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Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase—an extremely heat stable enzyme with proofreading activity

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ABSTRACT

We demonstrate that the DNA polymerase isolated from *Thermococcus litoralis* (Vent™ DNA polymerase) is the first thermostable DNA polymerase reported having a 3′→5′ proofreading exonuclease activity. This facilitates a highly accurate DNA synthesis *in vitro* by the polymerase. Mutational frequencies observed in the base substitution fidelity assays were in the range of 30×10^{-6}. These values were 5–10 times lower compared to other thermostable DNA polymerases lacking the proofreading activity. All classes of DNA polymerase errors (transitions, transversions, frameshift mutations) were assayed using the forward mutational assay (1). The mutation frequencies of *Thermococcus litoralis* DNA polymerase varied between $15-35 \times 10^{-4}$ being 2–4 times lower than the respective values obtained using enzymes without proofreading activity. We also noticed that the fidelity of the DNA polymerase from *Thermococcus litoralis* responds to changes in dNTP concentration, units of enzyme used per one reaction and the concentration of $MgSO_4$ relative to the total concentration of dNTPs present in the reaction. The high fidelity DNA synthesis *in vitro* by *Thermococcus litoralis* DNA polymerase provides good possibilities for maintaining the genetic information of original target DNA sequences intact in the DNA amplification applications.

INTRODUCTION

The increased number of applications utilizing polymerase chain reaction (PCR) (2,3) has generated increasing demands for thermostable DNA polymerases. Depending on the anticipated use of synthesized DNA, the fidelity of DNA synthesis by the polymerases can be of significant importance. Specifically, cloning and sequencing of cloned DNA utilizing amplified DNA generated by PCR requires information about the mutation rates created by the thermostable enzymes themselves. The fidelity of *Taq* DNA polymerase has already been widely studied (4,5). These studies indicate that *Taq* DNA polymerase lacks the 3′→5′ exonuclease activity and creates both single base substitution errors and transitions, transversions, frameshifts, and/or deletion mutations during *in vitro* DNA synthesis (4).

Our interest was to examine the fidelity of thermostable DNA polymerases isolated from other sources than the *Thermus* species (*Thermus aquaticus*, *Thermus thermophilus*, *Thermus flavis*). That is why we decided to study the fidelity of DNA synthesis by the Vent™ DNA polymerase isolated from *Thermococcus litoralis*, an extremely thermophilic marine archaebacterium (6). This organism originates from a submarine thermal vent and can grow at temperatures up to 98°C. The purified polymerase from this archaebacterium reflects thus high level of thermostability, with a half life of two hours at 100°C.

In this study we used three different forms of DNA polymerases originating from *Thermococcus litoralis*: a) a native DNA polymerase purified directly from *Thermococcus litoralis* cells ($Vent^{TM}_N$); b) a recombinant form of Vent™ DNA polymerase equal to native enzyme ($Vent^{TM}_R$); and c) a recombinant form of Vent™ DNA polymerase lacking the 3′→5′ exonuclease activity ($Vent^{TM}_{RExo-}$). Vent™ polymerases were purified to near homogeneity by standard ion exchange chromatography (7). Cloning and expression of recombinant forms of Vent DNA polymerase was carried out as described (8). As reference enzymes we used Replinase™ (isolated from *Thermus flavis*) and T7 DNA polymerase enzymes.

DNA synthesized by thermostable polymerases at high temperatures with concomitant higher rates of synthesis and decreased stability of the DNA helix makes data on the fidelity of this synthesis essential. If higher fidelity of DNA synthesis can be achieved by a thermostable DNA polymerase than those obtained by *Taq* DNA polymerase, the use of PCR for a variety of analyses in molecular biology can become even more attractive than it is now.

MATERIALS AND METHODS

Materials

The following reagents were kindly supplied by New England Biolabs: $Vent^{TM}_N$ DNA Polymerase (254S, Lot 2), $Vent^{TM}_R$ and $Vent^{TM}_{RExo-}$ DNA polymerases, T7 DNA polymerase (256S, form II, unmodified), Acetylated BSA, necessary restriction enzymes for the construction of the DNA substrates needed in the assays for fidelities (Ava II, Kpn I, Pvu I, Pvu II), buffers for the enzymes previously mentioned. M13mp2A89, M13mp2A103, M13mp2G103 and M13mpwt phages and E. coli strains CSH50 and NR9099 and 3′-end labeled oligonucleotide

substrate were kind gifts from Dr. Thomas A. Kunkel at NIEHS (NC, USA). Replinase™ (lot WFP1070) was purchased from Du Pont (with buffer); X-gal, IPTG, RNase and Proteinase K were purchased from 5′ Prime→3′ Prime; SeaPlaque GTG and SeaKem LE agaroses were purchased from FMC BioProducts; dNTPs and Sephacryl S-1000 were purchased from Phamacia, dGMP (D-9500),Tris (T-1503) and PEG 8000 (P-5413) were purchased from Sigma; *E.coli* MC1061 was purchased from the culture collection of the Helsinki University, Faculty of Agriculture and Forestry, Department of Microbiology; $CaCl_2$, $MgCl_2$, $MgSO_4$, NaCl, KH_2PO_4, Na_2HPO_4, NH_4Cl, Leucine and Thiamine-HCl were purchased from Merck; Agar, Yeast Extract and Glucose were purchased from Difco; Tryptone was purchased from Bio Kar, EDTA and SDS were purchased from Enprotech and phenol was purchased from Carlo Erba.

