Lazaro, J. M., Martin, G. & Salas, M. (1989) *Cell* 59, 219–228] that many DNA polymerases, including T4 DNA polymerase, share an extensively conserved 3′ → 5′ exonuclease motif. Therefore, extrapolation from *E. coli* DNA polymerase I sequence and structure to other DNA polymerases for which there is no structural information may not be valid.**

Bacteriophage T4 DNA polymerase is one of the best experimental systems for studying the role of DNA polymerase exonucleolytic proofreading in enhancing DNA replication fidelity (1–10). Wild-type T4 DNA polymerase has a potent 3′ → 5′ exonuclease activity (11), which is important for accurate DNA replication. Mutant DNA polymerases with reduced 3′ → 5′ exonuclease activity produce more DNA replication errors (mutator phenotype), whereas mutants with elevated 3′ → 5′ exonuclease activity, relative to polymerizing activity, increase DNA replication accuracy (antimutator phenotype) (3). The mutator phenotype was used to select mutant DNA polymerases with reduced 3′ → 5′ exonuclease activity; amino acid substitutions in the mutants were clustered between T4 DNA polymerase residues 255 and 363 (8–10, 12). Although amino acid changes within this region decreased 3′ → 5′ exonuclease activity, the mutant DNA polymerases still retained significant residual proofreading activity, which suggests that these particular residues do not function catalytically.

In the case of *Escherichia coli* DNA polymerase I (pol I), residues essential for 3′ → 5′ exonuclease activity have been identified. They include four metal ion binding residues; Asp-355, Glu-357, and Asp-501 of metal ion site A and Asp-424 of metal ion site B (13–15). Because both T4 and *E. coli* DNA polymerases require Mg^{2+} for 3′ → 5′ exonuclease activity, it is reasonable to suppose that T4 DNA polymerase also has essential metal ion binding residues. The primary structure of T4 DNA polymerase (16) is very different from the amino acid sequence of *E. coli* pol I; nevertheless, some sequence similarities exist [i.e., T4 DNA polymerase residues 188–195 and *E. coli* pol I residues 354–361, which includes part of pol I metal ion site A (Fig. 1) (8)]. Similar sequences were also noted for human DNA polymerase α and several viral DNA polymerases (8, 9, 16) in a conserved region found in eukaryotic and eukaryotic-like DNA polymerases that is called conserved region IV by Wang *et al.* (21). Other DNA polymerases were also found to have these sequence similarities (23, 24), and the regions of proposed sequence conservation were extended by Bernad *et al.* (23) to include most of the *E. coli* pol I 3′ → 5′ exonuclease domain. The hypothesis drawn from these sequence comparisons was that many eukaryotic, viral, and bacteriophage DNA polymerases have a conserved 3′ → 5′ exonuclease domain similar to that of *E. coli* pol I. T4 genetic studies are consistent with this proposal because mutations that reduce 3′ → 5′ exonuclease activity are located near the proposed conserved metal ion binding residues. The hypothesis was tested directly in phage φ29 DNA polymerase by substituting alanine residues for proposed conserved metal ion binding residues, which resulted in a 1000-fold reduction in 3′ → 5′ exonuclease activity without affecting polymerization activity (23). The φ29 DNA polymerase result compares favorably with the 10^5-fold reduction observed when alanine residues were substituted for *E. coli* pol I residues Asp-355 and Glu-357 (14).

We present here *in vitro* mutagenesis and genetic studies that were designed to test if proposed T4 DNA polymerase metal ion binding residues, identified on the basis of sequence similarities to those of *E. coli* pol I (8, 23), are required for 3′ → 5′ exonuclease activity. In contrast to the φ29 DNA polymerase studies, alanine substitutions for proposed conserved T4 DNA polymerase residues, Asp-189 and Glu-191 (Fig. 1), produced only slight decreases in 3′ → 5′ exonuclease activity and little or no increase in spontaneous mutation frequencies. Because of our results with T4 DNA polymerase, speculations about conserved residues that might serve as metal ion binding sites in eukaryotic and other viral DNA polymerases, based solely on protein sequence comparisons, must necessarily be viewed with caution.

