Functional Consequences and Exonuclease Kinetic Parameters of Point Mutations in Bacteriophage T_4 DNA Polymerase[†]

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ABSTRACT: Three groups of T_4 DNA polymerase mutants were prepared and characterized. In the first group, Ala and Asn were substituted for four acidic residues in the exonuclease domain that were chosen on the basis of their sequence alignment with the Klenow fragment from *Escherichia coli* DNA polymerase I. Two divalent metal ions required for catalyzing the 3′−5′ exonuclease reaction are ligated by carboxyl groups from these conserved Asp and Glu residues. The Ala and Asn replacements have a profound effect on the exonuclease activity of T_4 DNA polymerase and also have a significant, but less pronounced influence on its polymerase activity which is located in a domain distal to the exonuclease region. The k_{cat} values for the exonuclease reaction were reduced by 3−4 orders of magnitude by these replacements, but the values of $K_{m(app)}$ did not differ greatly from the wild-type enzyme. The second group consists of replacements of other residues, that are conserved in the exonuclease domain of eukaryotic DNA polymerases, but do not contribute to divalent metal ion coordination. Many of these alterations resulted in decreased exonuclease and/or polymerase activity. Mutants in the third group have substitutions of conserved residues in the polymerase domain which diminished polymerase and altered exonuclease activities. Our results, combined with structural data on crystals of protein N388, a truncated form of T_4 DNA polymerase (Wang et al., 1996), show that: (i) the reduction in the relative specific exonuclease activities of mutants in the first group was significantly less than that of mutants in the Klenow fragment, despite the nearly identical geometric arrangement of the metal liganding groups in two proteins; (ii) altered residues, that affect exonuclease and/or polymerase activities in mutants of the second group, cluster within a small area of the exonuclease domain, suggesting that this area may be directly or indirectly involved in polymerase activity; (iii) mutations in the third group, which affect polymerase and exonuclease activities, may participate in DNA and dNTP binding. Our results point to the functional interdependence of the polymerase and exonuclease domains in T_4 DNA polymerase, a property not observed with the Klenow fragment.

The T_4 DNA replication system has provided a unique opportunity to combine genetic and biochemical approaches to study structural and mechanistic aspects of DNA replication because of its relative simplicity and its lack of dependence on host proteins (for reviews see Nossal & Alberts, 1983; Nossal, 1992; Young et al., 1992). T_4 DNA polymerase plays a central role in this process since it catalyzes both template directed 5′−3′ synthesis of DNA and excision of nucleotides from 3′ termini, an activity that is crucial for maintaining fidelity of DNA replication (Kunkel & Loeb, 1984; Kunkel, 1988; Carroll & Benkovic, 1990; Reddy et al., 1992; Goodman et al., 1993). In contrast to *Escherichia coli* pol I which functions as a repair enzyme, T_4 DNA polymerase (898aa) is essential for phage replication. It has both polymerase (pol) and 3′−5′ exonuclease (exo) activities in the same polypeptide chain and differs in this respect from the essential DNA replication polymerase of *E. coli*, pol III, where pol and 3′−5′ exo activities reside in different subunits (Scheuerman & Echols, 1984; Maki & Kornberg, 1987; Brenowitz et al., 1991). Remarkably, T_4 DNA polymerase shares a greater degree of sequence homology with eukaryotic (B family) polymerases despite its existence in prokaryotes (Spicer et al., 1988; Ito & Braithwaite, 1991). Alignments among the exonuclease domains of different DNA polymerases including the Klenow fragment (KF)[1] and T_4 DNA polymerase have been proposed. These sequence alignments predicted that D112, E114, D219, and D324 in T_4 DNA polymerase would be equivalent to D355, E357, D424, and D501 in KF, respectively (Morrison et al., 1991; Blanco et al., 1992). This alignment was consistent with the results of Reha-Krantz and Nonay (1993) and has subsequently been verified by the 2.2 Å crystal structure (Wang et al., 1996) of the cloned N-terminal region of T_4 DNA polymerase, protein N388 (388aa). A portion of this protein (residues 102−359) had a remarkable structural resemblance to the exo domain of KF (Joyce & Steitz, 1994). It is worth noting that protein N388 has the same specific exonuclease activity as the full length enzyme

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[1] Abbreviations: KF: Klenow fragment of *E. coli* DNA polymerase I; WT: wild-type; dNTP: deoxynucleoside 5′-triphosphate; IPTG: isopropyl β-D-1-thiogalactopyranoside; PMSF: phenylmethanesulfonyl fluoride; EDTA: ethylenediaminetetraacetic acid disodium salt; DTT: dithiothreitol; SDS: sodium dodecyl sulfate; PAGE: polyacrylamide gel electrophoresis; ss: single-stranded; ds: double-stranded.

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when $p(dT)_4$ was used as a substrate (Lin et al., 1994). Despite conservation in the geometry of the residues that provide ligands for the two essential metal ions in the A and B sites of the exonuclease active center in T_4 DNA polymerase and KF, the sequence identity is only 15% in this region. In addition, there are also substantial functional differences between the two proteins (Spicer et al., 1988; Joyce & Steitz, 1994).

In this paper, we report the effect of single and double amino acid substitutions on critical steady state kinetic parameters of the 3′−5′ exonuclease reaction, using single- and double-stranded synthetic deoxyoligonucleotide substrates. We have also examined the effects of these mutations on the pol activity of the altered enzymes and determined the effect on exo and pol activities of substitutions at conserved sites not involved in metal binding. We have shown that among these there are some mutations in the exo domain that affect pol activity and others in the pol domain that influence exo activity. These results support the proposal that the exo and pol domains are functionally *interdependent* (Lin et al., 1994). The implications of these findings are discussed in light of our previous work on the kinetic properties of truncated forms of T_4 DNA polymerase (Lin et al., 1994), our current knowledge of the crystal structure of T_4 protein N388 (Wang et al., 1996), as well as the information available on the structure/activity relationships of the 3′−5′ exonuclease domain of the Klenow fragment where a mechanism for nucleotide excision has already been proposed (Derbyshire et al., 1991; Beese & Steitz, 1991).

MATERIALS AND METHODS

Materials. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from International Biotechnologies, Inc. Restriction endonucleases, T_4 polynucleotide kinase, and oligonucleotides were obtained from either Boehringer Mannheim, Pharmacia, or New England Bio Labs. [γ-^{32}P]-ATP was purchased from Amersham Corp. DEAE-cellulose (DE-52) and phosphocellulose (P11) were from Whatman, and Q-Sepharose was from Pharmacia. Affigel-10, protein A, prestained protein molecular weight markers, and all electrophoresis reagents were from Bio-Rad. Other chemicals were analytical grade. Oligonucleotides used for site-directed mutagenesis and for exo and pol assays were synthesized by the W. K. Keck Foundation Biotechnology Resource Laboratory. DNA sequencing services were also provided by W. K. Keck Foundation Biotechnology Resource Laboratory.