BTX ECM 600 electroporation device was used for all electroporations. Beckman LS7800 liquid scintillation counter was used for terminal mismatch excision assay analysis.

Methods

Preparation of the gapped M13mp2 DNA substrates. M13mp2 gapped substrates were constructed as earlier described (1,9,10) with minor modifications. The replicative form DNA was purified using S-1000 column chromatography the linear flow rate being 14 cm/h (11). To prepare single stranded DNA the phage particles were first PEG precipitated (1) after which the phage particles were Proteinase K treated (50 mg/ml Proteinase K with 0.5% SDS, 1.5 h at 37°C) and applied to the S-1000 column. Finally the pooled fractions were ethanol precipitated.

Transfection of competent cells and plating. A 60 ml aliquot of frozen *E.coli* MC1061 cells prepared according to the BTX ECM 600 operating manual were thawed. 1−1.5 μl of the reaction mixture (1:10 diluted, exept for the reversion assay where undiluted reaction mixture was used) was added and the mixture was transferred to an ice cold BTX electroporation cuvette P/N 620 (2 mm gap). A single pulse was delivered at 2.5 kV and 129 Ω. The resultant pulse lengths varied around 6 ms and the transfection efficiencies varied between $1-6\times10^9$ transfectants/μg DNA. Immediately after the pulse 2 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 20 mM glucose) was added. After thorough mixing the cells were plated with *E.coli* CSH50 cells (12).

Scoring of α-complementation mutants was performed as described (1,12,13). The DNA polymerase reactions were prewarmed to 72°C (for Vent™ DNA polymerases and Replinase™) or 37°C (for T7 DNA polymerase) for five minutes prior to the addition of the DNA polymerases. dNTPs were pH balanced in solution. After incubation at the indicated temperatures (72°C or 37°C) for 15 minutes, the reactions were stopped by the addition of EDTA to achieve a final concentration of 15 mM. The composition of the reaction mixtures for each enzyme assay are described in the legends to the tables. Each reaction was analyzed for the extent of gap-filling synthesis by agarose gel electrophoresis (1). All reactions reported here yielded products that migrated coincident with a double-stranded nicked (RF II) DNA standard (data not shown).

3′→5′ Exonuclease Assay I. The assay measures the 3′→5′ exonucleolytic removal of a mismatched base from a primer terminus (13). In the assay, DNA from two mutant derivatives of bacteriophage M13mp2 containing single base changes at position 103 are used to construct a gapped heteroduplex molecule. This molecule contains a 3′-terminal cytosine residue *in the primer (i.e., minus) strand opposite an adenine residue* in the template (i.e., plus) strand. Expression of the cytosine-containing sequence results in a medium-blue plaque phenotype, whereas expression of the adenine-containing sequence results in faint-blue plaque phenotype. If the mispaired cytosine is removed prior to extension by the polymerase, subsequent correct incorporation of thymidine opposite the template adenine will yield a homoduplex molecule having exclusively a faint-blue plaque phenotype. However, polymerization to fill the gap without excision of the cytosine will produce a doublestranded heteroduplex which, upon transfection, will yield medium-blue plaques (≈ 50−60%) and faint-blue plaques (≈ 40−50%) (4,9). The proportion of medium- and faint-blue plaques obtained upon the transfection of the reaction products is a function of the extent of terminal mismatch excision.

3′→5′ exonuclease assay II: Terminal Mismatch Excision by Gel Electrophoresis. In the assay the ability of the 3′→5′ exonuclease activity of a DNA polymerase is used to excise a 3′-terminal base from a mismatched end of a synthetic oligonucleotide (9). A 20-base oligonucleotide labeled with ^{32}P at the 5′-end was hybridized to a 27-base template oligonucleotide to create an incorrect A·G mispair at the 3′-OH end. Exonuclease assay was performed in 50 μl reaction containing 50 mM Tris (pH 8.0 at 25°C),10 mM $MgCl_2$, 300 ng of 5′-end-labeled DNA (30,000 cpm/μg) and 0,25 units of Vent™ DNA polymerase. The reaction mixture was prewarmed at 37°C for 3 min before the addition of the enzyme to allow primer annealing. Incubations were for 2, 5, 10, 30 and 60 min at 37°C, respectively, and 5 μl aliquots were taken at the indicated times. (Incubation was carried out at 37°C in order to avoid the melting of primer from the template). The reactions were stopped by the addition of EDTA to achieve a final concentration of 15 mM. Excision by the DNA polymerase was monitored by gel electrophoresis of 3 μl aliquots in a 20% denaturing polyacrylamide gel at the said time intervals. The gel was then dried and used to expose Kodak XAR film. The radioactivity in each band was quantitated by cutting the bands from the gel and counting the radioactivity in a liquid scintillation counter.