In the course of assessing the functional significance of the proposed conserved residues on T4 DNA polymerase 3′ → 5′ exonuclease activity, a 3′ → 5′ exonuclease-deficient DNA polymerase was generated. In contrast to 3′ → 5′ exonuclease-deficient DNA polymerases that have been studied

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Abbreviation: pol I, *Escherichia coli* DNA polymerase I.

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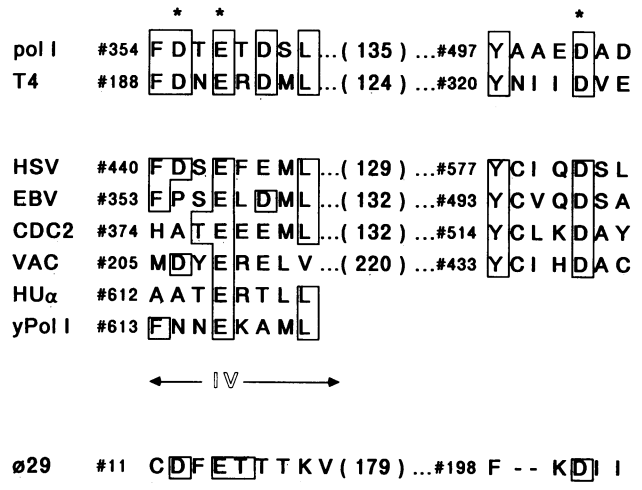

FIG. 1. *E. coli* pol I metal ion site A and proposed metal ion binding sites in other DNA polymerases. Pol I carboxylate-containing residues important for metal binding are indicated by asterisks (*) (14). Proposed similar sequences are observed for bacteriophage T4 (16), herpes simplex virus type 1 (HSV; ref. 17), Epstein–Barr virus (EBV; ref. 18), yeast DNA polymerase III (CDC2; ref. 19), vaccinia virus (VAC; ref. 20), human DNA polymerase α (HUα; ref. 21), yeast DNA polymerase I (yPol I; ref. 22), and φ29 DNA polymerase (φ29; ref. 23). Residues identical to *E. coli* pol I residues are boxed. The numbers in parentheses indicate the number of amino acids between the two regions in pol I that form metal ion site A and between proposed analogous regions in other DNA polymerases. The N-terminal portion of proposed metal binding sites corresponds to part of a previously recognized conserved region found in these polymerases, which has been called region IV by Wang *et al.* (21). Residues analogous to the C-terminal portion of metal ion site A are not suggested for human DNA polymerase α or yeast pol I because of insufficient protein sequence similarity; however, two sequences in the proposed 3′ → 5′ exonuclease region in these DNA polymerases (23) have the same spacing of aromatic and carboxylate-containing residues as found for *E. coli* pol I residues Tyr-497 and Asp-501: human DNA polymerase α residue 706 (YHLSELV) and yeast pol I residue 714 (WDLSEMY) or human DNA polymerase α residue 801 (YIVPDKQ) and yeast pol I residue 810 (FIVPDKE). Phage φ29 DNA polymerase sequence alignments are those of Bernad *et al.* (23).

from other organisms, it was possible with T4 to observe the consequences of severe reduction in DNA polymerase exonucleolytic proofreading *in vivo*. As expected, loss of 3′ → 5′ exonuclease activity increased spontaneous mutation frequency substantially, 500- to 1800-fold above the wild-type level. In *in vitro* reactions, the mutant DNA polymerase was observed to misincorporate nucleotides, to extend mispaired primer termini, and to exhibit increased activity on a nicked DNA template.

MATERIALS AND METHODS

***In Vitro* Mutagenesis.** Standard oligonucleotide mutagenesis procedures (25) were used to introduce mutations directly into the cloned T4 DNA polymerase gene (26). Mutations in the cloned gene were introduced into T4 phage by homologous recombination and marker rescue (8).

Protein Purification. Wild-type and mutant T4 DNA polymerases were purified to apparent homogeneity or to 80% or greater purity from T4-infected cells and from the *E. coli* expression vector by using standard procedures (26, 27). Most contaminating proteins, including nucleases, were removed by phosphocellulose (P-11; Whatman) chromatography (3). Purified DNA polymerases were free of contaminating endonuclease activity as measured in a plasmid nicking assay. Also, DNA was not degraded exonucleolytically by the 3′ → 5′ exonuclease-deficient mutant even after prolonged incubation (see *Results*). Protein concentrations of purified DNA polymerases were determined by quantitative amino acid analysis, comparative Coomassie blue staining, and A_{280} absorbance (for T4 DNA polymerase, $\varepsilon = 1.492 \times 10^5\ M^{-1}\cdot cm^{-1}$).

