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High-level Expression, Purification, and Enzymatic Characterization of Full-length *Thermus aquaticus* DNA Polymerase and a Truncated Form Deficient in 5′ to 3′ Exonuclease Activity

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The *Thermus aquaticus* DNA polymerase I (Taq Pol I) gene was cloned into a plasmid expression vector that utilizes the strong bacteriophage λ P_L promoter. A truncated form of Taq Pol I was also constructed. The two constructs made it possible to compare the full-length 832-amino-acid Taq Pol I and a deletion derivative encoding a 544-amino-acid translation product, the Stoffel fragment. Upon heat induction, the 832-amino-acid construct produced 1–2% of total protein as Taq Pol I. The induced 544-amino-acid construct produced 3% of total protein as Stoffel fragment. Enzyme purification included cell lysis, heat treatment followed by Polymin P precipitation of nucleic acids, phenyl sepharose column chromatography, and heparin–Sepharose column chromatography. For full-length 94-kD Taq Pol I, yield was 3.26×10^7 units of activity from 165 grams wet weight cell paste. For the 61-kD Taq Pol I Stoffel fragment, the yield was 1.03×10^6 units of activity from 15.6 grams wet weight cell paste. The two enzymes have maximal activity at 75°C to 80°C, 2–4 mM $MgCl_2$ and 10–55 mM KCl. The nature of the substrate determines the precise conditions for maximal enzyme activity. For both proteins, $MgCl_2$ is the preferred cofactor compared to $MnCl_2$, $CoCl_2$, and $NiCl_2$. The full-length Taq Pol I has an activity half-life of 9 min at 97.5°C. The Stoffel fragment has a half-life of 21 min at 97.5°C. Taq Pol I contains a polymerization-dependent 5′ to 3′ exonuclease activity whereas the Stoffel fragment, deleted for the 5′ to 3′ exonuclease domain, does not possess that activity. A comparison is made among thermostable DNA polymerases that have been characterized; specific activities of 292,000 units/mg for Taq Pol I and 369,000 units/mg for the Stoffel fragment are the highest reported.

The thermostable DNA polymerase I (Taq Pol I) from *Thermus aquaticus* (*Taq*) greatly improves the yield, specificity, automation, and utility of the polymerase chain reaction (PCR)[1,2] method of amplifying DNA fragments.[3] Furthermore, because of the high turnover number, lack of proofreading activity, high temperature optimum, and ability to incorporate 7-deaza-2-deoxyguanosine efficiently, Taq Pol I yields long stretches of readable DNA sequence that are uniform in intensity and free of background.[4]

A 94-kD Taq Pol I has been purified from *Taq*, but growing the organism is more difficult than growing *Escherichia coli*. Although the activity yield is high (40–60%), the expression level of Taq Pol I in the native host is quite low (0.01–0.02% of total protein). The cloning and expression of full-length 94-kD Taq Pol I in *E. coli* under control of the *E. coli lac* promoter[5,6] or the *tac* promoter[7] has been reported. Because polymerase yields in these constructs were low (~0.01% of total protein in our initial construct; see ref. 5), we sought to improve the expression level of the enzyme by mutagenizing the 5′ and 3′ ends of the gene and cloning the mutagenized gene into a more suitable expression vector. We also constructed a truncated Taq Pol I gene, deleted for the first 867 bp of the gene, which yields a predicted 61-kD translation product. The 61-kD derivative, Taq Pol I Stoffel fragment, is active in polymerase assays and PCR and is devoid of the inherent 5′ to 3′ exonuclease activity of Taq Pol I. A similarly truncated gene deleted for the first 705 bp, has recently been constructed, yielding an approximately 67-kD translation product, KlenTaq DNA polymerase.[8] Protein produced by the two constructs described here provides a plentiful supply of the two forms of Taq Pol I enzyme, thereby enabling their biochemical properties to be investigated. The study of these enzymes not only provides key insights into nucleic acid metabolism, but also provides useful information to those investigators who exploit DNA polymerases in specialized molecular biology techniques, including the PCR. Advancements in PCR methodology

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depend in part on an increased understanding of the biochemistry of thermostable DNA polymerases, as such information aids substantially in the constant refinement of this and other research techniques.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strain DG98 (*thi-1 endA1 hsdR17 lacI*^{Q} *lacZΔM15 proC*::Tn*10 supE44*/ F'*lacI*^{Q} *lacZΔM15 proC*$^{+}$) has been described.[9] *E. coli* strain DG116 [*thi-1 endA1 hsdR17 supE44* (λ*cI857 bioT76 ΔH1*)] is a derivative of strain MM294[10] containing a defective λ prophage. DG116 was prepared using bacteriophage P1 transduction[11] and *E. coli* strain N6590 [C600 *rK*$^{-}$ *mK*$^{-}$ *thr leu pro lacZXA21*::Tn*10* (λ*cI857 bioT76 ΔH1*), provided by M. Gottesman, Columbia University] as the source of the defective prophage. Plasmid pBS^{+} was purchased from Stratagene. Plasmid pFC54.T has been described.[12] Plasmid pDG160, a derivative of pFC54.T, contains a convenient restriction site polylinker between the P_L promoter and retroregulator cassettes.

Reagents

Restriction endonucleases [New England Biolabs (NEB)], *E. coli* DNA polymerase I, large fragment (Klenow) (U.S. Biochemicals), T4 DNA ligase,[13] calf intestine alkaline phosphatase (CIAP) (Boehringer-Mannheim), and polynucleotide kinase (kinase) (NEB) were used according to standard procedures. Oligonucleotides were phosphorylated as described.[14] Molecular weight standards for SDS-polyacrylamide gel electrophoresis (PAGE) were purchased from Pharmacia [γ-^{32}P]ATP (3000 Ci/mmole), [α-^{32}P]dCTP (800 Ci/mmole), and [^{3}H]dCTP (22.8 Ci/mmole), were purchased from New England Nuclear. Oligonucleotide synthesis was performed on a Biosearch Model 8750 DNA synthesizer using controlled-pore glass and *O*-cyanoethyl-*N*, *N*-diisopropyl phosphoramidites.[15,16] Reagents and synthesis protocols were obtained from Milligen/Biosearch (Novato, CA).

Crude salmon sperm DNA was obtained from Sigma (#D-1626, Type III) and "activated" by incubation at 4°C for 24 hr at 1.3 mg/ml in 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$.[17] Single-stranded template DNA was prepared by conventional methods[14] from M13 phage TSYC657 (7.359 kb), a derivative of M13mp10 containing a 129-nucleotide insert derived from a *Streptomyces limosus* α-amylase gene.[18] The 24-mer synthetic oligodeoxynucleotide primer SC64 (Table 1) is complementary to the *S. limosus*-derived insert in TSYC657 and has a calculated melting temperature of 95°C at 0.12 μM in 100 mM NaCl. Primer and template were annealed at 583 nM SC64 and 256 nM TSYC657(2.2:1 primer: template) in 10 mM Tris-HCl (pH 8.0) 6 mM $MgCl_2$, and 50 mM KCl. The mixture was heated at 95°C for 4 min, incubated at 70°C for 10 min, and cooled to room temperature.

Cloning Procedures

For oligonucleotide site-directed mutagenesis, single-stranded DNA was prepared from pBS^{+} plasmid derivatives pLSG1 and pLSG2 as described.[5] Competent cells were prepared and transformations were carried out by the method of Hanahan.[19] Alkaline-SDS miniplasmid DNA preparations were carried out by the method of Birnboim and Doly.[20] Enzymatic dideoxynucleotide DNA sequence analysis[21] confirmed all DNA sequence alterations.

Oligonucleotide Site-directed Mutagenesis[22]

For pLSG1 mutagenesis, 0.24 pmole of single-stranded pLSG1, 0.36 pmole of *Pvu*II-digested CIAP-treated vector pBS^{+}, and 0.48 pmole of primer DG26 (Table 1) were annealed. The mixture was put on ice and TTP, dGTP, dATP, [^{3}H]dCTP were each added to 200 μM along with 1 unit of Klenow fragment. The extension mixture was incubated at 0°C for 30 min and then at 30°C for 30 min. The reaction products were transformed into DG98. Nitrocellulose filters containing ampicillin-resistant transformants were hybridized according to the method of Woods et al.[23] with ^{32}P-labeled DG26 probe. Positive colonies were picked and screened by restriction analysis of alkaline-SDS miniplasmid preparations. A representative correct candidate was designated pLSG2 (Fig. 1).