Reversion Assay for Base Substitution Fidelity. Base substitution errors occurring during *in vitro* DNA synthesis can be monitored in the A89 opal codon reversion assay, which detects eight of nine possible single-base substitution errors at this codon (1,4, 9,13). An M13mp2 DNA molecule is constructed having a 361-nucleotide gap containing a single-base change (G→A) in the template (i.e., plus) strand at position 89 of the lacZα coding sequence. This change creates an opal (TGA) codon, resulting in a colorless plaque phenotype under the appropriate plating conditions. The gap is filled by a single cycle of *in vitro* DNA synthesis using the desired DNA polymerase and appropriate reaction conditions. Complete synthesis across the gap by the DNA polymerase is confirmed by gel electrophoresis (1). The complete M13mp2 phage is then used for the transfection of competent α-complementation host cells to ascertain the fidelity of the DNA polymerase by assessing base changes in the opal codon (12). Base substitution errors at the opal codon during the *in vitro* gap-filling DNA synthesis are detected as blue plaques. The proportion of blue plaques to total plaques (i.e., the reversion

frequency) reflects the error rate for the single round of gap-filling DNA synthesis by the polymerase.

Forward Mutational Assay. The fidelity of *in vitro* DNA synthesis is determined for a 250-base target sequence in the lacZα gene, scoring for any error causing loss of a non-essential gene function (α-complementation). This forward mutational assay is thus capable of detecting frameshift (14), deletion (12, 15), duplication, and more complex errors (16) in addition to a larger number of different base substitution errors at more sites (1) than the reversion assay for base substitution. A gapped M13mp wild-type DNA substrate is constructed having a 390-nucleotide gap. However, as already mentioned earlier, the target for mutations is the 250-nucleotide sequence, and the assay scores the loss of α-complementation of °-galactosidase activity and the mutants are thus identified as light blue or colorless. Over 200 different base substitution errors at over 100 different sites can be scored within the 250-nucleotide lacZα sequence (17). The number of light blue and white plaques relative to the total number of plaques scored (i.e., the mutant frequency) reflects the error rate, which can be precisely calculated as described (14,17).

RESULTS

3′→5′ Exonuclease Assay

Thermococcus litoralis and *Thermus flavis* DNA polymerases were examined for the presence of proofreading exonuclease activities with a highly sensitive assay that scores the excision of a single mispaired base from a C(medium blue)·A (faint blue) mispair located at the 3′-hydroxyl end of a gapped M13 molecule. The results shown in Table I compare the DNA polymerases from *Thermococcus litoralis* and *Thermus flavis* to T7 DNA polymerase, an enzyme having an associated 3′→5′ exonuclease activity (18, 19, 20).

Primer extension by T7 DNA polymerase creates a product yielding 6.0−8.6% medium-blue plaques, suggesting that the mismatch in the primer terminus is excised as expected prior to synthesis by T7 DNA polymerase under stated reaction conditions. Transfection of the products of the Vent$^{TM}_{N}$ and the recombinant Vent$^{TM}_{R}$ DNA polymerase reactions resulted in 9.8−12.3% and 5.0−9.4% medium-blue plaques, respectively, indicating excision of 94−100% of the mispaired cytosine prior to the extension of the primer-strand. Reactions carried out using recombinant Vent$^{TM}_{REXO-}$ DNA polymerase lacking the 3′→5′ exonucleolytic activity created products yielding 55.9−57.1% medium-blue plaques. The Replinase^{TM} reaction products resulted in 57.5−54.6% medium-blue plaques upon transfection. These data demonstrate that the 3′→5′ exonuclease activity associated with both Vent$^{TM}_{N}$ and Vent$^{TM}_{R}$ DNA polymerase very efficiently removes the terminal mispair in the primer terminus and is a proofreading exonuclease activity. On the other hand, there is little or no terminal mismatch excision prior to DNA synthesis by Replinase^{TM} or the Vent$^{TM}_{REXO-}$ DNA polymerases, reflecting their lack of a 3′→5′ exonuclease moeity. An interesting result is that the proofreading activity of the DNA polymerases used in this study were not affected by the different concentrations of dNTPs under the stated assay conditions as has been shown to affect fidelity with *Taq* DNA polymerase (5). For reference information,Thermus aquaticus DNA polymerase lacks detectable 3′→5′ exonucleolytic proofreading activity (4).