Enzyme Assays. DNA polymerase activity was determined by measuring the amount of radioactively labeled dNMP incorporated into DNA as described (3); 3′ → 5′ exonuclease activity was detected in the same DNA synthesis reactions by determining the amount of radioactively labeled dNTP converted to dNMP as a result of incorporation and subsequent excision (3). Exonuclease activity on single-stranded or duplex DNA was determined by using ^{3}H-labeled DNA at 60 μM (100 cpm/pmol of nucleotide) (3).

Bacteriophage T4 Strains. New strains are described in *Results*. The *tsL56* strain is a classical T4 DNA polymerase mutator strain (1, 10). The tsL56 DNA polymerase has less 3′ → 5′ exonuclease activity compared to wild type (3).

Mutator Activity. Mutator activity was measured in a forward assay system by determining the number of acriflavin-resistant mutants (28) and by two *rII* reversion assay systems (8, 12). The median mutation frequency value from five or more cultures was used as an index of mutator activity.

Burst Size and Temperature Sensitivity. Growth and viability of T4 bacteriophage carrying DNA polymerase mutations were determined by measuring the number of phage produced per bacterium in a single growth cycle (burst size) (10).

RNA Sequencing. DNA polymerase genes for all strains listed in Table 1 were sequenced completely by using DNA polymerase mRNA as described (8, 29). Specific primers complementary to the T4 DNA polymerase gene were annealed in separate reactions to total RNA isolated from infections terminated 10 min postinfection. Primed templates were extended by avian myeloblastosis virus reverse transcriptase in modified Sanger dideoxynucleotide sequencing reactions.

RESULTS

Site-Directed Mutagenesis of T4 DNA Polymerase Residues 189 and 191. Mutations that encode alanine substitutions at residues that are potentially analogous to *E. coli* pol I residues Asp-355 and Glu-357—namely, Asp-189 (D189A mutation) and Glu-191 (E191A mutation)—were produced by *in vitro* mutagenesis of the cloned T4 DNA polymerase gene. Mutations in the plasmid T4 DNA polymerase gene were transferred by homologous recombination to T4 phage (8, 12). DNA polymerase mRNA was sequenced to confirm presence of the mutations (see *Materials and Methods*). Surprisingly, in view of the protein sequence similarities, only small decreases in 3′ → 5′ exonuclease activity were observed with alanine substitutions for either Asp-189 or Glu-191 (Table 1). *In vivo* exonucleolytic proofreading by the mutants was also examined. A decrease in DNA polymerase 3′ → 5′ exonuclease activity results in an increase in spontaneous mutation frequency. In line with the small decreases in 3′ → 5′ exonuclease activity observed for the D189A and E191A mutant DNA polymerases, only small increases in spontaneous mutation were detected (Table 1). The double mutant DNA polymerase (D189A/E191A) had less 3′ → 5′ exonuclease activity than the single mutant DNA polymerases, but the double mutant still retained about 50% as much activity as that found in wild-type enzyme, and spontaneous mutation frequency was increased only 11- to 30-fold (Table 1).

Selection of Bacteriophage T4 DNA Polymerase 3′ → 5′ Exonuclease-Deficient Mutants. Genetic selection to isolate mutants with high mutator activity (12) was used to isolate 3′

Table 1. Effects of bacteriophage T4 DNA polymerase mutations on spontaneous mutation, 3′ → 5′ exonuclease activity, DNA polymerase activity, and phage viability

Mutation	Mutator activity*			3′ → 5′ exonuclease activity†	DNA polymerase activity‡		Burst size§	Temperature sensitivity
	ac	*rUV199*	*rP7oc*		Gapped	Nicked		
Wild type	1	1	1	1	1	1	43 (62)	None
D189A	4	1	1	0.91	0.8	ND	35	None
E191A	5	2	6	0.69	0.8	ND	36	Total
D189A/E191A	16	11	30	0.54	1	ND	9	Total
D324G	56	63	280	ND	ND	ND	9	Partial
E191A/D324G	650	514	1800	0.01	0.03	0.45	2 (12)	Total
tsL56	36	74	ND	0.12	ND	ND	29 (55)	Total

ND, not determined in this study.