Site-Directed Mutagenesis. To obtain T_4 DNA polymerase gene 43 mutants, a pBR322-derived plasmid, pTL7-g43, containing an M13 single-stranded DNA replication origin, a T7 promoter, and T_4 gene 43 with its own ribosome binding site was used as described (Lin et al., 1994). M13 helper phage was used to prepare single-stranded DNA, and oligonucleotide-directed mutagenesis was then performed according to the method of Kunkel et al. (1987). With each mutant, a unique restriction enzyme site was either created or abolished within or near the mutation site, so that the presence of the altered sequence could be identified by restriction analysis of DNA obtained from cells grown from single colonies prior to its confirmation by DNA sequencing.

Expression and Purification of T_4 DNA Polymerase and Its Mutants. Expression and purification of T_4 DNA polymerase mutants cloned into plasmid pTL7-g43 were carried out essentially as described previously (Lin et al., 1994) except for the inclusion of an additional chromatographic step after the P11 column. For this purpose, a Q-Sepharose column (2.5 × 7 cm) equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 25% glycerol) was used. Elution was carried out with a 250 mL linear gradient from 0 to 500 mM KCl in buffer A. Fractions containing highly purified T_4 DNA polymerase, as judged by SDS−PAGE, were pooled and dialyzed against modified buffer A that contained 50% rather than 25% glycerol. Protein concentrations were determined spectrophotometrically using a calculated molar extinction coefficient at 280 nm (E_m at 280 nm = 1.28×10^5 M^{-1} cm^{-1}), based on the amino acid composition of T_4 DNA polymerase (Spicer et al., 1988). Purified enzyme was stored at −70 °C in aliquots, and then thawed immediately before use.

Purification of Rabbit Anti-T_4 DNA Polymerase Antibody. The procedure described previously (Lin et al., 1994) was used to prepare T_4 DNA polymerase monospecific polyclonal antibody, except that the antibody was concentrated by ultrafiltration using a Centricon-10 membrane.

Limited Proteolysis of T_4 DNA Polymerase and Its Mutants. Wild-type and mutant T_4 DNA polymerases were examined for overall structural integrity by performing limited proteolysis as a function of time. A typical reaction mixture (15 μL) contained 10 μg of wild-type or mutant T_4 DNA polymerase in 20 mM Tris-HCl, pH 8.0, 1.7 mM 2-mercaptoethanol, and 10% glycerol. Chymotrysin (0.4 μg) was added, and the reaction mixture was incubated at 30 °C for various times and terminated with 7.5 μL of stop buffer (50 mM Tris-HCl, pH 8.5, 100 mM 2-β-mercaptoethanol, 0.1% sodium dodecyl sulfate, 20% glycerol, 0.01% bromophenol blue) containing 4 mM PMSF. Proteolysis products were separated using SDS−PAGE (12.5% polyacrylamide) and visualized by staining with Coomassie Brilliant Blue.

Preparation of Substrates. A 5′-^{32}P-labeled $p(dT)_{16}$ was used as the single-stranded DNA substrate. A 5′-^{32}P-labeled 16-mer primer (5′-GCAGCACACGACCAAC-3′) and a 24-mer template (3′-CGTCGTGTGCTGGTTGCCCCTTTT-5′) were annealed and used as the ds-DNA substrate. All synthetic oligonucleotides were purified by electrophoresis on 20% polyacrylamide gels containing 50% urea and 100 mM Tris−acetate buffer, pH 8.0, with 2.2 mM EDTA. Oligonucleotides were 5′-radiolabeled with [γ-^{32}P]ATP and T_4 DNA polynucleotide kinase, as described by Sambrook et al. (1989). Unincorporated nucleotides were removed with Sephadex G25 spin columns. Annealing of the primer to the template was carried out with a template to primer molar ratio of 1.1:1 in 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 20 mM $MgCl_2$; the resulting solution was held at 70 °C for 5 min and allowed to cool slowly to 25 °C. The extent of annealing was checked by electrophoresis using a 7.5% polyacrylamide gel in 25 mM Tris−acetate, 60 mM NaOAc, and 1 mM EDTA, pH 7.5.

Neutralization of T_4 DNA Polymerase Mutants with Polyclonal Antibodies. Wild-type or T_4 DNA polymerase mutants were incubated with affinity-purified polyclonal anti-T_4 DNA polymerase antibodies for 15 h at 4 °C in a 20 μL of solution containing 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and 1% glycerol.

Table 1: Relative Pol and Exo Activities of T_4 DNA Polymerase Mutants Involving Conserved Acidic Residues in the Exo Domain

altered residues in T_4 DNA pol	relative polymerase act.[a]	relative 3′−5′ exonuclease act. on ss-DNA[a]	equivalent metal binding in KF	exo domain metal binding sites[b]
none	1.0	1.0	none	
D112A	0.28	0.3×10^{-3}	D355	A + B
D112N	1.00	0.3×10^{-3}	D355	
E114A	0.20	190×10^{-3}	E357	A
D219A	0.48	1.6×10^{-3}	D424	B
D219N	0.50	1.3×10^{-3}	D424	
D324A	0.56	0.1×10^{-3}	D501	A
D324N	0.30	1.7×10^{-3}	D501	
D112A/D219A	0.08	0.3×10^{-3}	D355A/D424A	A + B, B
D219A/D324A	0.28	0.1×10^{-3}	D424A/D501A	B, A
D112A/D324A	0.34	0.02×10^{-3}	D355A/D501A	A + B, A

[a] Assays for polymerase activity were carried out as described in the Materials and Methods. Values for the specific activities of the wild-type enzyme were normalized to 1.0, and the specific activities of the mutants were expressed as the ratio of the activity of the mutant to the activity of wild-type. Assays were repeated with freshly thawed aliquots of the enzymes until at least three determinations gave values that agreed within ±20%. The average value from these sets is reported here. [b] The assignments of the A and B metal ion sites in KF were from Beese and Steitz (1991). The assignment of sites in T_4 DNA Pol were based on the structure of the T_4 protein N388 (Wang et al., 1996).