For mutagenesis of pLSG2, 0.5 pmole of single-stranded pLSG2, 0.5 pmole of *Pvu*II-digested, CIAP-treated pBS^{+}, and 2.5 pmoles of oligonucleotide SC107 (Table 1) were annealed. Extension, transformation, and screening were performed as described above, using ^{32}P-labeled SC107 as the probe. A representative correct candidate was designated pSYC1578 (Fig. 1).

Assembly of a Full-length Taq Pol I Gene in High-level Expression Vectors

An *Sph*I and *Bgl*II-digest of pSYC1578 DNA was ligated with *Hin*dIII- and *Bam*HI-digested vector pFC54.T and an annealed duplex oligonucleotide, DG27/28 (Table 1). The oligonucleotide duplex provided *Hin*dIII and *Sph*I cohesive ends and codons one, two, and two of three nucleotides for codon three of Taq Pol I. The resulting plasmid was designated pLSG5 (Fig. 1).

Assembly of a Truncated Taq Pol I Gene

A *Bst*XI digest of pSYC1578 was treated with Klenow to generate blunt ends. The DNA was then treated with *Bgl*II and a 1619-bp *Bst*XI-blunt–*Bgl*II fragment (encoding Taq Pol I codons 294–832) was isolated via gel electrophoresis and electroelution. The isolated DNA fragment was ligated to *Hin*dIII- and *Bam*HI-digested vector pFC54.T and an annealed

TABLE 1 Oligonucleotides Used in These Studies

DG26	5'-CCCTTGGGCTCAAAAAGTGGAAGCATGCCTCTCATAGCTGTTTCCTG
DG27	5'-AGCTTATGAGAGGCATG
DG28	ATACTCTCC-5'
DG29	5'-AGCTTATGTCTCCAAAAGCT
DG30	ATACAGAGGTTTTCGA-5'
DG48	5'-GGGAAGGGCGATCGGTGCGGGCCTCTTCGC
DG67	5'-CCCGGGCGGCGCCGCAGCGGCGGGp
SC64	5'-CCCGGGCGGCGCCGCAGCGGCGGG
SC107	5'-GCATGGGGTGGTAGATCTCACTCCTTGGC

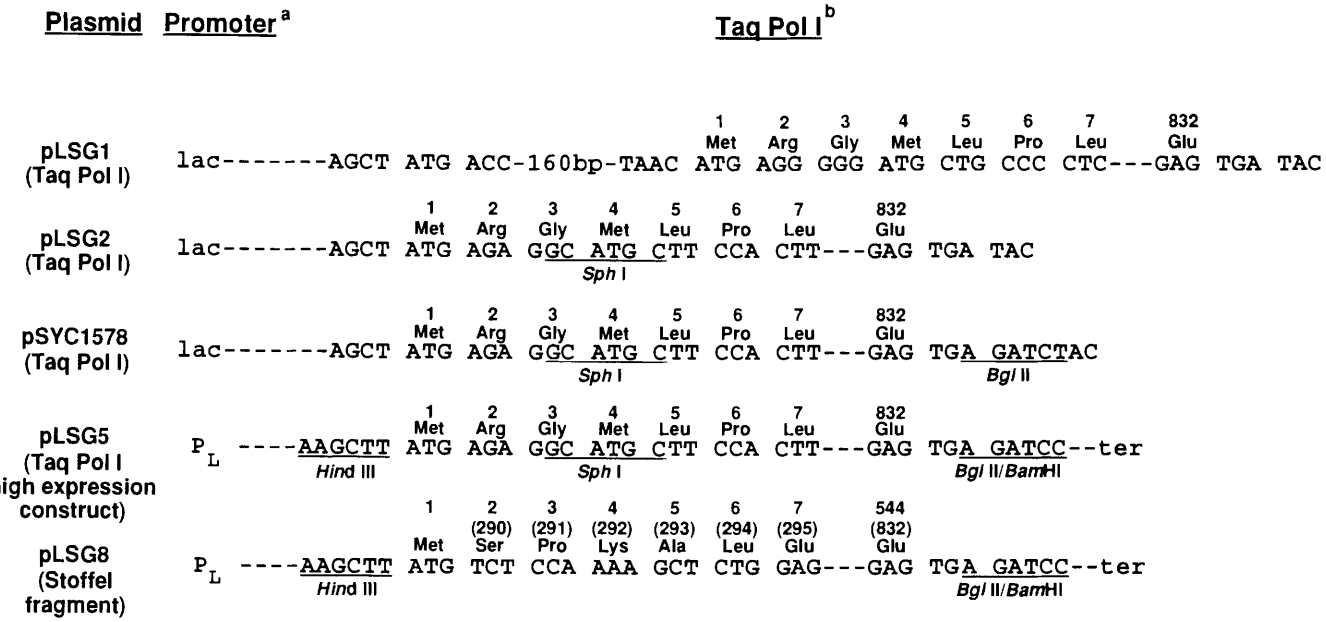

FIGURE 1 Taq Pol I expression constructions. Plasmids are described in the text. [a]Promoter-ribosome binding sites are: *lac*, β-galactosidase; P_L λ P_L promoter/gene *N* ribosome binding site. [b]Taq Pol I codons are numbered above each sequence to show the amino-terminal codons and carboxy-terminal codons and how they were altered in the mutagenesis and recloning described in the text. Restriction endonuclease cleavage sites are underlined and labeled. The *B. thuringiensis* δ-toxin retroregulator is indicated by ter. Numbers appearing in parentheses in pLSG8 refer to the corresponding codon numbering of the full-length gene.

duplex oligonucleotide DG29/DG30 (which provides a Met start codon as well as codons 290–293 of Taq Pol I; see Table 1). The truncated gene assembled in *Hin*dIII/*Bam*HI-digested vector pFC54.T was designated pLSG8 (Fig. 1).

Inductions

Isopropyl-β-D-thiogalactopyranoside (IPTG)-inductions of pLSG1 and pLSG2 were as described.[5] For heat inductions of plasmids pDG160, pLSG5, and pLSG8, cultures were inoculated to OD_{600} = 0.05 from a fresh 30°C overnight culture in Bonner–Vogel salts[24] plus 0.25% casamino acids, 0.2% glucose, 10 μg/ml thiamine, and 100 μg/ml ampicillin. Cultures were incubated at 30°C until the OD_{600} of the culture reached 0.7, then induced at 37°C or 41°C for 2.5–21 hr. For enzyme assays of small-scale inductions, frozen pellets were thawed, suspended in 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.5 μg/ml leupeptin, 2.5 mM phenylmethylsulfonyl flouride (PMSF), and sonicated. Heat-treated extracts were prepared by adding $(NH_4)_2SO_4$ to 0.2 M, heating at 75°C for 20 min, chilling on ice, and clarifying by centrifugation.

PCR Using Heat-treated Cell Extracts

Heat-treated extracts from 37°C, 9-hr heat inductions of cultures of pLSG5 (Taq Pol I) and pLSG8 (Stoffel fragment) were assayed for enzyme activity. The extracts were diluted to 4 units/μl in 1× storage buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween 20 [Pierce, "Surfact-Amps"], and 50% glycerol).The dilutions were used in the PCR under standard conditions[3] at 4, 2, 1, 0.5, or 0.25 units per reaction. Reactions were subjected to 30 cycles of PCR amplification using human globin gene primers, PC03 and PC04, and template DNA isolated from the human cell line Molt 4.[3]

Purification of Recombinant Taq DNA Polymerase

All operations were carried out at 0–4°C unless otherwise specified. All glassware was baked and all solutions were autoclaved where possible. Induced pLSG5/DG116 cells (165 grams wet cell weight) were thawed in 165 ml of 2× TE buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA), containing 2.4 mM PMSF and 0.5 μg/ml leupeptin, and homogenized at low speed in a blender. The thawed cells were lysed in an Aminco French Pressure Cell (20,000 psi), sonicated to reduce viscosity, and diluted to 935 ml with 1× TE containing 2.4 mM PMSF and 0.5 μg/ml leupeptin. This lysate (Fraction I; see Fig. 2A) contained 18.4 grams of protein and 45,000,000 units of DNA polymerase activity.

Ammonium sulfate was added to 0.2 M and the lysate centrifuged at 25,000*g* for 20 min. The supernatant, Fraction II, was incubated at 75°C for 15 min to denature *E. coli* host proteins, then cooled to 0°C and adjusted to 0.6% Polymin P, precipitating approximately 90% of the A_{260} absorbing material. The mixture was stirred at 0°C for 3 hr and centrifuged at 25,000*g* or 30 min. The heat-inactivation/Polymin P step removed 93% of the total soluble protein and yielded a greater than 14-fold purification (Fraction III, Fig. 2A).