*Mutator activity was determined by measuring the number of acriflavin-resistant mutants (ac) in 10 phage cultures and by measuring the number of *rII* revertants at the *rUV199* and *P7oc* sites in 5–7 cultures as described (8, 12, 28). Mutator activity values are reported relative to wild type; the number of wild-type mutations is 4×10^{-6} as determined by the number of acriflavin-resistant mutants, 1×10^{-6} as determined by the number of revertants at the *rUV199* site, and 4×10^{-9} as determined by the number of revertants at the *P7oc* site.

†Exonuclease activity was measured under DNA synthesis conditions (3) with 0.2 mM $p(dT)_n$ annealed to an equivalent nucleotide amount of $p(dA)_{12}$ (Pharmacia) and with 0.1 mM [^{3}H]dATP (NEN) at 111 cpm/pmol. DNA polymerase incorporates dAMP, but some of the incorporated dAMP is removed by 3′ → 5′ exonuclease activity (turnover). Turnover values are reported relative to wild-type DNA polymerase, which excised 24% of dAMP incorporated under these reaction conditions. The 3′ → 5′ exonuclease value for tsL56 DNA polymerase is from Muzyczka *et al.* (3) for production of TMP on $p(dA)_n$ primed with $p(dT)_{10}$.

‡DNA polymerase activity was measured on partially degraded DNA ("gapped") as described (3) and on duplex DNA with "nicks." Both DNA templates were at 833 μM under standard reaction conditions (3). DNA polymerase activity values are reported relative to wild-type DNA polymerase; specific activity of wild-type DNA polymerase preparations ranged from 4 to 5.9 units/μg, where 1 unit corresponds to 10 nmol of TMP incorporated in 30 min at 30°C on gapped DNA template. Wild-type and mutant DNA polymerases were similarly active as measured on gapped DNA except for the E191A/D324G DNA polymerase purified from the expression vector, which was >30-fold less active than wild type. Wild-type DNA polymerase had similar activity on the "nicked" DNA template, but the E191A/D324G DNA polymerase was 15-fold more active on this template, which presumably has nicks and some small gaps.

§Burst size is equal to the number of progeny phage produced per infected bacterium. Burst sizes were determined at 30°C in the *optA1* host HR44 and in the standard phage T4 host, CR63; burst sizes in CR63 are given in parentheses.

→ 5′ exonuclease-deficient mutants, but the search was targeted to residues that may function synergistically with proposed conserved T4 residues (residues 188–195) by beginning the selection with T4 phage carrying the DNA polymerase mutation that encodes an alanine substitution at codon 191 (E191A mutation). Approximately 3×10^8 infective centers were screened, and one of the isolates had exceptionally strong mutator activity and was characterized further. Two mutation sites were discovered after sequencing the entire DNA polymerase transcript: the parental E191A mutation and a second mutation at codon 324, Asp (GAC) to Gly (GGC).

Characterization of the E191A/D324G T4 DNA Polymerase Double Mutant. Mutator activity of this mutant strain was 500- to 1800-fold higher than that of wild type and ≈10-fold higher than observed for previously studied T4 DNA polymerase mutants with reduced 3′ → 5′ exonuclease activity [note tsL56 (10); Table 1]. Phage carrying only the mutation for the Asp-324 to Gly substitution (D324G mutation) were isolated by recombination from double mutant E191A/D324G phage. The D324G mutation increased spontaneous mutation 50- to 280-fold above that of wild type (Table 1).