Samples were centrifuged at 3000*g* for 5 min, and the supernatants were tested for 3′−5′ exonuclease activity by assaying with 5′-^{32}P-labeled p(dT)$_{16}$. The exonucleolytic activities were compared to T_4 DNA polymerase or its derivatives that were incubated as above, but in the absence of the antibody. Aliquots of T_4 DNA polymerase antibody were also incubated with 5′-^{32}P-labeled p(dT)$_{16}$ under the same conditions to test for contaminating nucleases in the antibody preparations.

Exonuclease Assays. 5′-^{32}P-labeled p(dT)$_{16}$ and the 5′-^{32}P-labeled 16-mer primer annealed to a 24-mer template were used as substrates to test for the 3′−5′ exonuclease activity of the wild-type T_4 DNA polymerase and its mutants on ss- and ds-DNA. p(dT)$_{16}$ (1250 pmol/mL) or the 16/24-mer primer-template (600 pmol/mL) was incubated with the enzymes and assayed as described by Lin et al. (1994). The rates of excision of the 3′-terminal nucleotides were calculated as previously described (Lin et al., 1994). Assays were repeated until at least three determinations gave values that agreed within ±20%. The values from these sets were averaged and reported as the specific excision rate for each enzyme and substrate studied.

Primer Extension (Pol) Assay. The template (24-mer) and the complementary 5′-^{32}P-labeled primer (16-mer) were annealed and used as the substrate for primer extension. The reaction mixture contained 10 μL: 66 mM Tris-HCl, pH 8.8, 17 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 6.5 mM $MgSO_4$, 10% glycerol, 166 μg/mL bovine serum albumin, 0.6 μM of the 16/24-mer primer-template, 100 μM of each of the dNTPs, and various concentrations of wild-type or mutant T_4 DNA polymerases. After incubation for 10 min at 30 °C, the reactions were terminated by addition of 5 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). The aliquots were boiled for 2 min and subjected to electrophoresis on a 20% polyacrylamide−50% urea gel in TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) buffer, pH 8.0. The length of the 5′-labeled primer after the reaction was determined, and the amount converted was estimated after autoradiography by densitometric scanning. The rate of the polymerase reaction was calculated essentially as described for exonuclease reaction by Lin et al. (1994) except that nucleotide incorporation rather than nucleotide removal rates was determined. The specific polymerase activities were determined from the rate of extension of the 3′-terminus of the primer of duplex DNA (16/24-mer primer-template). The values obtained from at least three consistent (±20%) assays at various incubation times or enzyme concentrations were then averaged and reported as the specific polymerase activity for each enzyme.

Kinetic Analyses. Determination of $K_{m(app)}$ and k_{cat} for the exonuclease reactions were carried out under steady state conditions as described by Lin et al. (1994). For each K_m, k_{cat} determination, five to eight different substrate concentrations within a 5-fold range on either side of the K_m were used. Duplicate measurements were made at each substrate concentration. Product analysis and rate profiles were determined as previously described (Lin et al., 1994). Kinetic data were analyzed by Lineweaver−Burk double-reciprocal plots.

RESULTS AND DISCUSSION

Replacement of Conserved Acidic Residues in the Exonuclease Domain of T_4 DNA Polymerase Severely Impairs Exo Activity and Moderately Reduces Pol Activity. Structural studies on the Klenow fragment (KF) led to the identification of four conserved acidic residues, which furnish carboxylate ligands for two divalent metal ions, one in site A and the other in site B (Derbyshire et al., 1991; Joyce & Steitz, 1994).

To characterize mutant T_4 DNA polymerases where these four acidic residues (D112, E114 of exo motif I, D219 of motif II, D324 of motif III) were replaced by Ala or Asn, we determined their specific polymerase (pol) and exonuclease (exo) activities relative to wild-type T_4 DNA polymerase (Table 1). Radioautograms of typical gel patterns are shown for the exo and pol assays using a ss-DNA substrate, p(dT)$_{16}$ (Figure 1, panel A), and a ds-DNA substrate, 16/24-mer primer-template, for both the exo (Figure 1, panel B) and pol assays (Figure 1, panel C). In the pol assays, no degradation of the 5′-labeled 16-mer primer was observed because the dNTP's needed for primer extension were maintained in a large enough excess over the primer-template concentration to favor polymerization. Even though the

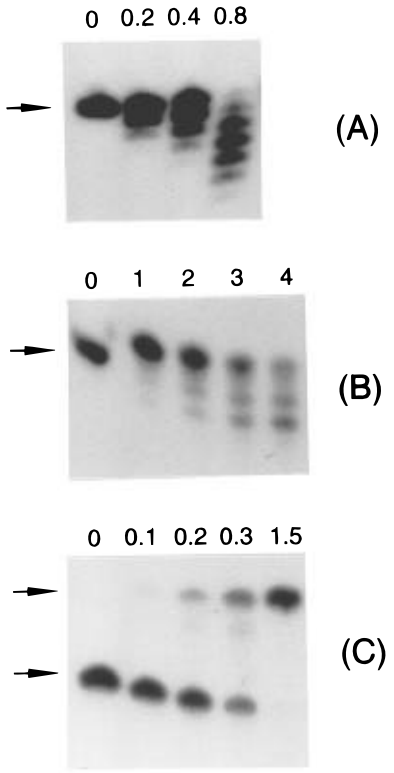

FIGURE 1: Autoradiograms showing polymerase and exonuclease activities of T4 DNA polymerase. Experiments were carried out as described in the Materials and Methods, and also in the legends of Tables 1, 2, and 4. The autoradiograms are for the WT enzyme but represent typical gels used routinely for assaying: (A) exonuclease activity on $p(dT)_{16}$; (B), exonuclease activity on a 16/24-mer primer-template; and (C) polymerase activity using a 16/24-mer primer-template in the presence of dNTPs. The concentration of T_4 pol (nM) increased from left to right (shown on top of each panel). The concentration of the substrates was 1.25, 0.6, and 0.6 μM, respectively, for each of these assays. Reaction mixtures were incubated at 30 °C for 5, 10, and 10 min, respectively, for each assay. Arrows in (A) and (B) and the lower arrow in (C) indicate the remaining ss-DNA $p(dT)_{16}$ or ds-DNA (16/24-mer) substrates of the exonuclease reactions. Exonuclease degradation products in (A) and (B), and primer extension products (indicated by upper arrow) in (C), increased from left to right with increasing enzyme concentrations. The concentration of the T_4 pol mutants varied from the nanomolar to the micromolar range for assaying the exonuclease activities.

targeted residues are located in the exo domain, pol activities were determined in order to obtain information about the structural integrity of the entire protein and the extent to which changes in the exo domain affected the pol function. Some of these exo mutants had diminished pol activities (10−80% of WT), but the decreases were never as large as the losses observed in the exo activity nor did they correlate with the extent of the decrease in exo activity relative to WT (Table 1). The relative specific exo activities, using $p(dT)_{16}$ as the substrate, for D112A, D219A, and D324A were 0.3×10^{-3}, 1.6×10^{-3}, and 0.1×10^{-3}, respectively, compared to the specific activity of WT, which we arbitrarily set at 1.0.