Fraction III was loaded onto a 2.2 × 11-cm (42 ml) phenyl–Sepharose CL-4B (Pharmacia-LKB, Lot #MI 02547) column (equilibrated in TE containing 0.2 M ammonium sulfate) at 40 ml/hr. The column was washed with 400 ml of the same buffer (A_{280} to baseline), followed by 180 ml of TE at 50 ml/hr, and finally 105 ml of 20% (wt/vol) ethylene glycol

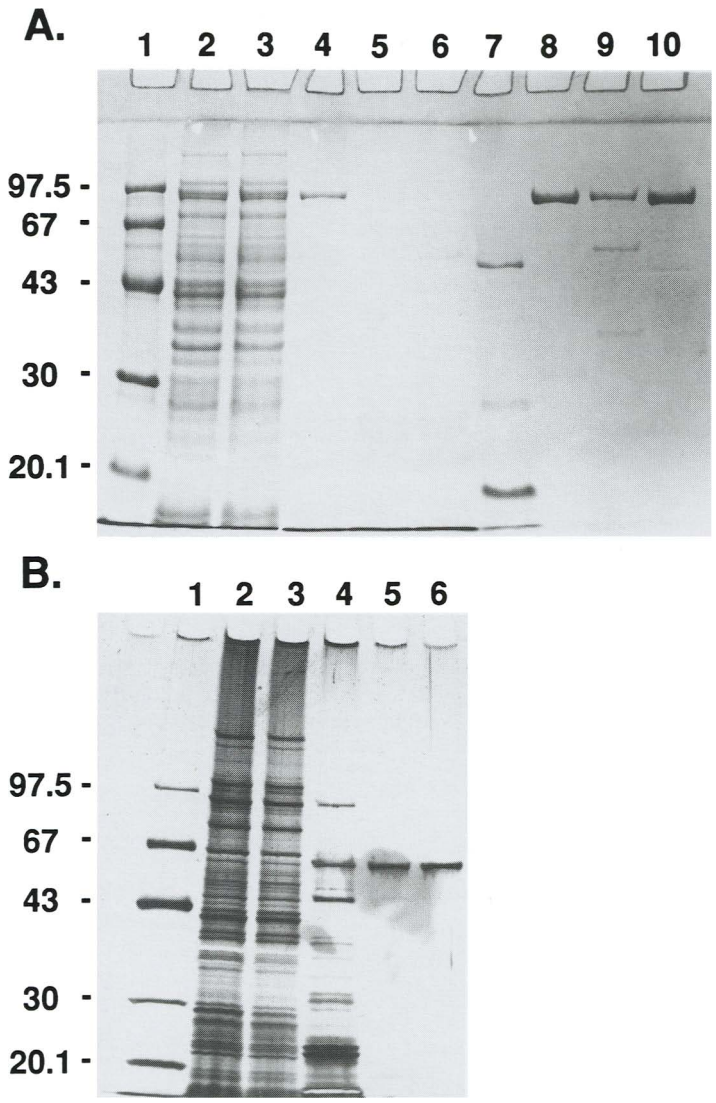

FIGURE 2 Purifications of full-length of Taq Pol I and Stoffel fragment. (*A*) Full-length Taq Pol I purification fractions analyzed via SDS-PAGE on a 10% running gel, 4.75% stacking gel, stained with Coomassie brilliant blue. (Lane *1*) Molecular weight standard, molecular weights (in thousands) shown at left; (lane *2*) 20 μg of Fraction I; (lane *3*) 20 μg of Fraction II; (lane *4*) 3 μg of Fraction III; (lane *5*) 3 μg of early phenyl–Sepharose flow-through; (lane *6*) 3 μg of late phenyl–Sepharose flowthrough; (lane *7*) 3 μg of phenyl–Sepharose low-salt eluate; (lane *8*) 1 μg of Fraction IV; (lane *9*) control from a previous purification; (lane *10*) 1 μg (230 units) of Fraction V. (*B*) Stoffel fragment purification fractions analyzed via SDS-PAGE on a 4–20% gradient gel and stained with silver (Integrated Separation Systems). (Lane *1*) Molecular weight standard, molecular weights (in thousands) shown at left; (lane *2*) 1.1 μg of Fraction I; (lane *3*) 0.9 μg of Fraction II; (lane *4*) 2.4 μg of Fraction III; (lane *5*) 0.1 μg of Fraction IV; (lane *6*) 0.1 μg (22.5 units) of Fraction V.

in TE, removing most of the residual nucleic acid. The Taq Pol I activity was eluted with a 600-ml linear gradient of 0–4 M urea in TE containing 20% ethylene glycol. Fractions (5.5 ml) eluting between 0.25 and 2.7 M urea were pooled forming Fraction IV (Fig. 2A), which contained 73% of the applied activity and 12% of the applied protein.

Fraction IV was loaded onto a 3.2 × 9-cm (72 ml) heparin–Sepharose column (equilibrated with 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 0.2% Tween 20) at 70 ml/hr. The column was washed with 170 ml of the same buffer containing 150 mM KCl and eluted with a 700-ml linear gradient of 150–650 mM KCl in the same buffer. Fractions eluting between 275 mM and 325 mM KCl were pooled (98 ml total) and the pooled fractions concentrated/diafiltered on an Amicon YM30 membrane to a final volume of 20 ml in a 2.5× storage buffer preparation lacking glycerol. Thirty milliliters of 80% glycerol were added to the concentrated and diafiltered sample forming Fraction V (Fig. 2A), which was stored at −20°C.

Purification of 61-kD Taq Pol I, Stoffel Fragment

Purification of the Stoffel fragment from induced pLSG8/DG116 cells proceeded as the purification of full-length Taq Pol I (above) with some modifications: Induced pLSG8/DG116 cells (15.6 grams) were homogenized and lysed (Fraction I; see Fig. 2B), as above, yielding 1.87 grams of protein and 1,046,000 units of DNA polymerase activity. Following heat treatment (Fraction II, Fig. 2B), as above, Polymin P (pH 7.5) was added slowly to 0.7%. The heat-inactivation/Polymin P step removed 92% of the total soluble protein and yielded a greater than 14-fold purification. The supernatant, Fraction III (Fig. 2B), was loaded onto a 1.15×3.1-cm (3.2 ml) phenyl–Sepharose column at 10 ml/hr. All of the applied activity was retained on the column. The column was washed with 15 ml of the equilibration buffer and then with 5 ml of 100 mM KCl in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (TE). The polymerase activity was eluted with 2 M urea in TE containing 20% ethylene glycol. Fractions (0.5 ml each) with polymerase activity were pooled (8.5 ml) and dialyzed into heparin sepharose buffer containing 100 mM KCl, forming Fraction IV (Fig. 2B). This fraction contained 87% of the applied activity and 3.6% of the applied protein, yielding a 24-fold purification. Fraction IV was loaded onto a 1.0-ml bed volume heparin–Sepharose column equilibrated as above. The column was washed with 6 ml of the same buffer (A_{280} to baseline) and eluted with a 15-ml linear gradient of 100–500 mM KCl in the same buffer. Fractions (0.15 ml) eluting between 165 and 255 mM KCl were pooled (2.5 ml) and diafiltered on a Centricon 30 membrane into a 2.5× storage buffer preparation lacking glycerol, forming Fraction V (Fig. 2B).

Enzyme Purification Activity Assays

DNA polymerase assays were performed

in a 50-µl volume at 75°C essentially as described.[5] For full-length Taq Pol I, assay conditions were 25 mM *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS) (pH 9.5), 1 mM β-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl, 200 µM each dATP, TTP, and dGTP, 100 µM [α-^{32}P]dCTP (0.05 Ci/mmole), and 250 µg/ml activated salmon sperm DNA template. For the Stoffel fragment, assay conditions at 75°C were 25 mM Tris-HCl (pH 8.3), 1 mM β-mercaptoethanol, 4 mM $MgCl_2$, 15 mM KCl, 200 µM each dATP, TTP, and dGTP, 100 µM [α-^{32}P]dCTP (0.1–0.2 Ci/mmole), and 250 µg/ml of activated salmon sperm DNA template.