A large reduction in 3′ → 5′ exonuclease activity, to ≤1% of wild type activity, was observed for the purified mutant enzyme (Table 1), which could account for the high level of spontaneous mutation observed for the E191A/D324G double mutant strain. Although 1% represents significant residual exonuclease activity, little or no exonucleolytic proofreading was observed with the double mutant in a misincorporation assay using a 5′ ^{32}P-labeled 17-oligonucleotide primer annealed to a 37-nucleotide template (Fig. 2). When DNA synthesis reactions were carried out with this primer–template without dGTP, wild-type DNA polymerase extended the primer only two nucleotides (Fig. 3*A*). At position +3, dGTP was required (Fig. 2), but when DNA synthesis could no longer proceed, the intrinsic 3′ → 5′ exonuclease activity of the wild-type DNA polymerase degraded the primer (Fig. 3 *A* and *C*). In reactions with more wild-type DNA polymerase (up to 0.8 μg), the rate of primer extension to the +2 position increased, but the rate of primer degradation also increased, and there was no extension to the +3 or +4 positions (results not shown). In contrast, the E191A/D324G DNA polymerase extended the primer four nucleotides with little or no primer degradation (Fig. 3 *B* and *D*). In the first 30 sec, the mutant DNA polymerase extended some of the primer three nucleotides, which indicates stable incorporation of a wrong nucleotide at position +3. With time, the primer was extended four nucleotides, indicating that the E191A/D324G DNA polymerase can extend a mispaired primer–terminus. Further extension did not occur, probably because two adjacent stable misincorporations were required at positions +5 and +6 (Figs. 2 and 3). The absence of primer degradation, stable incorporation of wrong nucleotides, and extension of a mispaired primer–terminus indicates that the E191A/D324G DNA polymerase has little or no 3′ → 5′ exonuclease proofreading activity. The *in vitro* misincorporation assay may well approximate E191A/D324G DNA polymerase misincorporation *in vivo*. We predict that *in vivo* many misincorporated nucleotides are not corrected because

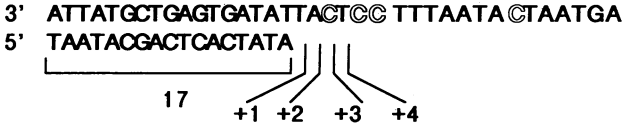

FIG. 2. Primer–template for DNA replication misincorporation assay. The 17-nucleotide primer was ^{32}P-labeled at the 5′ end by T4 polynucleotide kinase prior to annealing to the 37-nucleotide template. Primer-extension reactions were carried out in 20 μl, which contained 67 mM Tris·HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 6.7 mM $MgSO_4$, bovine serum albumin at 167 μg/ml, 0.51 μM primer–template, and 100 μM dATP, 100 μM dCTP, 100 μM dGTP, and 100 μM TTP for complete reactions; dGTP was omitted for misincorporation assays. Equal units of wild-type and E191A/D324G DNA polymerase (0.01 unit; 1 unit incorporates 10 nmol of TMP at 30°C in 30 min) were added to start reactions. Reactions were maintained at 30°C.