The result summarized in Table 1 demonstrate that substitution of Ala or Asn for each of the conserved Asp residues reduces the 3′ to 5′ exonuclease activity by 3−4 orders of magnitude relative to the WT enzyme. Asn behaved like Ala when substituted for D112 or D219; however, when Asn replaced Asp in the D324N mutant, its exo activity on ss-DNA was 17 times higher than that found with the D324A mutant. Since only one carboxylate oxygen of D501 in KF (Derbyshire et al., 1991) is known to contribute to the coordination of metal ion A, the equivalent residue D324 in T_4 may also contribute only one oxygen. The presence of the β-carboxamide oxygen in Asn 324 of D324N mutant can still coordinate metal ion A; therefore, D324N retains much higher exo activity than D324A.

We also asked whether the exo activity could be reduced still further by simultaneously removing two acidic ligands. Accordingly, we prepared double Asp to Ala mutants and found that their exo activity was equal to that of the single Asp to Ala mutant which displayed the lower activity; i.e., D219A/D324A with a 10^4-fold decrease had the same activity as D324A alone. The only exception was the D112A/D324A double mutant which had 5-fold lower activity than its single D324A counterpart and had a 15-fold decrease relative to D112A (Table 1). It should be noted that both D112 and D324 participate in coordinating metal ions in the A site.

Protein Misfolding Cannot Account for the Reduction in Pol Activity Caused by Mutations in the Exo Domain. As described in the previous section, replacement of the conserved acidic residues in the exo domain not only drastically reduced exo activity but also caused a moderate reduction in pol activity. To rule out the possibility that the reduction in pol activity was due to misfolding or perturbations in the overall structure as a result of these substitutions, we examined the behavior of each mutant, after partial chymotryptic digestion as a function of incubation time. We then compared the patterns obtained after SDS−PAGE with those found with wild-type T_4 DNA polymerase when it was subjected to the same treatment. Examples of these chymotryptic digestion patterns are shown in Figure 2. The time course of the appearance of proteolysis products for all mutants investigated were nearly identical to each other and to that of WT within the range of variation of the proteolytic digestion patterns of the WT enzyme observed in repeated experiments. If a reasonable fraction of the T_4 DNA polymerase mutants were misfolded or structurally destabilized, we would expect to observe substantially altered patterns for the proteolysis products. The fact that this was not observed suggests that there was no significant structural perturbation or misfolding caused by the single or double amino acid replacements.

Residual Exo Activity of the Exo Domain D to A Mutants Is Not Due to Host Cell Nuclease Contamination. It should also be noted that the relative decrease in exo activity that we found with the D219A mutant was not as great as that observed by Frey et al. (1993), who were unable to detect any exo activity for the same mutant using a 20-mer ss-DNA as a substrate. Within the limits of their assay, the decrease in activity relative to WT was greater than 10^7. The apparent discrepancy between our results and theirs could be explained by different assay conditions. Frey et al. used the 20-mer ss-DNA to preform the enzyme−substrate complex before adding Mg^{2+} to start the reaction, which was then continued for up to 3 s. The preincubation of the 20-mer oligonucleotides with the mutants in the absence of Mg^{2+} may result in a nonproductive complex which cannot be converted quickly to a productive complex upon the addition of Mg^{2+}. However, the question of possible contamination with extraneous exonucleases in our study also had to be considered and was addressed. We titrated WT and the D219A, D112A, and D324A polymerase mutants with

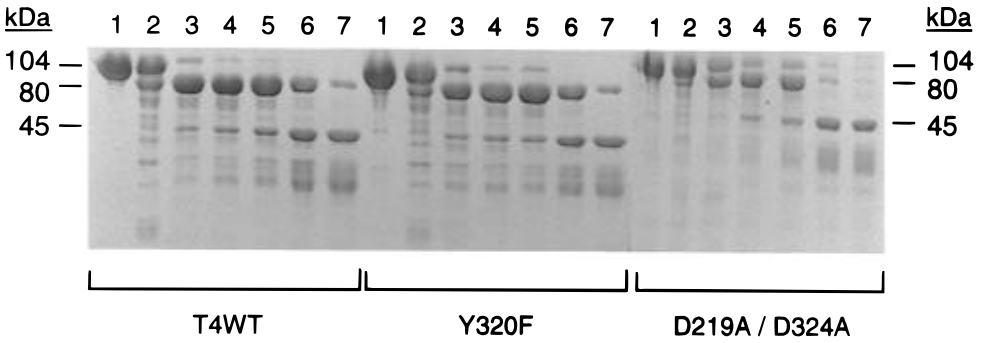

FIGURE 2: Chymotryptic digestion patterns of the mutant T4 DNA polymerases. SDS−PAGE of the chymotryptic digested wild-type and mutant T_4 DNA polymerases shown as a function of digestion time at 30 °C. Lane 1, undigested T_4 pol; lanes 2−7 contain T_4 pol or mutant T_4 pol incubated with chymotrypsin in w/w ratio of 25:1 for 0, 2.5, 4, 7, 30, and 60 min, respectively. Approximately 3 μg of protein was loaded in each lane. The major chymotryptic digestion products are the 80 and 45 kDa fragments.

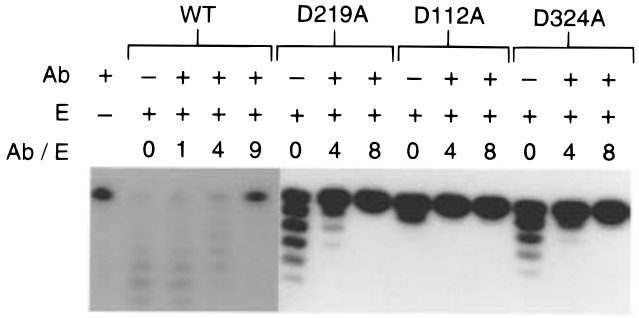

FIGURE 3: Inhibition of 3′−5′ exonuclease activity of T4 pol and its mutants by anti-T4 pol polyclonal antibodies. T_4 pol or its mutants were preincubated with increasing concentrations of T_4 pol antibody for 15 h at 4 °C, and the exonuclease activity was assayed using a 5′-^{32}P-labeled p(dT)$_{16}$ as described in Materials and Methods. The symbol (+) denotes the presence of antibody or enzyme; the symbol (−) denotes the absence of antibody or enzyme; Ab, antibody; E, enzyme; and Ab/E, the antibody to enzyme ratio used in the preincubation. The residual exonuclease activity of the enzyme was completely abolished when pretreated with about 8−9-fold molar excess of antibody over the enzyme in each case.

affinity purified, T_4 DNA polymerase monospecific polyclonal antibody and showed that we could completely eliminate the exo activity (Figure 3), effectively ruling out contamination with other exonucleases at levels that would significantly affect our results.