Divalent Metal Ion Optimization

A Titrisol standard solution of $MgCl_2$ (VWR Scientific) was used to titrate a solution of EDTA colorimetrically.[25] The EDTA was then used to determine the actual concentration of previously prepared stocks of $MgCl_2$, $MnCl_2$, $CoCl_2$, and $NiCl_2$. All optimization assays used activated salmon sperm DNA template (250 µg/ml). Optimizations were performed in 25 mM Tris-HCl (pH 8.3) (25 mM TAPS, pH 9.4, for Taq Pol I $MgCl_2$ optimizations), 55 mM KCl, and 11.2 fmoles polymerase (21 ng/ml for Taq Pol I and 13.6 ng/ml for the Stoffel fragment) per reaction, in otherwise standard 50-µl assays.[5]

KCl Optimization

Optimization reactions (50 µl) for KCl concentration with Taq Pol I (11.2 fmoles enzyme; 21 ng/ml) using both the M13 primer-template (53 nM) and activated DNA template (250 µg/ml) contained 2.5 mM $MgCl_2$ in an otherwise standard TAPS, pH 9.4 buffer.[5] For KCl optimization in assays using the Stoffel fragment, activated DNA template (250 µg/ml) assays (50 µl) were performed in 25 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$ with 11.2 fmoles enzyme (13.7 ng/ml), whereas M13 primer-template (53 nM) assays (50 µl) were performed in 25 mM Tris-HCl (pH 8.3), 4.5 mM $MgCl_2$ with 27.9 fmoles polymerase (34 ng/ml). KCl concentration was varied from 0 to 200 mM.

Thermal Activity Profile Assay

Taq Pol I thermal activity assay conditions were either 2 mM $MgCl_2$, 12 fmoles polymerase (22.5 ng/ml), and 250 µg/ml activated template or 3 mM $MgCl_2$, 74.4 fmoles polymerase (140 ng/ml), and 60 nM primer-template, in an otherwise standard 50-µl TAPS, pH 9.4 reaction. Assay conditions of the thermal activity of the Stoffel fragment were 25 mM Tris-HCl (pH 8.3), 25 mM KCl, and either 4.0 mM $MgCl_2$, 11.2 fmoles polymerase (13.7 ng/ml), and 250 µg/ml activated DNA template or 4.5 mM $MgCl_2$, 74.4 fmoles polymerase (90.8 ng/ml), and 60 nM primer-template.

Thermal Inactivation Assay

Thermal inactivation assays of Taq Pol I and Stoffel fragment were carried out in a standard PCR mixture: 10 mM Tris-HCl (pH 8.3), 0.5 ng λ DNA template, 200 µM each of dATP, TTP, and dGTP, 0.2 µM each of two oligonucleotide primers, and either 50 mM KCl, 2 mM $MgCl_2$, and 1.25 units (4.3 ng) of Taq Pol I or 10 mM KCl, 3 mM $MgCl_2$, and 5 units (13.5 ng) of Stoffel fragment in a 50-µl reaction volume. Reaction mixtures were overlaid with paraffin oil and incubated at either 95°C or 97.5°C for 0, 2, 5, 10, 30, or 60 min. Aliquots (5 µl) were held on ice until assayed in the presence of 100 µM [α-^{32}P]dCTP for polymerase enzyme activity on activated salmon sperm DNA in the enzyme purification activity assay described above.

5′ to 3′ Exonuclease Activity Assay

Five picomoles of 5′-^{32}P-labeled, 3′-phosphorylated oligonucleotide DG67 (Table 1) was annealed to 6 pmoles of single-stranded TSYC657 DNA in the presence (to form a 133-nucleotide gap between the 3′ end of DG48 and the 5′ end of DG67) or absence of 15 pmoles of the synthetic oligonucleotide primer DG48 (Table 1) in a 60-µl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.1 mM EDTA. The annealing mixture was heated to 95°C for 4 min, incubated at 70°C for 10 min, and then cooled to room temperature. Exonuclease activity was assayed at 70°C for 6 min in a 30-µl reaction containing 1 mM β-mercaptoethanol, 25 mM TAPS (pH 9.4), 50 mM KCl, 2 mM $MgCl_2$, 200 µM each TTP, dATP, dGTP, and dCTP, 6 µl (0.6 pmole single-strand DNA) of the above annealing mixture, and either 0.05 pmole (157 ng/ml) or 1 pmole (3.1 µg/ml) of Taq Pol I, or 2 pmoles (4.1 µg/ml) of Stoffel fragment. Reactions were stopped by the addition of 6 µl of 60 mM EDTA. The reaction products were fractionated electrophoretically on a 20% polyacrylamide gel and analyzed by autoradiography. Radioactivity was quantitated by liquid scintillation spectrometry of excised reaction products.

RESULTS

DNA Sequence Manipulations

Figure 1 shows a summary of the plasmids described below and the pertinent DNA sequence changes among them. As previously described,[5] plasmid pLSG1 contains the full-length Taq Pol I gene under transcriptional control of the *E. coli lac* promoter. IPTG-induced cultures harboring pLSG1 produce 14–37 units of Taq Pol I per milligram of total crude extract protein (Table 2 and ref. 5) or ~0.005–0.015% of total protein. To increase expression levels of Taq Pol I, several manipulations were performed. Oligonucleotide mutagenesis of the 5′ end of the gene resulted in a 170-bp deletion of *Taq* and vector DNA, moving the ATG of Taq Pol I into the position of the ATG codon of β-galactosidase. In addition, a *Sph*I site (in italics, below) was introduced near the Taq Pol I ATG start codon by changing a G to a C in the third codon. Also, codons two, five, six, and seven were altered in their third positions to be more AT rich, changing the DNA sequence from ATG AGG GGG ATG CTG CCC CTC to ATG AGA G*GC ATG* CTT CCA CTT without altering the amino acid sequence of the translated protein. The resulting plasmid was designated pLSG2. An IPTG-induced pLSG2 culture has Taq Pol I activity of 639 units/mg crude extract protein, a 38-fold increase over an uninduced culture (Table 2). The 3′ end of the Taq Pol I gene in pLSG2 was mutagenized to introduce a *Bgl*II site (in italics, below) at the stop codon, changing that sequence from TGATAC to TG*AGATCT*AC. The resulting plasmid, with the Taq Pol I gene altered at both the 5′ and 3′ ends was designated pSYC1578.

Cloning and Expression of Taq Pol I in P_L Expression Vector

The plasmid expression vector into which we cloned the Taq Pol I gene uti-

TABLE 2 *Taq* Polymerase Activity in *E. coli* Extracts

Experiment	Extract	Uninduced[a] (−)/ Induced (+)	Specific activity[b]
I	pDG160	+	≤0.06[c]
	pLSG1	−	1.1
		+	14.3
	pLSG2	−	16.7
		+	639
	pLSG5	−	97.6
		+ (37°C)	2790
		+ (41°C)	362.7
II	pDG160	+	≤0.07
	pLSG8	− (5 hr, 30°C)	61.9
		+ (5 hr, 37°C)	1037.4
		+ (5 hr, 41°C)	3163.7
		− (21 hr, 30°C)	506.7
		+ (21 hr, 37°C)	9503.4
		+ (21 hr, 41°C)	12,310.1

[a]Experiment I: pDG160 and pLSG5 were heat-induced as described in Materials and Methods for 9 hr. Experiment II: pDG160 and pLSG8 heat inductions were 5 or 21 hr. Uninduced cultures were incubated at 30°C for the same amount of time. IPTG induction of pLSG1 and pLSG2 is also described in Materials and Methods.

[b]Specific activity in units/mg total crude extract protein when assayed, as described[5] on clarified, heat-treated extract, for Experiment I and as described in Materials and Methods for Experiment II.

[c]A background of 0.01% input counts has been subtracted. The specific activity represents less than two times background.

lizes the bacteriophage λ P_L promoter and gene *N* ribosome binding site. The Taq Pol I cloning preserved the natural spacing between the gene *N* ribosome binding site and ATG start codon. In addition, the vector provides the *Bacillus thuringiensis* positive retroregulator and point mutations in RNA II, rendering the plasmid temperature sensitive for copy number as described.[12] A culture harboring pLSG5 was heat-induced, as described in Materials and Methods. Taq Pol I activity in a heat-treated crude extract of induced pLSG5 was approximately four-fold higher than in pLSG2 (Table 2). A corresponding increase in induced Taq Pol I protein was also seen upon SDS-PAGE analysis (data not shown).

Others have observed[26,27] that proteins produced upon heat induction of P_L promoter constructs at 42°C are insoluble. By lowering the induction temperature to 37°C or 41°C, these investigators found they could increase the solubility of induced proteins significantly. We compared heat-treated crude extracts of pLSG5 that had been induced at 37°C or 41°C. Enzyme assays show that we recovered 8- to 13-fold less DNA polymerase activity in the heat-treated extract from the 41°C induction compared to that from the 37°C induction (Table 2). In addition, we observed more degraded forms of Taq Pol I in a Western blot of total protein from the 41°C-induced culture compared to that from the 37°C-induced culture (data not shown). Based on the specific activity of the polymerase and protein determination on total crude lysate, the induced activity in the pLSG5/DG116 culture at 37°C represented 1–2% of total protein being expressed as Taq Pol I.