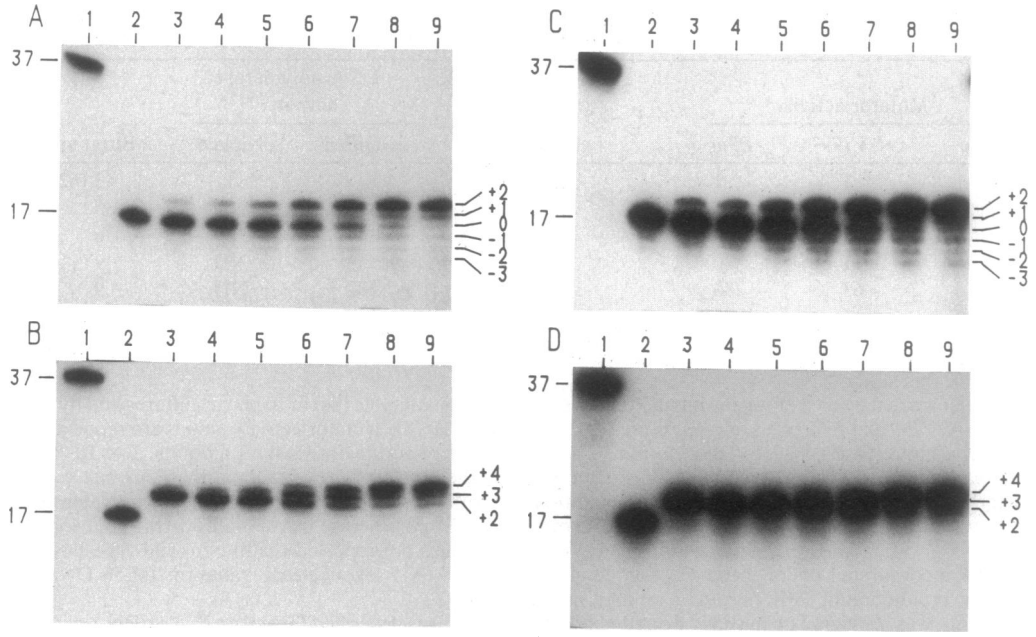

FIG. 3. DNA replication misincorporation assay. Reaction products with the primer–template described in Fig. 2 were analyzed on DNA sequencing gels (12% polyacrylamide/8.3 M urea). (*A*) Reaction products from assays with wild-type enzyme. (*B*) E191A/D324G DNA polymerase assays. (*C* and *D*) Longer exposures of *A* and *B*, respectively. Lane 1, products from complete (all four dNTPs) reactions after 5 min. Lane 2, control reaction without enzyme. Lanes 3–9, products from reactions with no dGTP. Samples were taken at 0.5 min (lane 3), 1 min (lane 4), 2 min (lane 5), 5 min (lane 6), 10 min (lane 7), 20 min (lane 8), and 60 min (lane 9). Because the specific activity of the E191A/D324G DNA polymerase is low compared to wild type, control experiments with equal concentrations of protein were also done. In these experiments, 0.8 μg of wild-type or E191A/D324G DNA polymerases were incubated in reaction conditions given for Fig. 2. For the E191A/D324G DNA polymerase, results identical to those in *B* were observed. For wild-type DNA polymerase, by 1 min all primer was extended two nucleotides, and by 10 min significant primer degradation was observed (data not shown). Even with this high concentration of wild-type DNA polymerase, there was no evidence of primer extension to the +3 or +4 positions. Sizes of bands (in nucleotides) are given at left.

of the lack of exonucleolytic proofreading; the misincorporated nucleotides are then extended and become mutations.

The E191A/D324G DNA polymerase was 15-fold more active on a nicked DNA template than on DNA with gaps (Table 1). Nicked DNA has breaks or nicks in the phosphodiester backbone, but DNA polymerases cannot use nicks as sites of DNA replication initiation unless the DNA polymerase can displace DNA from the template strand. Wild-type T4 DNA polymerase, without accessory proteins, cannot initiate strand displacement synthesis at nicks (30). Thus, nicked DNA is expected to be a poor template for wild-type DNA polymerase, but wild-type T4 DNA polymerase was moderately active with our preparation of nicked DNA, presumably because the nicked DNA also contained some gaps. The large stimulation in DNA synthesis at nicks observed for the mutant DNA polymerase suggests that T4 DNA polymerase can efficiently elongate primer–termini at nicks by strand displacement if exonucleolytic proofreading is absent.

DISCUSSION

Bacteriophage T4 DNA Polymerase 3′ → 5′ Exonuclease-Deficient Mutant. T4 DNA polymerase exonucleolytic proofreading was nearly eliminated by amino acid substitutions for two carboxylate-containing residues, E191A and D324G mutations. This double mutant has properties predicted for a 3′ → 5′ exonuclease-deficient DNA polymerase: high spontaneous mutation frequency, increased stable incorporation of wrong nucleotides, and increased extension of mispaired primer–termini. The double mutant, like T7 DNA polymerase 3′ → 5′ exonuclease-deficient mutants (31, 32), also initiates strand displacement DNA synthesis at nicks. Thus, initiation of strand displacement synthesis at nicks may be an intrinsic activity of DNA polymerases that is not ordinarily detected in *in vitro* reactions because of the normally high level of 3′ → 5′ exonuclease activity.

Is There a Conserved 3′ → 5′ Exonuclease Site in T4 DNA Polymerase? The exonuclease-deficient T4 DNA polymerase mutant E191A/D324G has amino acid substitutions for two carboxylate-containing residues that reside in sequences with similarity to *E. coli* pol I sequences that encode metal ion site A (Fig. 1). Alone, the D324G mutation produces a substantial increase in spontaneous mutation and, thus, the D324G mutation could possibly have a catalytic function. Alanine substitutions for Glu-191 and Asp-189, however, produced only small decreases in 3′ → 5′ exonuclease activity and equally small increases in spontaneous mutation frequency (Table 1). These results were unexpected in view of the sequence comparisons cited previously and because alanine substitutions for *E. coli* pol I metal binding ligands Asp-355 and Glu-357 reduced its 3′ → 5′ exonuclease activity 10^5-fold (14), whereas alanine substitutions for proposed analogous residues in phage φ29 DNA polymerase reduced its 3′ → 5′ exonuclease activity 10^3-fold (23). Because alanine substitutions in T4 DNA polymerase at residues Asp-189 and Glu-191 produced only a small effect on exonucleolytic proofreading, the significance of previously proposed protein sequence similarities must be reevaluated.