Comparison of the Reduction in Exo Activity between T_4 DNA Polymerase Mutants and KF Mutants. While it was important to determine the specific exo activity of the T_4 mutants using a ss-DNA substrate to avoid the complexity that might arise from binding and melting of ds-DNA, we nevertheless wanted to compare their relative exo activities with the corresponding mutants in KF. Since the only exonuclease data available with KF mutants are based on results with ds-DNA (Derbyshire et al., 1991), we decided to determine the relative specific activities of the T_4 DNA polymerase mutants with a 16/24-mer primer template so that we could compare the effect of the different substitutions with those corresponding to the conserved acidic residues in KF. Table 2 shows that replacement of conserved Asp residues to Ala decreases the T_4 DNA polymerase exo activity from 0.8×10^{-2} to 6.6×10^{-2} of WT. Table 2 also shows that replacement of corresponding Asp residues in KF to Ala also decreases the exo activity, but the range is from 0.13×10^{-4} to 0.97×10^{-4} of WT. It should be noted, however, that the substrates used in these two studies are slightly different (see Table 2, footnotes). It is interesting

Table 2: Comparison of the Relative 3′−5′ Exonuclease Activities of T_4 DNA Polymerase Mutants with Corresponding Klenow Fragment Mutants Acting on a ds-DNA substrate

T_4 DNA pol	exo act. relative to WT[a]	Klenow fragment	exo act. relative to WT[b]
WT	1.0	WT	1.0
D112A	6.6×10^{-2}	D355A	1.16×10^{-4}
D112N	0.07×10^{-2}	D355N	0.70×10^{-4}
E114A	11×10^{-2}	E357A	23×10^{-4}
D219A	0.11×10^{-2}	D424A	0.13×10^{-4}
D219N	0.86×10^{-2}	D424N	0.32×10^{-4}
D324A	0.08×10^{-2}	D501A	0.97×10^{-4}
D324N	1.0×10^{-2}	D501N	0.55

[a] The substrate used for the T_4 DNA pol assays was a 5′-end-labeled 16-mer primer annealed to a complementary 24-mer template. Assays were carried out as reported in the Materials and Methods. Values for the specific activity of WT were normalized to 1.0, and the values for the mutants were expressed as the ratio of the activity of the mutant to the activity of WT. Assays were repeated with freshly thawed aliquots of the enzymes until at least three determinations gave values that agreed within ±20%. The average value from these sets is reported here.[b] The ds-DNA substrate for KF assays was a heterogeneous mixture of 3′-end-labeled fragments; values for the specific activity of the wild-type (WT) enzyme were normalized to 1.0, and values for the mutants were expressed as the ratio of the activity of the mutant to the activity of WT as described by Derbyshire et al. (1991).

that not only does T_4 DNA polymerase have a higher intrinsic k_{cat} than KF (Lin et al., 1994), but its exo activity is not reduced as much compared to KF when the metal coordinating Asp residues are changed to Ala. Further experiments are needed to fully explain this observation.

There are three comparisons in Table 2 that require further comments: (i) When E114 was changed to Ala, there was less than a 10-fold drop in exo activity of the E114A mutant. Similarly, with the corresponding KF mutant, E357A, there was only a 500-fold decrease in exo activity. With either enzyme the reduction of exo activities of the E to A mutation was much less than the D to A mutations. While it was once thought that the carboxyl group of E357 in KF was important for positioning the substrate correctly within the active site (Beese & Steitz, 1989), it is now believed that this residue participates indirectly in catalysis, perhaps by hydrogen bonding to a water molecule that may be a ligand for metal A (Derbyshire et al., 1991). (ii) There is a 100-fold further decrease in exo activity when D112N is compared to D112A; this phenomenon is seen only when ds-DNA is the substrate (compare the values in Tables 1

and 2). Furthermore, this difference does not show up in the corresponding KF mutants, as the values for D355A and D355N are very similar when ds-DNA is used as the substrate. One possible explanation is that, in the case of the T_4 DNA polymerase D112N mutant, occupancy of the 16/24-mer primer-template in the pol site is highly favored over the exo site, compared to D112A mutant. This would not apply to ss-DNA substrates, and as predicted, no difference in excision rates between D112A and D112N was observed (Table 1). (iii) The relatively large decrease in exo activity of the T_4 DNA polymerase mutant D324N (to 1% of WT) vs the relatively small decrease for the Klenow fragment D501N mutant (to 55% of WT) is the reverse of what is usually observed; i.e., T_4 mutants usually exhibit a smaller reduction in exo activity than KF mutants. This difference is even more dramatic when the ratios of the exo activities of D324A to D324N in T_4 DNA polymerase are compared with the corresponding D501A to D501N ratios in KF; namely, 8×10^{-2} for T_4 DNA polymerase vs 1.76×10^{-4} for KF (Table 2). With KF it was proposed that only one of the two carboxylate oxygens of D501 is required for binding to metal A (Derbyshire et al., 1991). With T_4 DNA polymerase this explanation is not adequate to account for the almost 100-fold drop in exo activity found with the D324N mutant acting on the 16/24-mer substrate. Thus, despite the remarkable similarities in the architecture of the exonuclease active centers in KF and T_4 protein N388 (Wang et al., 1996), the detailed architecture is sufficiently different to be reflected in their specific exo activities. A possible clue as to the difference in the behavior of the T_4 mutant with the Asn substitution as compared to the mutant with the equivalent Asn substitution in KF comes from the crystal structure of T_4 protein N388 where the side chain carboxylate oxygen of D324 forms a salt bridge with the amino group of K299 (Wang et al., 1996). In the T_4 D324N mutant it is likely that the side chain carboxamide oxygen of Asn 324 is hydrogen bonded to the amino group of K299, preventing rotation of the β-carboxamide around the adjacent methylene group. In the wild-type T_4 protein, the side chain oxygens of D324 are fixed, as a consequence of the salt bridge to K299, and thus have the correct geometry for ligand binding to the metal ion in the A site. When Asp 324 is replaced by Asn, the position of the side chain amide may still be fixed with nitrogen pointing toward the metal; however, since amide nitrogens are poor ligands for coordinating metal ions, this could account for the large drop in exo activity of the D324N mutant compared to WT. In KF there is no equivalent salt bridge, thus there is nothing to prevent the side chain carboxamide oxygen of Asn 501 from serving as a coordinating ligand for metal A in KF. If this is the case, it would account for the ability of the D501N mutant in KF to retain nearly half of its WT exo activity (Table 2). This interpretation is also consistent with the decrease in exo activity when Lys 299 in T_4 DNA polymerase is changed to Ala (see below).