Terminal Mismatch Excision by Gel Electrophoresis

We next examined the ability of the 3′→5′ exonuclease of *Thermococcus litoralis* DNA polymerase to excise a 3′-terminal

Table I. Presence of 3′→5′ Exonuclease Activity with the *Thermococcus litoralis* DNA Polymerase and *Thermus flavis* DNA polymerase

Polymerase	Reaction Temp. °C	[dNTP]* μM	Number of Plaques Scored Total	Medium blue (%)	% Terminal Mismatch Excised**
None			5890	595 (10.1)	
Vent$^{TM}_{N}$[a]	72	50	1883	184 (9.8)	100
Vent$^{TM}_{N}$[a]	72	1000	3535	434 (12.3)	94
Vent$^{TM}_{R}$[b]	72	50	1328	66 (5.0)	100
Vent$^{TM}_{R}$[b]	72	1000	1512	143 (9.4)	100
Vent$^{TM}_{REXO-}$[b]	72	50	1820	1017 (55.9)	0
Vent$^{TM}_{REXO-}$[b]	72	1000	1708	976 (57.1)	0
T7 DNA Pol[c]	37	50	2231	135 (6.0)	100
T7 DNA Pol[c]	37	1000	694	60 (8.6)	100
Replinase^{TM}[d]	72	50	2976	1711 (57.5)	0
Replinase^{TM}[d]	72	1000	2201	1202 (54.6)	0

* [dNTP] = concentration of each dNTP

** To calculate percent excision the method described by Tindall and Kunkel (4) was used

[a] Vent$^{TM}_{N}$ reactions (20μl) contained 20 mM Tris (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml acetylated BSA, 100 ng gapped M13mp2A103 DNA and 1 unit of Vent$^{TM}_{N}$ DNA polymerase (Lot 2 NEB).

[b] Reaction conditions were the same as for Vent$^{TM}_{N}$.

[c] T7 DNA pol (Form II, unmodified) reactions (20 μl) contained 20 mM Tris (pH 7.5 at 25°C), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 100 ng gapped M13mp2A103 DNA and 1 unit of T7 DNA Pol (Lot 1 NEB).

[d] Replinase^{TM} Reactions (20 μl) contained 50 mM Tris (pH 9.0 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 100 ng gapped M13mp2A103 DNA and 1 unit of Replinase^{TM} (Lot WFP1070, DuPont).

Values presented in Table I are the mean of two to four experiments. Four experiments were made for Vent^{TM} DNA polymerase, two for the reference enzymes. The standard deviations varied between 2−3% of the mean values.

base from a mismatched end of an oligonucleotide primer as described in the methods section. The results are presented in fig. 1. There is only 4.7% and 2.3% of the 20-mer oligonucleotide with the A·G mismatch remaining after 10 min and 30 min incubation times, respectively, while there is still 38.2% and 22.0% of the 19-mer with the respective C·G match remaining in the reaction mixture at the same time intervals. The results also indicate the absence of a 5′→3′ exonuclease activity, since this activity would remove the label and the resulting products would not be detected. Furthermore, there is no loss of total radioactivity upon counting all the the bands for any lane shown in fig. 1. These data confirm the presence of 3′→5′ exonucleolytic activity, since the mismatched base in the primer terminus is being excised faster than the respective match after mismatch.

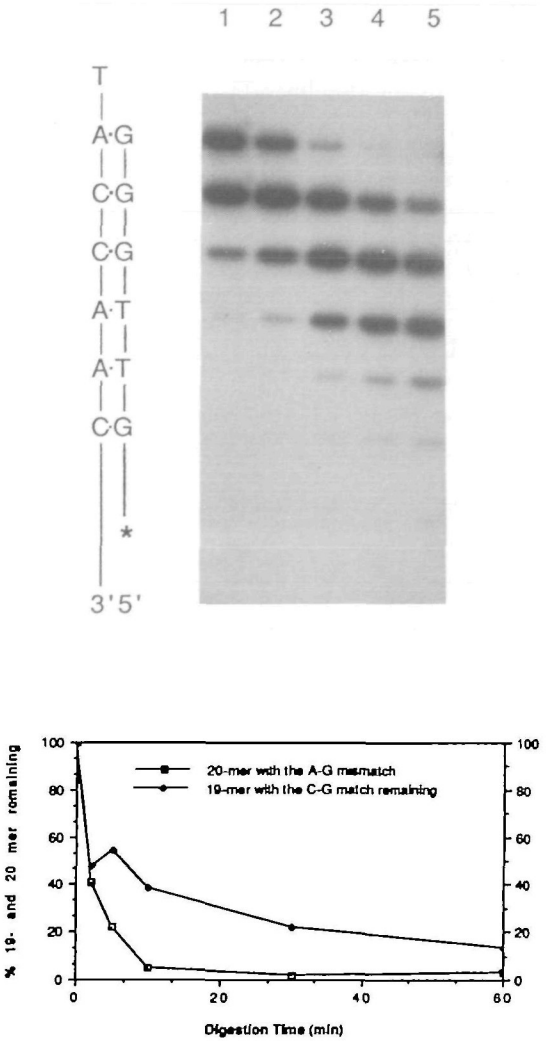

Figure 1. Electrophoretic analysis of terminal mismatch excision by the 3′→5′ exonuclease in the *Thermococcus litoralis* DNA polymerase. The experimental conditions are described under 'methods'. Lanes 1–5 in the autoradiogram represent the Vent$^{TM}_{N}$ DNA polymerase reactions in which the oligonucleotide with the A·G mismatch in the 3′-prime end of the primer terminus was used as substrate for the exonuclease. Incubation was for 2, 5, 10, 30 and 60 min, respectively. The lower part of the picture is a plot of the data obtained by excising each fragment from the gel and counting the radioactivity with a scintillation counter.