Cloning and Expression of Truncated Taq Pol I

We had observed immunoreactivity and enzymatic activity in induced cultures harboring pFC84 and pFC85, which were deleted for the first 615 nucleotides of the Taq Pol I gene.[5] Thus, there was reason to believe that other truncated forms of Taq Pol I could be active. The *Bst*XI recognition site in the Taq Pol I gene at nucleotide 872 (amino acid 291) is directly upstream of the region corresponding to the end of *E. coli* Pol I small fragment. In addition, the *Bst*XI recognition site is approximately 570 bp (190 amino acids) proximal to the beginning of the region that, when translated, shows high amino acid similarity to the polymerase domain of *E. coli* Pol I. Because the *E. coli* Pol I Klenow fragment is active as a polymerase, we made a deletion of Taq Pol I that we hoped would express a thermostable polymerase molecule analogous to Klenow fragment.

Unlike its full-length counterpart, the Stoffel fragment activity is not lost upon heat-induction at 41°C (Table 2). After 21 hr at 41°C, a heat-treated crude extract from a culture harboring pLSG8 had 12,310 units of heat-stable DNA polymerase activity per milligram of crude extract protein, a 24-fold increase over an uninduced culture. A heat-treated extract from a 21-hr, 37°C-induced pLSG8 culture had 9503 units of activity per milligram of crude extract protein. Table 2 shows the difference in accumulated levels of polymerase activity between 5 hr and 21 hr of induction—a ninefold increase between 5 hr and 21 hr at 37°C and a nearly fourfold increase between 5 hr and 21 hr at 41°C.

Activity of Crude Extracts in PCR

To determine whether both the full-length and Stoffel fragment forms of Taq Pol I (encoded by pLSG5 and pLSG8, respectively) were active in PCR, we performed PCR assays using heat-treated crude extracts from induced cultures of pLSG5 and pLSG8. Heat-treated extracts containing approximately 4, 2, 1, 0.5, or 0.25 units of enzyme activity were used in otherwise standard PCR amplifications.[3] The results, shown in Figure 3, indicated that both the full-length (lanes 1–5) and Stoffel fragment (lanes 7–11) Taq Pol I enzymes were thermostable and functioned well in PCR. The full-length enzyme functioned well in PCR at 0.5–2 units of enzyme. Faint PCR products were visible from reactions carried out with 0.25 unit of enzyme. Four units of enzyme in the reactions yielded high-molecular-weight nonspecific products, as expected.[28] In contrast, PCR product was only observed in the reactions utilizing 4 units and 2 units of Stoffel fragment (lanes 7 and 8). Interestingly, a significantly higher yield of intended specific product was obtained with 4 units of Stoffel fragment. In addition, nonspecific higher-molecular-weight products were not visible.

Enzyme Purification

The purification of full-length *Taq* DNA

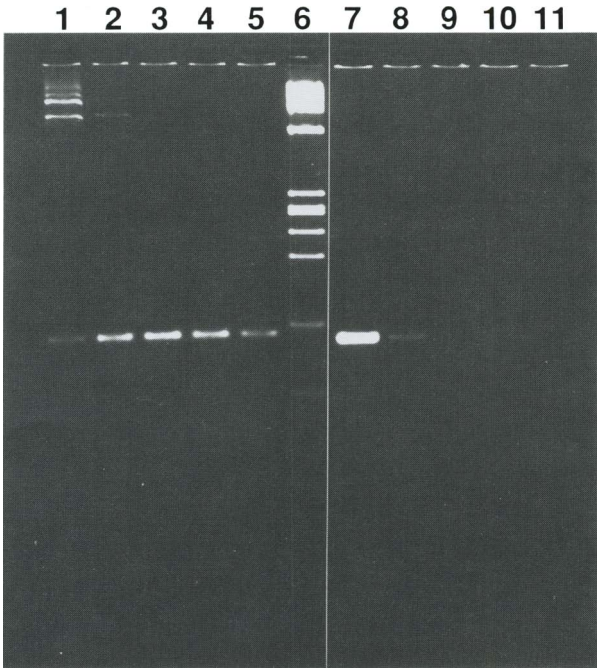

FIGURE 3 PCR comparing the two forms of Taq Pol I. PCR using heat-treated extracts of induced cultures is described in Materials and Methods. (Lanes *1–5*) Taq Pol I from pLSG5; (lanes *7–11*) Stoffel fragment from pLSG8; (lane *6*) molecular weight marker; (lanes *1* and *7*) 4 units of enzyme; (lanes *2* and *8*) 2 units; (lanes *3* and *9*) 1 unit; (lanes *4* and *10*) 0.5 units; (lanes *5* and *11*) 0.25 units.

polymerase (Fraction V, Fig. 2A) resulted in the recovery of 32,600,000 units (73%) of polymerase activity. Between 11 mg (protein concentration determined by amino acid composition) and 14 mg (protein concentration determined by Lowry assay using BSA as a standard) of protein were obtained, resulting in a calculated specific activity between 230,000 and 290,000 units/mg protein, and representing a 97- to 122-fold purification. The predicted molecular weight of Taq Pol I (based on the determined DNA sequence of the 832-codon open reading frame) is 93,920 daltons. A specific activity of 290,000 units/mg protein yields a calculated molecular turnover number of 150 nucleotides per second at 75°C.

The purification of the Stoffel fragment (Fraction V, Fig. 2B) yielded 1,291,588 units of DNA polymerase activity and 2.8 mg of protein. The calculated specific activity of the Stoffel fragment is 369,000 units/mg protein, representing a 451-fold purification. The predicted molecular weight of the Stoffel fragment (based on the determined DNA sequence of the 543- or 544- codon open reading frame, see below) is 61.3 kD, yielding a calculated molecular turnover number of 130 nucleotides per second at 75°C. For comparison, the specific activity of *E. coli* DNA polymerase I Klenow Fragment is between 8,000 and 30,000 units/mg,[29–31] and the specific activity of T7 DNA polymerase (T7 gene 5 protein and *E. coli* thioredoxin) is 10,000–55,000 units/mg.[29,32] The ranges are a reflection of the dependence of the enzyme activity on the nature of the DNA substrate in the assay.

Based on amino acid analysis (data not shown), recombinant Taq Pol I (in contrast to native *Taq* DNA Polymerase) was not blocked at the amino terminus and retained the initiating methionine residue, as would be predicted from the properties of *E. coli* methionine amino peptidase.[33] Amino acid analysis of the purified Stoffel fragment yielded a complex result. The expected amino acid sequence at the amino terminus of the protein is Met-Ser-Pro-Lys-Ala. Unlike Taq Pol I, the majority of the purified Stoffel fragment did not retain the initiating methionine residue. The amino terminus of the major species was Ser-Pro-Lys-Ala. However, there were two minor species in the analysis, one of which had the amino-terminal Met, the other of which had a Pro at its amino terminus.

Optimization Studies

Figure 4 shows divalent metal ion titration data for Taq Pol I (A) and Stoffel fragment (B) enzymes using $MgCl_2$, $MnCl_2$, and $CoCl_2$. Assays using $NiCl_2$ yielded little, if any, detectable polymerase activity for either enzyme (data not shown). For Taq Pol I, magnesium chloride stimulated enzyme activity maximally at 2–3 mM (Fig. 4A) and 10 mM $MgCl_2$ yielded only 60% of the maximum activity, at 200 μM each TTP, dATP, and dGTP and 100 μM dCTP. Maximal stimulation of activity with $MnCl_2$ (0.6 mM) and $CoCl_2$ (0.6 mM) was approximately 80% of the maximal activity stimulated by $MgCl_2$. Similar to Taq Pol I, the Stoffel fragment showed a prefer-