Kinetic Analysis of the 3′−5′ Exonuclease Activities of T_4 DNA Polymerase Mutants Acting on ss- and ds-DNA. To determine whether the reduced exo activity of the T_4 DNA polymerase mutants was due to decreased binding or a lower catalytic efficiency, we estimated $K_{m(app)}$ and k_{cat} with $p(dT)_{16}$ as the ss-DNA substrate and a 16/24-mer primer-template as the ds-DNA substrate (Table 3). Similar $K_{m(app)}$ values were obtained for the WT enzyme and the D to A mutants using ss-DNA substrates with the exception of D219A where the $K_{m(app)}$ was twice the value found for the WT enzyme. Thus, the decrease in exo activity of the Asp to Ala mutants is due almost entirely to drastically reduced k_{cat} values which are several orders of magnitude lower than wild-type T_4 DNA polymerase. These results indicate the importance of the β-carboxyl groups in helping to correctly position the catalytic divalent metal ions. The γ-carboxyl group of Glu 114 probably does not act directly as a ligand for the A metal as mentioned above, since there is only about a 30-fold decrease in k_{cat} for E114A. This is consistent with the location of the E114 carboxylate in the structure of T_4 protein N388 (Wang et al., 1996).

Table 3: Kinetic Parameters for 3′−5′ Exonuclease Activities of T_4 DNA Polymerase Mutants Acting on ss- and ds-DNA Substrates[a]

	$p(dT)_{16}$		16/24-mer	
protein	$K_{m(app)}$ (μM)	k_{cat} (s^{-1})	$K_{m(app)}$ (μM)	k_{cat} (s^{-1})
WT	6.9	65.1	0.4	0.16
D112A	7.7	7.8×10^{-3}	1.6	0.37×10^{-2}
D112N	ND[b]	ND	4.0	0.01×10^{-2}
E114A	5.0	2.0	2.0	0.08
D219A	12.8	9.6×10^{-2}	0.3	0.06×10^{-3}
D324A	6.3	6.2×10^{-3}	0.4	0.07×10^{-3}
D219A/D324A	6.7	3.4×10^{-3}	ND	ND

[a] Kinetic parameters were determined as described in the Materials and Methods. We used double-reciprocal plots having at least five data points at concentrations within a 5-fold range on either side of the K_m values. Experiments were repeated until at least three determinations gave values that were within ±30%, and the average value from these sets is reported here. [b] Not determined.

The $K_{m(app)}$ values for the D219A and D324A mutants with the ds-DNA substrate are nearly identical to WT whereas the D112A, D112N, and E114A mutants have up to 10-fold higher $K_{m(app)}$ (Table 3). These higher values suggest that binding in the exo mode is less favorable for these mutants than for WT. The $K_{m(app)}$ values for ds-DNA are clearly lower than those for ss-DNA. Similar differences in $K_{m(app)}$ for ss- vs ds-DNA were found with KF and were attributed to the higher affinity of the enzyme for ds-DNA (Derbyshire et al., 1991).

The differences in k_{cat} values for excision of the 3′ nucleotide of ss- vs ds-DNA by the WT and mutant are quite dramatic, i.e., 500-fold for WT and 100-fold for D324A (Table 3). Differences in this range are observed for nearly all the mutants except for D112A where the k_{cat} for $p(dT)_{16}$ is just twice that estimated for hydrolysis of the phosphodiester linkage of the 3′-terminal nucleotide in the primer component of the 16/24-mer. Given the structure of the 16/24-mer, where 4 out of 5 base pairs at the 3′ end of the primer are G:C, the tendency of the 3′ end of the primer to melt will be small, which will reduce the residence time of the primer-template in the exo mode, consequently lowering the effective concentration of the catalytically competent E−S complex for the exo reaction, and will result in a decreased k_{cat} for ds-DNA. This is because the k_{cat} for the exo reaction is a composite rate constant which includes a translocation rate constant for the ds substrate as it moves from the pol to the exo site as suggested by Catalano et al. (1990). The higher k_{cat} for $p(dT)_{16}$ probably reflects the rate of the chemical step as suggested by Derbyshire et al. (1991) for KF.

Table 4: Sequence Alignment and Activities of T_4 DNA Polymerase Mutants That Do Not Directly Involve Divalent Metal Ion Binding Residues for Exo Function[a]

(A) Sequence Alignment of Altered Residues in T_4 DNA Polymerase with DNA Polymerases from Phage RB69 and *E. coli*[b]

	Exo I			Exo II				Exo III	
	• •			• •		• •	•	• •	•
T_4	112 DIE	····120	FPDPMK	·····214	**N**IEG**F**D	····284	**S**FS**LE**SVAQHETKKG**K**	·····320	**YN**IID
RB69	114 DIE	····123	FPEPSQ	·····217	NVESFD	····287	SYSLDYISEFELNVGK	·····323	YNIID
KF	355 DTE	····360	SLDNIS	·····420	NL-KYD	····455	RHDMDSLAERWLKHKT	·····497	YAAED

(B) Relative Pol and Exo Activities of T_4 DNA Polymerase Mutants

	mutants										
act.	WT	N214S[f]	F218A	S284A	L287A	E288A	K299A	Y320A	Y320Q	Y320F	N321A
Pol[c]	1.0	0.5	1.12	1.0	0.29	0.50	1.0	0.02	0.02	0.15	0.67
ds.exo[d]	1.0	1.0	1.0	0.52	0.36	0.88	0.50	0.068	0.07	0.018	0.83
ss.exo[e]	1.0	1.0	1.0	0.35	0.19	1.02	0.17	0.019	0.013	0.006	1.0

[a] Assays for the enzyme activities were carried out as described in the experimental section. Values for the specific activities of the WT were normalized to 1.0, and the specific activities of the mutants were expressed as the ratio of the activity of the mutant to the activity of WT. Assays were repeated with freshly thawed aliquots of the enzymes until at least three determinations gave values that agreed within ±20%. The average value of each set is reported here. [b] (•) Shows the location of T_4 mutations. Altered residues other than those serving directly as metal ligands in T_4 polymerase are in bold type. [c] 5′-end-labeled 16-mer primer annealed to a complementary 24-mer template and dNTPs were used as substrates for the polymerase activity assay. [d] 5′-end-labeled 16-mer annealed to a complementary 24-mer template was used as a substrate for the double-stranded exonuclease activity assay. [e] 5′-end-labeled $p(dT)_{16}$ was used as a substrate for the single-stranded exonuclease activity assay. [f] Spicer et al. (1988).