Reversion Assay for Base Substitution Fidelity

The base substitution fidelity of thermostable DNA polymerases from *Thermococcus litoralis* and *Thermus flavis* were determined by the opal codon reversion assay (Table II). The reversion assays were performed with two different dNTP concentrations, 200 μM and 4 mM (total dNTP conc.). The reversion frequencies of DNA polymerases (Vent$^{TM}_{N}$, Vent$^{TM}_{R}$ and T7 DNA Pol) having the 3′→5′ proofreading exonuclease activity varied between 3×10^{-6} and 58×10^{-6}. The reversion frequency was lowest for T7 DNA polymerase ($3-15\times10^{-6}$) and equal for Vent$^{TM}_{N}$ and Vent$^{TM}_{R}$ DNA polymerases ($29-58\times10^{-6}$). A 2-fold increase in the reversion frequency was detected for Vent$^{TM}_{N}$ and Vent$^{TM}_{R}$ when the total concentration of dNTPs was increased from 200 μM to 4 mM. The reversion frequencies of DNA polymerases lacking the 3′→5′ exonuclease activity (Vent$^{TM}_{RExo-}$ and Replinase^{TM}) were generally 2–150 times higher than the respective values of DNA polymerases having the proofreading activity. An interesting finding was that the reversion frequency of both Vent$^{TM}_{RExo-}$ and Replinase^{TM} decreased 2-fold when the total concentration of dNTPs was increased from 200 μM to 4 mM. This may be due to the fact that the total 4 mM dNTP concentration is closer to equimolar Mg^{2+}/dNTP concentration than the respective 200 μM total dNTP concentration. As earlier described (5) the equimolar concentrations of Mg^{2+} and dNTPs result in higher fidelities among DNA polymerases without proofreading exonuclease activity. Comparative values obtained for Taq DNA polymerase regarding the reversion assay for base substitution fidelilty vary between $260-310\times10^{-6}$ (assayed in 10 mM Mg2+ and 1 mM total dNTP concentration) (4).

Forward Mutational Assay

All classes of DNA polymerase errors (trasitions, transversions, frameshift, and deletion mutations) are detected in the M13mp2 forward mutation assay as a result of a single round of *in vitro* DNA synthesis to fill a 390 base gap located opposite the wild-type M13mp2 lac Za target sequence (1). The fidelities were assayed in 200 μM and 4 mM total dNTP concentrations (Table III). The mutation frequency of DNA synthesis by the enzymes with proofreading activity (Vent$^{TM}_{N}$, Vent$^{TM}_{R}$ and T7 DNA polymerase) varied from 11×10^{-4} to 34×10^{-4}. A 2-fold increase in the mutation frequency was noticed when the concentration of dNTPs was raised from 200 μM to 4 mM. The mutation frequencies generated by exonuclease free enzymes (Vent$^{TM}_{RExo-}$ and Replinase^{TM}) varied from 59×10^{-4} to 127×10^{-4}, the frequency being 2–11 times higher than the respective value of enzymes with proofreading exonuclease activity. The increase in dNTP concentration did not significantly affect the mutation frequencies of enzymes without 3′→5′ exonuclease activity in these assays. For Taq DNA polymerase it has been reported that the forward mutational frequency varies between 120–130×10–4 (assayed in 10 mM Mg2+ and 1 mM total dNTP concentration) (4).

Effect of Various Reaction Conditions on the Fidelity of Vent^{TM} DNA Polymerase

Error rates observed with *Taq* DNA polymerase during PCR can vary considerably being a function of both target DNA sequences and reaction conditions (5, 21, 22). The fidelity of *Taq* DNA polymerase is influenced by the variations in the reaction conditions (5). We wanted to examine the effects of different reaction conditions on the fidelity of *Thermococcus litoralis* DNA

Table II. Fidelity of *Thermococcus litoralis* and *Thermus flavis* DNA polymerases in a Base substitution Reversion Assay.