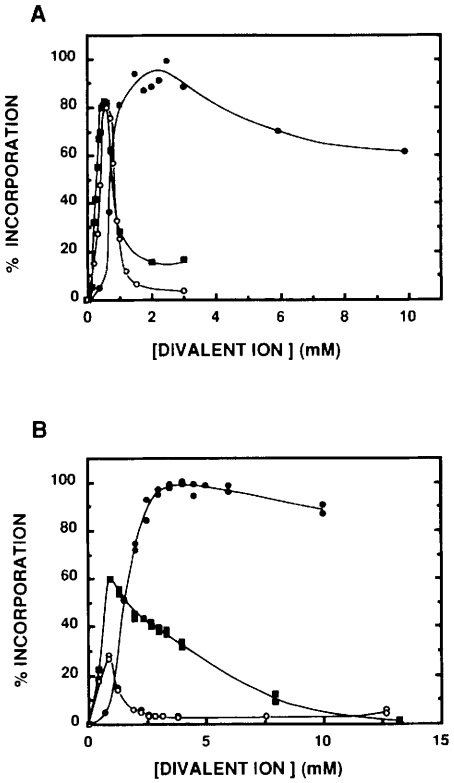

FIGURE 4 Divalent metal ion titration. The influence of $MgCl_2$(●), $MnCl_2$ (■), and $CoCl_2$ (○) on Taq Pol I(*A*), and Stoffel fragment (*B*) enzyme activities. Assays and titration are as described in Materials and Methods and results are plotted as the percent of maximum dCMP incorporation with respect to increasing concentration of divalent metal ion.

ence for $MgCl_2$. Unlike the full-length form, Stoffel fragment enzyme activity was maximally stimulated over a broad concentration range of $MgCl_2$, with a maximum of 4 mM (Fig. 4B). Similarly, Stoffel fragment activity was stimulated over a broad concentration range of $MnCl_2$, with a maximum of 0.92 mM (60% of the maximal activity achieved with $MgCl_2$). Cobalt chloride stimulated Stoffel fragment activity maximally at 0.88 mM (27% of the maximal activity achieved with $MgCl_2$).

Figure 5 shows that the influence of KCl concentration differs between Taq Pol I and the Stoffel fragment and also depends on the template used in the assay. For Taq Pol I assayed with activated DNA template, the optimum KCl concentration was 55 mM with approximately 58% activity at 0 and 150 mM. In contrast, with Taq Pol I and the M13 primer-template, maximum activity was achieved at 10 mM KCl (activity in the absence of KCl was not determined) with 48% activity at 50 mM KCl. For the Stoffel fragment with activated DNA template, maximum activity was achieved with 0 KCl, although polymerase activity remained above 90% of maximum up to 45 mM KCl and remained over 50% at 100 mM KCl. In contrast, with M13 primer-template and the Stoffel fragment, activity declined rapidly at greater than 10 mM KCl. Assaying the M13 primer-template with 40 mM KCl yielded approximately 50% maximum activity and activity declined to near 0 by 100 mM KCl.

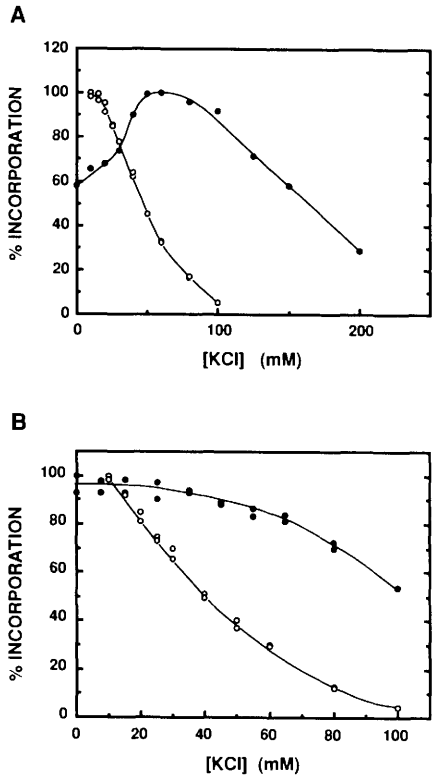

FIGURE 5 KCl titration. The influence of KCl on Taq Pol I,(*A*) and Stoffel fragment (*B*) enzyme activities using either activated salmon sperm DNA template (●) or the defined M13 primer-template (○), as described in Materials and Methods. One-hundred percent incorporation represents 155 pmoles of dCMP incorporated for Taq Pol I with the activated template, 106 pmoles for Taq Pol I with the M13 primer-template, 110 pmoles for Stoffel fragment with the activated template, and 92 pmoles for Stoffel fragment with the M13 primer-template.

Thermoactivity and Thermal Inactivation

Thermal activity profiles of Taq Pol I and the Stoffel fragment on M13 primer-template and activated salmon sperm DNA were compared. Figure 6 shows that the optimal temperature depends on the nature of the DNA template. DNA polymerase activity increased faster, initially, with increasing temperature on the M13 primer-template than on the activated DNA. For both forms of the enzyme, the primer-template substrate is primarily single-stranded DNA (approximately 390 μM total single-stranded template nucleotide and 53 nM primer duplex). In contrast, the activated DNA (approximately 750 μM total DNA nucleotide) substrate is primarily duplex DNA containing an undefined, but probably high, concentration of nicks and short gaps.[17] The pronounced difference in thermal activity with inherently different substrates could reflect differences either in DNA binding constants or stability constants for single-stranded or double-stranded DNA as a function of temperature. Alternatively, the relative increase in activity on M13 primer-template DNA at 65°C and lower may accurately reflect the difference in extension rate for the polymerase when there is no blocking strand. If the extension rate is limited by the presence of a blocking strand (e.g., limited 5′ to 3′ exonuclease activity during synthesis or limited ability to catalyze strand-displacement synthesis), the sharp and pronounced transition in activity on salmon sperm DNA at 70–75°C could reflect denaturation of the template blocking strand.

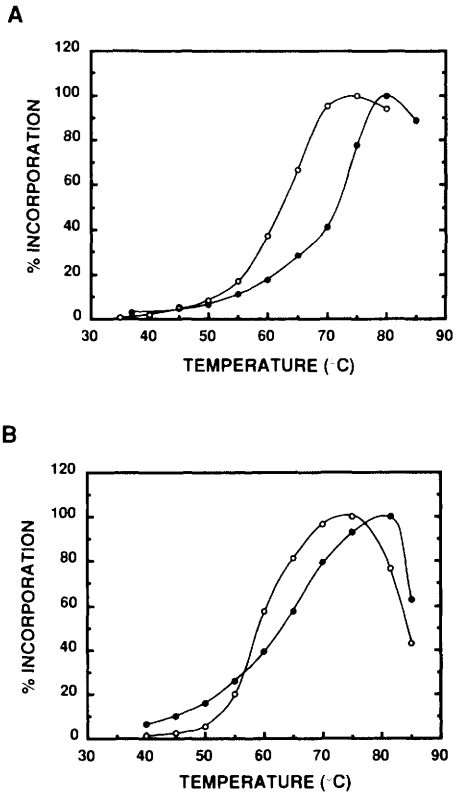

FIGURE 6 Thermal activity profiles of purified recombinant *Taq* DNA polymerases. Activity is represented as percent of maximum dCMP incorporation for each template and enzyme (assays performed under previously determined optimum conditions). (*A*) Taq Pol I with: (●) activated template (100% represents 114 pmoles of dCMP incorporated); (○) M13 primer-template (100% represents 80 pmoles of dCMP incorporated). (*B*) Stoffel fragment with: (●) activated template (100% represents 150 pmoles of dCMP incorporated); (○) M13 primer-template (100% represents 130 pmoles of dCMP incorporated).

Figure 7 shows the steady-state thermal inactivation of recombinant Taq Pol I and the Stoffel fragment at 95°C and 97.5°C under PCR buffer conditions. Taq Pol I has an activity half-life of approximately 45–50 min at 95°C, and 9 min at 97.5°C. Native Taq Pol I was inactivated with similar kinetics (data not shown). The half-life of the Stoffel fragment at 97.5°C is approximately 21 min. The thermal inactivation of the Stoffel fragment was unaffected by KCl concentration over a range of 0–50 mM (data not shown). These results do not measure thermoresistance of the enzymes in ther-

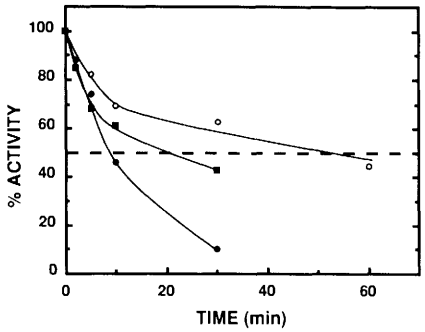

FIGURE 7 Thermoresistance of purified recombinant *Taq* DNA polymerases. Percent remaining enzyme activity as a function of time. (●) Taq Pol I, 97.5°C; (○) Taq Pol I, 95°C; (■) Stoffel fragment, 97.5°C. The intersection of each solid line with the dashed line indicates the approximate $t^{1/2}$ for each condition.

mal cycling or otherwise repeated short exposure to high temperatures.