The $K_{m(app)}$, k_{cat} values for the D112A vs the D112N mutants suggest that the translocation step from the pol site to the exo site in the case of D112N may be responsible for reducing the observed k_{cat} for D112N to 2.7% that of the D112A mutant. The partitioning of a primer-template between pol and exo sites has been well documented for T_4 DNA polymerase and KF (Cowart et al., 1989; Reddy et al., 1992; Gopalakrishnan & Benkovic, 1994). This difference between D112A and D112N suggests that occupancy by the 16/24-mer in the pol site might be highly favored in the D112N mutant. This is in agreement with the fact that the pol acitivity of D112A is lower than that of D112N (28% vs 100% of WT, see Table 1). The properties of the D112N mutant are distinctly different from the corresponding D355N KF mutant, which exhibits almost the same relative decrease in exo activity as the D355A mutant (Derbyshire et al., 1991). An adequate explanation for the behavior of the T_4 D112N pol mutant, however, will require determination of the crystal structure of the complex of this mutant polymerase with the 16/24-mer or with a similar primer-template. Since the DNA polymerase from a related T-even phage RB69 (Wang, C. C., et al., 1995) has been crystallized,[2] we anticipate that the structure of a DNA polymerase−primer-template complex will be solved.

The k_{cat} value for D219A was more than 10-fold higher than for D112A and D324A with $p(dT)_{16}$. This difference may be due to the fact that alterations which perturb coordination of ligands to the A metal or to shared metal sites damage the catalytic center more than mutations which affect the B metal site.

Alteration of Conserved Residues Other Than Asp and Glu in the Exo Domain Can Affect Pol and/or Exo Activities. The presence of highly conserved residues (Table 4, panel A) other than those required for divalent metal ion binding in the exo I, II, and III motifs of family B polymerases prompted us to examine the effect of single substitutions at some of these sites. Unexpectedly, only L287A, K299A, S284A, and substitutions of various amino acids for Y320 resulted in significantly diminished exo activity (Table 4, panel B). Both Lys 299 and Tyr 320 may be involved in the exo active site but in different ways. K299 appears to form a salt bridge with the carboxylate oxygen of D324 (Wang et al., 1996), and disruption of this interaction may perturb the geometry of the D324 ligand enough to diminish the exo activity. The role of Tyr 320 is more puzzling. In protein N388, the hydroxyl group of Tyr 320 points away from the active site and is hydrogen bonded to the backbone carbonyl of Leu 300 (Wang et al., 1996). This situation differs from the equivalent conserved Tyr 497 in KF where the hydroxyl group points into the active site and assists in orienting the water molecule that attacks the phosphorus atom in the scissile bond (Derbyshire et al., 1991; Freemont et al., 1988; Beese & Steitz, 1991). Replacing Tyr 320 by Ala, Gln, or Phe in the intact T_4 DNA polymerase results in significantly lower exo and pol activities. The fact that partial proteolysis profiles of the mutants and the WT were identical (Figure 2) suggested that global misfolding of the mutants did not occur. We believe that the orientation of Y320 in the intact T_4 DNA polymerase is similar to Y497 in KF, and also participates in the active site. We base this on the crystal structure of RB69 DNA polymerase[2] where Tyr 323 has an orientation closely resembling Y497 in KF. The results obtained with the Y320 mutants, as well as with other mutants such as L287A, E288A, and N321A, show that substitutions in the exo domain can diminish pol activity even when the exo activity remains at WT levels; i.e., N321A, N214S, and E288A (Table 4, panel B).

It is noteworthy that all of the conserved residues that are important for preservation of exo and/or pol activity are located on one face of the exo domain (Figure 4). This is consistent with the idea that the pol and exo sites are functionally linked, perhaps because this region makes contact with the pol domain. This represents a major difference between T_4 DNA polymerase and KF since none of the reported exo mutants in KF affect pol activity (Derbyshire et al., 1991; Joyce & Steitz, 1994).

[2] J. Wang, A. K. M. A. Sattar, C. C. Wang, J. Karam, W. H. Konigsberg, and T. A. Steitz (unpublished results).

Table 5: Sequence Alignment and Activities of T_4 DNA Polymerase Mutants Involving Residues Conserved in the Pol Domain of B Family DNA Polymerases

(A) Sequence Alignment of Altered Residues in T_4 DNA Polymerase with Other DNA Polymerases from Pol B Family[a]

	Region I	Region II	Region III	Region IV
T4	408 **D**LTSLYPSII····557	**K**ILINSLYG ····616	AGDTDS ·····	702 **K**KR**Y**ALNVY
RB69	411 DLTSLYPSII····560	KLLINSLYG ····619	YGDTDS ·····	705 KKRYALNVW
Ø29	249 DVNSLYPAQM····383	KLMLNSLYG ····454	YCDTDS ·····	497 QKTYIQDIY
PRD1	220 DVNSMYPHAM····340	KLILNSSYG ····426	YCDTDS ·····	464 KKLYALYAG
Human α	860 DFNSLYPSII····950	KLTANSMYG ···1000	YGDTDS ·····	1052 KKKYAALVV

(B) Relative Pol and Exo Activities of T_4 DNA Polymerase Mutants Involving Residues in the Pol Domain[b]

	mutants			
act.	WT	D408A	K557A	K702S/Y705A
Pol[c]	1.0	0.20	0.40	*f*
ds.exo[d]	1.0	0.28	0.76	2.40
ss.exo[e]	1.0	0.15	0.34	0.11

[a] The sequence alignment except the one with RB69 was from Blanco et al. (1991). Locations of T_4 mutations are shown in bold type. [b] Assays for the enzyme activities were carried out as described in the experimental section. Values for the specific activities of the WT were normalized to1.0, and the specific activities of the mutants were expressed as the ratio of the activity of the mutant to the activity of WT. [c] 5′-end-labeled 16-mer primer annealed to a complementary 24-mer template and dNTPs were used as substrates for the polymerase activity assay. [d] 5′-end-labeled 16-mer annealed to a complementary 24-mer template was used as a substrate for the double-stranded exonuclease activity assay. [e] 5′-end-labeled $p(dT)_{16}$ was used as a substrate for the single-stranded exonuclease activity assay. [f] Competing exonuclease activity made the polymerase activity not detectable by the standard assay method.