Polymerase	Temp. °C	[dNTP]* μM	Number of Plaques Scored Total	Mutant	Reversion Frequency ($\times 10^{-6}$)**
Vent$^{TM}_{N}$[a]	72	50	500000	17	31
Vent$^{TM}_{N}$[a]	72	200	926000	30	29
Vent$^{TM}_{N}$[a]	72	1000	429000	26	58
Vent$^{TM}_{R}$[b]	72	50	341000	12	32
Vent$^{TM}_{R}$[b]	72	1000	795000	48	57
Vent$^{TM}_{RExo-}$[b]	72	50	432000	190	437
Vent$^{TM}_{RExo-}$[b]	72	1000	280000	54	190
T7 DNA Pol[c]	37	50	672000	4	3
T7 DNA Pol[c]	37	1000	436000	8	15
Replinase^{TMd}	72	50	152000	33	214
Replinase^{TMd}	72	1000	198000	21	103

* [dNTP] = concentration of each dNTP

** The background (3×10^{-6}) has been substracted from the reversion frequency values

[a] Vent$^{TM}_{N}$ reactions (50μl) contained 20 mM Tris (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml acetylated BSA, 250 ng gapped M13mp2A89 DNA and 1 unit of Vent$^{TM}_{N}$ DNA polymerase (Lot 2 NEB).

[b] Reaction conditions were the same as for Vent$^{TM}_{N}$ ([a]).

[c] T7 DNA pol (Form II, unmodified) reactions (50 μl) contained 20 mM Tris (pH 7.5 at 25°C), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 250 ng gapped M13mp2A103 DNA and 1 unit of T7 DNA Pol (Lot 1 NEB).

[d] Replinase^{TM} Reactions (50 μl) contained 50 mM Tris (pH 9.0 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 250 ng gapped M13mp2A103 DNA and 1 unit of Replinase^{TM} (Lot WFP1070, DuPont).

Product analysis, transfections and plating were performed as described (13).

Values presented in Table II are the mean of two to four experiments. Four experiments were made for Vent^{TM} DNA polymerase, two for the reference enzymes. The standard deviations were between 1.2−7% of the mean values. In similar studies (4, 5) 10−20% deviations have been observed.

Table III. Fidelity of *Thermococcus litoralis* and *Thermus flavis* DNA polymerases in the Forward Mutational Asssay.

Polymerase	°C	[dNTP]* μM	Number of Plaques Scored Total	Mutant	Mutant Frequency ($\times 10^{-4}$)**
Vent$^{TM}_{N}$[a]	72	50	17152	93	15
Vent$^{TM}_{N}$[a]	72	1000	10079	74	34
Vent$^{TM}_{R}$[b]	72	50	9634	50	13
Vent$^{TM}_{R}$[b]	72	1000	5883	35	20
Vent$^{TM}_{RExo-}$[b]	72	50	6940	115	127
Vent$^{TM}_{RExo-}$[b]	72	1000	4438	59	94
T7 DNA Pol[c]	37	50	10426	52	11
T7 DNA Pol[c]	37	1000	10002	54	15
Replinase^{TMd}	72	50	6246	61	59
Replinase^{TMd}	72	1000	8240	85	64

* [dNTP] = concentration of each dNTP

** The background (39×10^{-4}) has been substracted from mutant frequency values

[a] Vent$^{TM}_{N}$ reactions (30μl) contained 20 mM Tris (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml acetylated BSA, 200 ng gapped M13mp2wt DNA and 1 unit of Vent$^{TM}_{N}$ DNA polymerase (Lot 2 NEB).

[b] Reaction conditions were the same as for Vent$^{TM}_{N}$ ([a]).

[c] T7 DNA pol (Form II, unmodified) reactions (30 μl) contained 20 mM Tris (pH 7.5 at 25°C), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 200 ng gapped M13mp2wt DNA and 1 unit of T7 DNA Pol (Lot 1 NEB).

[d] Replinase^{TM} Reactions (30 μl) contained 50 mM Tris (pH 9.0 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 ng gapped M13mp2wt DNA and 1 unit of Replinase^{TM} (Lot WFP1070, DuPont).

The mutants were not verified by plaque purification and DNA sequencing; therefore, the reported mutant frequency represents an upper limit to the absolute mutant frequency.

Values presented in Table III are the mean of two to four experiments. Four experiments were made for Vent^{TM} DNA polymerase, two for the reference enzymes. The standard deviations were between 14−16% of the mean values. In similar studies (4, 5) 10−20% deviations have been observed.

polymerase (Vent$^{TM}_N$). We studied the effects of different amounts of the enzyme, concentration of dNTPs, concentration of Mg^{2+}, temperature and addition of dGMP on the fidelity of Vent$^{TM}_N$ DNA polymerase. The results are shown in tables IVa (reversion assay) and IVb (forward assay).