5′ to 3′ Exonuclease Activity

The ability of Taq Pol I and the Stoffel fragment to excise 5′-terminal nucleotides from a variety of DNA substrates was examined (Table 3). The 5′ to 3′ exonuclease activity of Taq Pol I was negligible on single-stranded DNA, i.e., a 5′-phosphorylated oligonucleotide. The activity was moderate on the 5′-phosphorylated recessed end of double-stranded DNA, which was a 5′-phosphorylated oligonucleotide annealed to single-stranded circular DNA in this assay. It is only in the presence of an upstream primer that the enzyme possessed substantial 5′ to 3′ exonuclease activity, yielding 78% release of 5′ nucleotides compared to 18% in the absence of a competent upstream primer. Thus, this activity is a polymerization-dependent 5′ to 3′ exonuclease. In contrast, as predicted from the amino acid sequence, the Stoffel fragment was completely devoid of exonuclease activity (Table 3).

TABLE 3 5′ to 3′ Exonuclease Activity

Enzyme	Substrate	Percent release[a]
Taq Pol I (0.05 pmole)	ss DNA[b]	1.7
	ds DNA[c]	ND
	ds DNA[d] with primer	64.4
Taq Pol I (1 pmole)	ss	6.4
	ds	18.0
	ds with primer	77.6
Stoffel fragment (2 pmoles)	ss	ND
	ds	ND
	ds with primer	0.5

[a]Release is percent of 5′-terminal nucleotides converted to mono- or dinucleotides, quantitated by liquid scintillation spectrometry of excised reaction products previously separated via PAGE.
[b]Oligonucleotide DG67, ^{32}P-phosphorylated at its 5′ end.
[c]DG67 (above) annealed with TSYC657.
[d]TSYC657/DG67 complex (above) additionally annealed with upstream DG48.

DISCUSSION

DNA polymerases isolated from mesophilic microorganisms have been extensively characterized, but much less is known about the properties of thermostable DNA polymerases. With the introduction of the PCR method of DNA amplification, considerable interest has been focused on the DNA polymerases of thermophilic organisms. A DNA polymerase isolated from *T. aquaticus* has been previously described.[34,35] The enzyme is reported to have an approximate molecular weight of 62,000–68,000, a temperature optimum of 70–80°C, and a pH optimum in the range of 7.8–8.3. Optimal activity is obtained with 60–200 mM KCl and 10 mM Mg^{2+}. Manganese is only 20–50% effective and shows an optimum of 1–2 mM. The enzyme as isolated by Chien et al.[34] has a specific activity[36] greater than 474.8 units/mg, whereas the enzyme isolated by Kaledin et al.[35] has a specific activity between 1612 units/mg and 5200 units/mg, depending on the nature of the substrate. In contrast, the *T. aquaticus* DNA polymerase we describe has a molecular weight of 94,000, and a specific activity of 292,000 units/mg. Optimal polymerization activity is achieved at 75–80°C, in the presence of 10–55 mM KCl and 2–3 mM Mg^{2+}. Manganese is approximately 80% as effective and shows an optimum of 0.6 mM. The Stoffel fragment behaves somewhat differently than the full-length enzyme in activity assays. It has a preference for low ionic strength regardless of template, and has a broader and higher (~4 mM) Mg^{2+} optimum. The optimum for manganese is also broader; however, it is only 60% as effective relative to $MgCl_2$.

Based on the physical and biochemical characterization of the polymerases purified by Chien et al.[34] and Kaledin et al.,[35] it is unclear whether these proteins are the products of a distinct gene (or genes) from that which encodes the DNA polymerase described here, or are partially purified proteolytic degradation fragments of the same translation product. The *Taq* DNA polymerase described by Engelke et al.[7] is the translation product of the gene initially identified[5] and further modified as reported in this manuscript. In the expressed protein isolated by Engelke, et al.,[7] the first two amino acids encoded for in the native gene (Met-Arg-) were replaced with three vector-encoded amino acids Met-Asn-Ser. Expression levels were approximately 3.5-fold lower than those achieved with pLSG5 (Table 2), as determined by activity assays of clarified heat-treated extracts. The purification resulted in an enzyme preparation that contained trace contamination from *E. coli* proteins and had a specific activity of 5263 units/mg. A clone expressing a *Taq* DNA polymerase gene has also been identified by Sagner et al.[6] This clone expresses a 94-kD *Taq* DNA polymerase that is presumably identical to the polymerase described here. An expression clone producing an active amino-terminally truncated *Taq* DNA polymerase, KlenTaq, has also been described,[8] with translational initiation occurring at the corresponding Met^{236} codon of the native gene.

The DNA polymerases from a number of other thermophilic eubacteria have also been isolated and partially characterized (Table 4). These include *Bacillus stearothermophilus* (*Bst*),[37,38] *Thermus ruber* (*Tru*),[39] *T. flavus* (*Tfl*),[40] *T. thermophilus* (*Tth*) HB-8,[41,42] and *Thermotoga* sp. (*Tsp*) strain FjSS3-B.1.[43] In addition to the eubacteria, thermophilic archaeal DNA polymerases have also been characterized. The Archaea constitute a group of prokaryotes with an intermediate phylogenetic position between eukaryotes and eubacteria. They comprise the most extreme thermophilic organisms known. A DNA polymerase has been purified and characterized from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* (*Sac*).[44–46] Similarly, DNA polymerases from *Sulfolobus solfataricus* (*Sso*),[47]

TABLE 4 Properties of Thermophilic DNA Polymerases

Enzyme	Optima				Molecular weight ($\times 10^{-3}$)	Specific activity[a]	Exonuclease activity	
	$MgCl_2$ (mM)	pH	KCl (mM)	Temperature (°C)			5′ to 3′	3′ to 5′
Taq Pol I	2	9.4	55	80	94	292,000	+	−
Stoffel fragment	4	8.3	10	80	61.3	369,000	−	−
Taq Pol (34)	10	7.8	60	80	63–68	>475	−	−
Taq Pol (35)	10	8.3	100–200	70	60–62	1,600–5,200	−	−
Bst Pol (37)	30	9.0	ND	65	ND	424	−	−
Bst Pol (38)	20	8–9	270	60	76	16,000	±	−
Tru Pol (39)	2.5	9.0	15	70	70	8,000	−	−
Tfl Pol (40)	10–40	10.0	50	70	66	12,000	−	−
Tth Pol (41)								
A	ND	ND	ND	50	110–150	58	−	−
B	ND	ND	ND	63	~110	922	−	−
C	ND	ND	ND	63	~110	1,380	−	−
Tth Pol (42)	ND	ND	ND	ND	67	4,000	−	ND
Tsp Pol (43)	10	7.5–8.0	0	>80	85	16	ND	ND
Sac Pol (44)	0.1–1	6.0–8.0	0	65	100	71,500	−	+
Sac Pol (45)	ND	ND	ND	70	100	60,000	−	−
Sso Pol (47)	3	6.8	0	75	110	42,360	−	−
Tac Pol (48)	4	8.0	5[b]	65	88	17,500	−	+
Mth Pol (52)	10–20	8.0	100	65	72	4,720	+	+

[a]Specific activities have been normalized such that one unit equals the amount of activity which will incorporate 10 nmoles of dNTPs into product in 30 min.
[b]NH_4Cl.

Thermoplasma acidophilum (*Tac*),[48] *Thermococcus litoralis* (*Tli*),[49,50] and *Pyrococcus furiosus* (*Pfu*)[51] have been isolated. The purification and characterization of a DNA polymerase from a methanogenic archaeon *Methanobacterium thermoautotrophicum* (*Mth*) has also been reported[52]

Table 4 shows that considerable variation exists among those thermostable DNA polymerases that have been characterized in the literature. A distinct feature of all these DNA polymerases is that they all appear to be monomeric. Whether or not a multimeric DNA polymerase similar to *E. coli* DNA polymerase III exists in thermophilic bacteria is unknown. It is also unknown whether these polymerases are involved in replication or repair. A substantial difference among the enzymes is the presence or absence of associated exonuclease activities. Only Taq Pol I[53,54] (Table 3), and *Mth* DNA polymerase[52] have been shown to contain an inherent 5′ to 3′ exonuclease activity, whereas only *Tac* DNA polymerase,[48] *Mth* DNA polymerase,[52] *Tli* DNA polymerase,[49,50] and *Pfu* DNA polymerase[51] have been shown to contain an inherent 3′ to 5′ exonuclease activity. Of the characterized thermostable DNA polymerases, only *Mth* DNA polymerase resembles *E. coli* DNA polymerase I, in that it contains both a 5′ to 3′ exonuclease and a 3′ to 5′ exonuclease.[52] The lack of any 5′ to 3′ exonucleolytic activity associated with the majority of the thermostable DNA polymerases may reflect a real difference among the enzymes, differences in the sensitivity of the assay procedures, or, alternatively, may be the consequence of proteolytic degradation resulting in an amino-terminal truncation of the protein. Considerable care in purification is required, as DNA polymerases appear to be particularly susceptible to proteolytic cleavage.[44,47,48,52] Preliminary evidence suggests that a 94,000-dalton DNA polymerase purified from *Tth* contains an inherent 5′ to 3′ exonuclease identical with that of Taq Pol I.[55] The absence of an inherent 3′ to 5′ exonuclease activity in a majority of the polymerases may again reflect a real difference between species; alternatively a 3′ to 5′ exonuclease may exist as a separate subunit or auxiliary protein.