The weak protein sequence similarity between T4 DNA polymerase residues flanking Asp-324 and pol I residues flanking Asp-501 would not have been recognized except for identification by genetic selection of the mutation encoding the Asp-324 to Gly substitution (Fig. 1). In fact, Bernad *et al.* (23) propose from their protein sequence comparisons that T4 DNA polymerase residues Phe-337 and Asp-339 correspond to pol I residues Tyr-497 and Asp-501, even though the spacing between these aromatic and carboxylate-containing residues differs from that of pol I. The protein sequence similarity between the region in T4 DNA polymerase that includes residues Asp-189 and Glu-191 to the region in *E. coli*

pol I that spans residues Asp-355 and Glu-357 appears to be more substantial (Fig. 1). Similar regions of sequence similarity were identified in other eukaryotic and viral DNA polymerases (8, 16, 21, 23, 24). Nevertheless, T4 DNA polymerase residues Asp-189 and Glu-191 are not essential for its 3′ → 5′ exonuclease activity. Thus, T4 DNA polymerase does not fit into the extensive 3′ → 5′ exonuclease motif proposed by Bernad *et al.* (23). Alternatively, it may be argued for T4 DNA polymerase that all metal ion binding ligands are not equal and, perhaps, two or more ligands (i.e., Asp-189 and Glu-191) may be eliminated without significant loss of function. Structural studies are required to test this possibility, but it is clear that Asp-189 and Glu-191 in T4 DNA polymerase do not appear to function in the same way as observed for *E. coli* pol I residues Asp-355 and Glu-357.

What is clear from this and other T4 DNA polymerase studies is that residues important for 3′ → 5′ exonuclease activity are located in the N-terminal portion of the enzyme and include at least the region spanned by residues 255–363. Also, because single amino acid substitutions have not yet been found that eliminate T4 DNA polymerase 3′ → 5′ exonuclease activity, two or more amino acid substitutions may be required to completely inactivate its extraordinarily potent 3′ → 5′ exonuclease activity.

Contribution of T4 DNA Polymerase Exonucleolytic Proofreading to DNA Replication Fidelity. Phage T4 is a good experimental system to study the role of DNA polymerase in DNA replication fidelity, because T4 DNA polymerase is the primary determinant of T4 spontaneous mutation rates (1–10, 12). Thus, the 500- to 1800-fold increase in spontaneous mutation frequency observed for the E191A/D324G T4 DNA polymerase suggests that exonucleolytic proofreading by T4 DNA polymerase may contribute about 1000-fold to DNA replication fidelity. Now that a 3′ → 5′ exonuclease-deficient T4 DNA polymerase has been generated, it will be possible to determine T4 DNA polymerase nucleotide misinsertion frequency, but a reasonable estimate is in the range of 10^{-4}–10^{-5} (33). DNA polymerase replication accuracy is believed to be achieved by a two-step process, with first discrimination in nucleotide incorporation and then exonucleolytic proofreading; for both steps, base pairing is examined (33). Thus, overall DNA replication accuracy is predicted to be the product of incorporation and proofreading steps, which are approximately 10^{-7}–10^{-8} [(10^{-4}–10^{-5}) × (10^{-3})] for T4 DNA polymerase. This value for T4 DNA polymerase accuracy is about equal to fidelity measurements determined from *in vitro* studies (10^{-6}–10^{-8}) (34–36), and it is comparable to the *in vivo* mutation frequency of about 10^{-8} (37). A 1000-fold contribution by exonucleolytic proofreading observed for T4 DNA polymerase is likely an upper limit because proofreading is costly due to removal of correct as well as incorrectly paired nucleotides (38). There may also be indirect effects on spontaneous mutation in phage with the E191A and D324G DNA polymerase mutations due to poor growth rates or by aberrant DNA structures that may be produced by strand-displacement synthesis and template switching. Mutant T4 DNA polymerases described here and in previous studies (8–10, 12) provide the basis for further examination of the mechanism of T4 DNA polymerase exonucleolytic proofreading.

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