Both in the reversion assay and in the forward assay the mutant frequency is higher when dGMP is added to the reaction mixture in addition to dNTPs. This is an indication of proofreading exonuclease activity which is at least partially inhibited by the addition of monophosphates (9). The amount of enzyme used per reaction increases both the reversion frequency and the forward mutation frequency of *Thermococcus litoralis* DNA polymerase. The increase in both assays is generally 1.5-fold, when the amount of enzyme used is increased from 0.5−1 unit to 5 units (other conditions as stated in Table IV.) There were no significant changes in the reversion frequency of Vent$^{TM}_N$ DNA polymerase at 55°C and 72°C. However, in the forward mutation assay the decrease in temperature from 72°C to 55°C increased the mutant frequency from 15×10^{-4} to 27×10^{-4}. The increase of total dNTP concentration from 200 μM to 4 mM increased both the reversion and forward mutational frequencies to two-fold. The most dramatic changes in the mutant frequencies in our studies were observed when the concentration of $MgSO_4$ was elevated in connection with dNTPs. The mutant frequencies were 4−6 -fold higher in the reactions with 10 mM Mg^{2+} and 16 mM total dNTP concentration in comparison to reactions carried out with 1 mM Mg^{2+} concentration. With low Mg^{2+} concentrations (1 mM) lowest mutation frequencies were achieved in both reversion and forward assays even if the total dNTP concentration varied from 1 mM to 16 mM. This suggests, that either high dNTP concentration or high Mg^{2+} concentration alone does not cause elevated mutation frequencies in either of the assays. Therefore, the fidelity of Vent$^{TM}_N$ DNA polymerase responds not only to the absolute Mg^{2+} or to the absolute dNTP concentration, but also to the relative concentrations of Mg^{2+} and dNTPs. However, the effect of absolute Mg^{2+} concentration on the fidelity is stronger than the respective effect of absolute dNTP concentration.

Table IV. Effect of Various Reaction Conditions on the Fidelity of Vent$^{TM}_N$ DNA Polymerase*

Table IVa. Reversion Assay

°C	Units of Vent$^{TM}_N$	[dNTP][a] μM	[dGMP] mM	[$MgSO_4$] mM	Plaques Total/ Mutants	Reversion Frequency[b]
72	1	50	20	2	230000/9	36×10^{-6}
55	1	50	–	2	217000/7	29×10^{-6}
72	0.5	50	–	2	96000/3	28×10^{-6}
72	5	50	–	2	106000/5	44×10^{-6}
72	1	50	–	2	500000/17	31×10^{-6}
72	1	250	–	1	489000/12	22×10^{-6}
72	1	200	–	2	926000/30	29×10^{-6}
72	1	1000	–	2	429000/26	58×10^{-6}
72	1	1000	–	10	257000/16	59×10^{-6}
72	1	4000	–	1	310000/8	23×10^{-6}
72	1	4000	–	10	172000/26	148×10^{-6}

* Reversion assay reactions (50 μl) contained 20 mM Tris (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 μg/ml acetylated BSA and 250 ng gapped M13mp2A89 DNA in addition to components above.
[a] [dNTP] = concentration of each dNTP
[b] The background value 3×10^{-6} has been substracted from the error frequencies
Values presented in Table IVa are the mean of two to four experiments. The standard deviations varied between 14−23% of the mean values. In similar studies (4, 5) 10−20% deviations have been observed.

Table IVb. Forward Mutational Assay

°C	Units of Vent$^{TM}_N$	[dNTP][a] μM	[dGMP] mM	[$MgSO_4$] mM	Plaques Total/ Mutants	Mutant Frequency[b]
72	1	50	20	2	6277/39	23×10^{-4}
55	1	50	–	2	8938/59	27×10^{-4}
72	0.5	50	–	2	11007/64	19×10^{-4}
72	5	50	–	2	3197/21	27×10^{-4}
72	1	50	–	2	17152/93	15×10^{-4}
72	1	250	–	1	5398/31	18×10^{-4}
72	1	1000	–	2	10079/74	34×10^{-4}
72	1	1000	–	10	7564/67	50×10^{-4}
72	1	4000	–	1	5157/30	19×10^{-4}
72	1	4000	–	10	9675/123	88×10^{-4}

* Forward assay reactions (30 μl) contained 20 mM Tris (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 μg/ml acetylated BSA and 200 ng gapped M13mp2wt DNA in addition to components above.
[a] [dNTP] = concentration of each dNTP
[b] The background value 39×10^{-4} has been substracted from the error frequencies.
Values presented in Table IVb are the mean of two to four experiments. The standard deviations varied between 3.4−15% of the mean values. In similar studies (4, 5) 10−20% deviations have been observed.

DISCUSSION

Using M13mp2-based fidelity systems we have been able to demonstrate the presence of 3′−5′ proofreading exonuclease activity associated with the native and recombinant forms of *Thermococcus litoralis* DNA polymerase (Vent$^{TM}_N$ and Vent$^{TM}_R$). On the other hand, we have also shown that there is no 3′−5′ proofreading exonuclease associated with the genetically engineered *Thermococcus litoralis* DNA polymerases (Vent$^{TM}_{REXO-}$) and *Thermus flavis* DNA polymerase (Replinase^{TM}). Variations in total dNTP concentrations of 200−4000 μM had little or no effect on the 3′−5′ exonuclease assay of the DNA polymerases with associated proofreading activities. Both Vent$^{TM}_N$ and Vent$^{TM}_R$ DNA polymerases were capable of excising the 3′-OH terminal mismatch in low (200 μM) and high (4000 μM) total dNTP concentrations. The results of the terminal mismatch excision by gel electrophoresis also confirms the presence of the 3′−5′ exonucleolytic activity of *Thermococcus litoralis* DNA polymerase (Fig. 1). The results are consistent with earlier results (9) in the proofreading studies of Chick Embryo DNA Polymerase-g. The data obtained in the gel electrophoretic assay also suggests that there is no 5′−3′ exonuclease associated with the *Thermococcus litoralis* DNA polymerase because there was no loss of total radioactivity upon counting all the bands for any lane shown in Fig. 1.