Alteration of Conserved Residues in the Polymerase Domain Can Affect Pol and/or Exo Activities. At least four regions (I−IV) of conserved amino acid motifs are located in the C-terminal portion of the B family DNA polymerases (Table 5, panel A) (Blanco et al., 1991). To test the functional role for some of these conserved residues, they were replaced by mutagenesis in various DNA polymerases: Based on studies with several B family DNA polymerases (Dong et al., 1993a,b; Blasco et al., 1992, 1993b), it appears that the motif DX_2SXYPX_3 (region I) plays a role in dNTP binding and/or catalysis of the nucleotidyl transferase reaction. Residues in the motif KX_3-NSXYG (region II) participate in primer-template binding and dNTP binding (Blasco et al., 1992, 1993a; Zhu et al., 1994). Residues in motif DTDS (region III), for example, D456 and D458 in ϕ29 DNA polymerase (Blasco et al., 1993a) and D1002 and D1004 in human DNA polymerase α, have been proposed as ligands for the metal ions that are essential for the nucleotidyl transferase activity (Copeland & Wang, 1993; Copeland et al., 1993). Recently, it has been shown that conserved residues in region IV (KKXY) in ϕ29 DNA polymerase are important for stabilization of the primer-terminus in the pol active site (Blasco et al., 1995). To extend our knowledge of the role of several invariant residues in the pol domain of T_4 DNA polymerase, we constructed the following mutants and determined their specific pol and exo activities: D408A, K557A, and K702S/Y705A (Table 5). Alterations of these conserved residues in regions I and II of the polymerase domain affect both pol and exo activities (Table 5, panel B). The diminished exo activity of the T_4 DNA pol domain mutants provide further support for the idea that the pol and exo sites are functionally linked, as mentioned in the previous section.

Interestingly, the double mutant K702S/Y705A involving conserved residues of region IV affects exo activity (Table 5, panel B) in an unexpected way, by increasing the ds exo activity compared to that of WT. This is similar to the behavior of the T_4 DNA pol mutant located downstream in the same region (A737V) (Spacciapoli & Nossal, 1994). Recently, other mutants with these properties have been reported. For example, the K498R, K498T, and Y500S mutants involving conserved residues in region IV of ϕ29 (Table 5, panel A) had an exo activity on ds-DNA that was

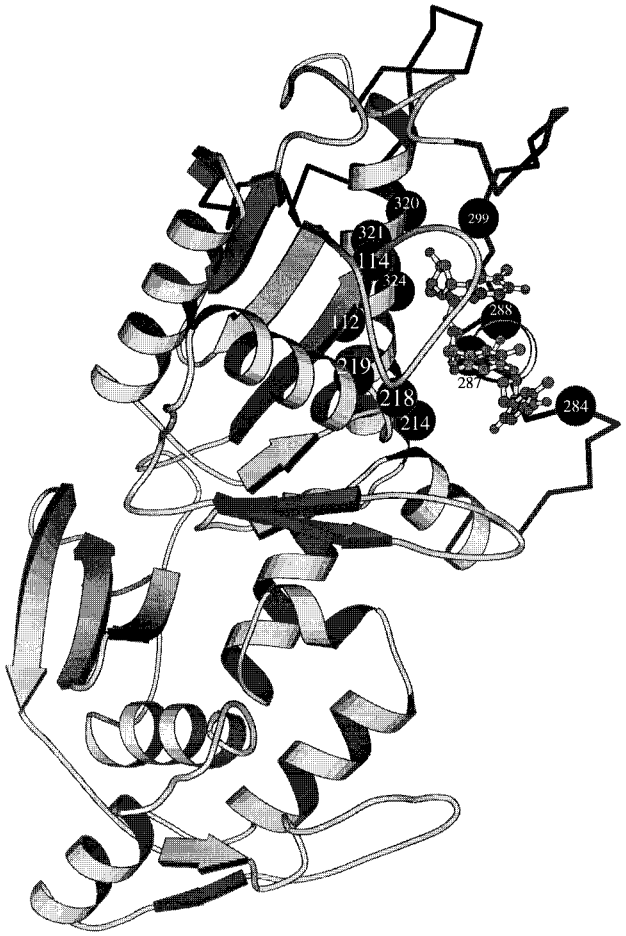

FIGURE 4: Positions of amino acid residues that were altered in the N-terminal region of T4 pol. The mutations are numbered in dark spheres on the ribbon representation of the T_4 protein N388 structure along with the bound $p(dT)_3$ substrate in the ball-and-stick drawing which was prepared by J. Wang based on the structure of T_4 protein N388 (Wang et al., 1996).

2.5−5 times higher than the WT enzyme (Blasco et al., 1995). The increase in exo activity of these mutants vs WT when acting on ds-DNA can be attributed to the importance of K702 and Y705 in stabilizing the primer-template in the pol vs the exo mode. Replacement of these residues with Ala probably shifts the equilibrium to favor the exo mode, enhancing the exo activity.

Differences between T_4 DNA Polymerase and Klenow Fragment. To summarize, our results support the concept that T_4 DNA polymerase and the Klenow fragment share the same basic design for the exonuclease active center: three Asp residues coordinating two metal ions that facilitate phosphodiester bond hydrolysis. However, the two enzymes differ in significant ways: (i) Mutations in either exo or pol domains of T_4 DNA polymerase frequently diminish both exo and pol activities. This implies that, in contrast to KF, the exo and pol domains in T_4 DNA polymerase are at least functionally interdependent. (ii) Some features in the exo domain of T_4 DNA polymerase such as the D324 to K299 salt bridge are not found in KF. This salt bridge may account for the greater reduction in exo activity shown by D324A compared to D112A or D219A, a situation that does not occur in the corresponding KF mutants. (iii) T_4 DNA polymerase not only has a higher k_{cat} than KF (Lin et al., 1994), but also the D to A mutations in the exonuclease domain of T_4 DNA polymerase does not reduce exo activity as much as the equivalent mutations in KF. Double D to A mutations such as D112A/D324A in T_4 DNA polymerase reduce its exo activity further to 2×10^{-5} of WT. However, this double mutant still retains a much higher exo activity than KF having a single D to A mutation, when compared under identical assay conditions (data not shown). Although the crystal structures of KF, protein N388, and RB69 DNA polymerase provide a picture of the exo active sites, they do not explain why the exo activity of T_4 DNA polymerase is so much greater than that of KF. Further kinetic and more detailed structural information is required to answer this question.

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