A unique feature of Taq Pol I is its exceptional thermostability. This is surprising considering that several of the above-mentioned organisms grow optimally at temperatures considerably higher than *Taq*; *Tsp* strain FjSS3-B.1 grows optimally at 80°C,[43] *Sac* grows at temperatures up to 85°C,[45] and *Sso* grows optimally at 87°C,[47] whereas *Taq* has an optimum temperature for growth of only 70°C.[56] Yet for those enzymes whose thermostabilities are reported in the literature, Taq Pol I, with an activity half-life of 45–50 min at 95°C and 9 min at 97.5°C, is only second in thermostability to that of the truncated Stoffel fragment, with an activity half-life of 21 min at 97.5°C. In comparison, *Tsp* strain FjSS3-B.1 has an activity half-life of 7 min at 95°C,[43] *Sac* has an activity half-life of less than 15 min at 90°C,[45] and *Sso* has an activity half-life of 5 min at 90°C.[47] This extraordinary thermostability makes Taq Pol I especially suited to those applications where repeated incubation at very high temperatures is desired. Subsequent to completion of this manuscript, the thermostability of *Tli* DNA polymerase has been described.[57] The polymerase, derived from a hyperthermophilic Archae which grows at temperatures up to 98°C, has an activity half-life of about 2 hr at 100°C.

Taq Pol I has many features in common with *E. coli* DNA polymerase I. Considerable amino acid similarity exists between Taq Pol I and *E. coli* DNA polymerase I.[5,58,59] The amino-termi-

nal region of Taq Pol I corresponds to the amino-terminal domain of *E. coli* DNA polymerase I shown to contain the 5′ to 3′ exonuclease activity.[60] The carboxy-terminal region of Taq Pol I corresponds to the *E. coli* DNA polymerase I domain shown to contain DNA polymerase activity.[61] For the 3′ to 5′ exonuclease domain of *E. coli* DNA polymerase I,[62] no meaningful alignment with Taq Pol I was obtained. The domain structure of *E. coli* DNA polymerase I is conserved in Taq Pol I. Similar to the Klenow fragment of *E. coli* DNA polymerase I,[60,63] Stoffel fragment retains all of the DNA polymerase activity of the full-length protein, but is completely devoid of any 5′ to 3′ exonuclease activity. Characterization of the 5′ to 3′ exonuclease activity of the two full-length proteins reveals that the mechanism of exonuclease action is very similar for the two enzymes.[64] Like *E. coli* DNA polymerase I, Taq Pol I requires a duplex structure for 5′ to 3′ exonuclease activity and is stimulated by concurrent polymerization.[65,66] The major difference between the two enzymes, aside from the exceptional thermostability and thermoactivity of Taq Pol I, appears to be the lack of an inherent 3′ to 5′ exonuclease activity in Taq Pol I. Although this is not a unique feature of all thermostable DNA polymerases, as discussed above, it appears to be a common feature among the DNA polymerases isolated to date from thermophilic eubacteria.

Subtle differences exist between the polymerase activity of the Stoffel fragment and full-length Taq Pol I. Whereas the Stoffel fragment is more thermostable than Taq Pol I, it is not as active at temperatures greater than 80°C (see Fig. 6). This may be due to a difference in the ability of the enzyme to bind to DNA at high temperatures. That a difference in DNA binding exists between the two enzymes is reflected in the approximately 10-fold lower processivity of the Stoffel fragment.[55] This difference in processivity also may explain the observed difference in sensitivity to ionic strength between the enzymes. A low salt concentration increases the stability of the protein-nucleic acid complex and decreases the rate of dissociation of the polymerase from the replicated template, resulting in an increase in processivity. Thus, in low salt the higher processivity results in increased activity on long templates (i.e., primed M13 template). For the replication of short stretches of DNA like those presumably seen with activated salmon sperm DNA template, the decreased dissociation rate of the polymerase-replicated template complex may cancel the gains from increased processivity, and result in a somewhat lower synthesis rate (see Fig. 5A). For the Stoffel fragment, however, with a much lower processivity, the increase in processivity dominates, and maximal activity is seen at low ionic strength with both templates (see Fig. 5B). An increased enzyme–substrate stability in low salt may increase the activity of the Stoffel fragment on activated salmon sperm template by favoring strand-displacement synthesis. Alternatively, the differences between full-length Taq Pol I and the Stoffel fragment observed on activated template may be the result of the effect of ionic strength on the 5′ to 3′ exonuclease activity of Taq Pol I, for which nick-translation synthesis presumably predominates over strand-displacement synthesis. Differences are also apparent in the response of the two enzymes to divalent cations. The Stoffel fragment is optimally active over a broader range of Mg^{2+} concentrations, but is less active when either Mn^{2+} or Co^{2+} is substituted for Mg^{2+} (see Fig. 4). The truncated *Taq* DNA polymerase, KlenTaq, was measured to have a twofold lower mutation rate as compared to the full-length enzyme.[8] The model for this comparative increase in fidelity derives from a consideration of the relative processivities of the two forms of the enzyme, and their subsequent abilities to extend a mispaired nucleotide (i.e., lower processivity results in suppression of mismatch extension, thereby decreasing the misincorporation rate of the enzyme). Analogously, the Stoffel fragment may also possess a lower comparative mutation rate.

The differences between the two enzymes can be exploited for a variety of applications. The Stoffel fragment may be preferred for performing dideoxy nucleotide sequencing, where the 5′ to 3′ exonuclease activity of Taq Pol I may produce unwanted artifacts. The Stoffel fragment also may prove superior in performing the PCR with certain templates that contain stable secondary structure. Extension of a primer on a template strand possessing a hairpin structure creates an ideal substrate for the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase at the site of the hairpin.[53,65,66] Extension with Taq Pol I could result in exonucleolytic cleavage of the template strand at the hairpin structure, rather than strand displacement, creating DNA fragments that are incapable of serving as templates in later cycles of the PCR. The Stoffel fragment may be useful in PCR where a large amount of product is desired. Standard PCRs with Taq Pol I reach a plateau stage in late cycle PCR, where product accumulation is no longer exponential. An effect contributing to plateau may be the renaturation of product strands during extension. This could result in a substrate for the 5′ to 3′ exonuclease activity of Taq Pol I, where the renatured strand can be cleaved, and synthesis may proceed under nick-translation conditions, resulting in no net gain in product. The Stoffel fragment, on the other hand, may proceed under strand-displacement conditions, with no destruction of product. The increased thermostability of the Stoffel fragment allows for the amplification of exceptionally G/C-rich targets, where high denaturation temperatures are required. The broader Mg^{2+} optimum for activity of the Stoffel fragment may prove useful in multiplex PCR, where multiple templates are being amplified simultaneously.[67] Alternatively, in applications such as random mutagenesis PCR[68] and reverse transcription,[69] where Mn^{2+} is desired as the divalent cation cofactor, full-length Taq Pol I may prove superior. The 5′ to 3′ exonuclease activity of Taq Pol I can be used in a PCR-based detection system to generate a specific detectable signal concomitantly with amplification.[53] Taq Pol I also may be preferable in the amplification of long templates, where increased processivity may be an advantage, whereas the Stoffel fragment, if shown to have higher fidelity than Taq Pol I, may be preferable for high-fidelity amplifications. The lower processivity of the Stoffel fragment also makes the enzyme useful for the amplification of rare mutant alleles in a background of normal DNA, using allele-specific primers where 3′ mismatch extension is suppressed relative to that of full-length Taq Pol I.[70,71]

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