The 2-fold differences in mutation frequencies observed in the reversion assay (Table II) and in the forward mutational assay (Table III) for Vent$^{TM}_N$ and Vent$^{TM}_R$ DNA polymerases at 200 μM and 4 mM total dNTP concentrations suggest that the 3′−5′ proofreading exonuclease activity is partially inhibited by higher dNTP concentrations affecting the fidelity of DNA synthesis of both enzymes. On the other hand, the decrease observed in reversion frequencies in reactions carried out with Vent$^{TM}_{REXO-}$ and Replinase^{TM} at elevated dNTP concentrations suggests that the fidelity of DNA synthesis by 3′−5′ exonuclease deficient DNA polymerases is affected by the relative dNTP and

Mg^{2+} concentrations (5). In the DNA polymerization reactions by Vent™$_{REXO-}$ and Replinase™ the concentration of dNTPs and Mg^{2+} were closer to equimolar in the high (4 mM) dNTP reactions rather than in the low (200 μM) dNTP reactions. The fidelity of DNA synthesis by *Taq* DNA polymerase was found to be highest at Mg^{2+} concentration that was equimolar to the total concentration of dNTP substrates present in the reaction (5). The reversion frequencies observed in our studies for DNA polymerases with and without associated 3′→5′ proofreading activities were of the same level obtained in the fidelity studies for DNA polymerase I Klenow fragment and Taq DNA polymerase, respectively (4). The mutant frequencies achieved with our enzymes of interest in the forward mutational assays were somewhat lower than those obtained earlier (4). However, interpretations of the data in Tables III and IVb (forward mutational assays) were slightly limited by higher background frequencies which may be due to higher mutant frequency of the starting DNA preparation. On the other hand, changes in mutant frequencies were very consistent after several repeats of assays which entitles us to make comparisons between different enzymes and assay conditions in each of the assay.

We have also demonstrated that fidelity of DNA synthesis *in vitro* by *Thermococcus litoralis* DNA polymerase responds to changes in the reaction conditions. The relative concentrations of dNTPs and Mg^{2+} were shown to have the highest impact on the fidelity of DNA synthesis by the native Vent™$_N$ DNA polymerase (Table IV). High dNTP concentration (16 mM total) together with high Mg^{2+} concentration (10 mM) caused reversion frequencies of 6-fold and forward mutation frequencies of 5-fold compared to reaction conditions of highest fidelities achieved (Table IV).

Furthermore, we were able to show that the addition of dGMP into the reaction mixture caused at least partial inhibition of the proofreading 3′→5′ exonuclease activity thus increasing the mutant frequencies in both reversion and forward assays. This was expected according to previous results (9). We also noticed an increase in the mutant frequencies in both reversion and forward assays when high enzyme amounts (5 units) were applied in the reactions. However, the mechanism of decreased fidelity by high amount of enzyme remains yet unknown. One possible explanation is that the enzyme binds its cofactor Mg^{2+} thus affecting the relative concentration of Mg^{2+} and dNTPs in favor of dNTPs. As already shown, the increase in dNTP concentration may inhibit the proofreading by the enzyme.

The thermostability of *Thermocccus litoralis* DNA polymerase (Vent™ DNA polymerase) and processivity of the enzyme with amplifications of 8−9 kb fragments (data not shown) is superior over the thermostability of *Taq* DNA polymerase. The 3′→5′ proofreading exonuclease activity present with the polymerase provides high fidelities under *in vitro* DNA synthesis conditions examined in our studies. Vent™ DNA polymerase appears to be a good alternative to *Taq* DNA polymerase for DNA synthesis reactions at high temperatures requiring high fidelity. What is more, the fidelity of DNA synthesis by Vent™ DNA polymerase can be influenced by optimizing the reaction conditions. Low relative concentrations of Mg^{2+} ions and dNTPs result in highest fidelities according to our studies. The effects of other parameters (e.g. pH, temperature) influencing the reaction conditions and the fidelity of DNA synthesis by Vent™ DNA polymerase remain to be demonstrated in further studies. Temperature and pH have been demonstrated to play a role in the fidelity of *Taq* DNA polymerase lacking the proofreading activity (5). Nevertheless, we have been able to show that at high temperature DNA synthesis reaction conditions, Vent™ DNA polymerase provides good possibilities for maintaining the genetic information of original target DNA sequences intact.

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