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Construction of a Fusion-Stoffel Fragment to Improve 3'-5' Exonuclease Activity

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Abstract *Taq* DNA polymerase exhibits a sizable drawback compared to the other thermophilic DNA polymerases in that it demonstrates lower proof-reading activity due to the deficiency of 3'-5' exonuclease activity. A study was undertaken to improve the 3'-5' exonuclease activity in the PCR of *Taq* DNA polymerase. The three-dimensional structural alignment of the polymerase and 3'-5' exonuclease domains from the pol I family DNA polymerases explains why *Taq* DNA polymerase has just a background level of 3'-5' exonuclease activity. A comparison indicated that the two polymerase domains are very similar in primary and tertiary conformations, even though *Taq* DNA polymerase carries a much shorter 3'-5' exonuclease domain than that of *E. coli* DNA polymerase I. Those two polymerase domains were interchanged between *Taq* DNA polymerase and *E. coli* DNA polymerase I. The 3'-5' exonuclease domain from *E. coli* DNA polymerase I was separated and pasted into the polymerase domain of *Taq* DNA polymerase I, which resulted in a functional fusion-Stoffel fragment. The 3'-5' exonuclease activity of the fusion-Stoffel fragment increased up to 48% of the value of the Klenow fragment, while that of *Taq* DNA polymerase remained at 6.0% of the Klenow fragment.

Key words: *Taq* DNA polymerase, PCR, fusion protein, 3'-5' exonuclease

Taq DNA polymerase from *Thermus aquaticus* is very useful in polymerase chain reactions (PCR). It shows an optimum reaction temperature at 75°C and maintains activity for about one hour at 94°C. The high optimum polymerization temperature at 75°C provides unique advantages when comparing *Taq* DNA polymerase with mesophilic DNA polymerases, such as *E. coli* DNA polymerase I [9, 19, 23, 24]. Not only is *Taq* DNA polymerase very useful commercially for PCR applications, but it is also valuable for studying DNA replication. *Taq* DNA polymerase is apparently homologous to *E. coli* DNA polymerase I, which has long been used for DNA replication studies. *Taq* DNA polymerase has a domain at its amino terminus (residues 1 to 291) that has 5'-3'exonuclease activity, a non-functional 3'-5'exonuclease domain (residues 292-423), and a domain at its C-terminus (residues 424-832) that catalyzes the polymerase reaction [17].

Research into *Taq* DNA polymerase commenced when it became a commercially useful DNA polymerase. One objective was to improve the processivity and fidelity of *Taq* DNA polymerase, primarily because it is the most popular enzyme in PCR. Another goal was to introduce a new thermophilic DNA polymerase to substitute for *Taq* DNA polymerase, which was mostly undertaken by biotech companies. Their efforts have launched several thermophilic DNA polymerases into the market, such as *Pfu* DNA polymerase (Stragene Company), Vent DNA polymerase (New England Biolab), and *Bca* DNA polymerase (Takara Shuzo Company). *Taq* DNA polymerase exhibits a sizable drawback compared to the other thermophilic DNA polymerases in that it demonstrates lower proof-reading activity due to the deficiency of 3'-5' exonuclease activity. *Taq* DNA polymerase does not carry 3'-5' exonuclease activity, possibly due to the shorter 3'-5' exonuclease domain, even though there is a corresponding domain [7]. High fidelity DNA synthesis is a very important factor in thermophilic DNA polymerase being a preferred enzyme for PCR reaction [6]. DNA polymerization fidelity is influenced by several factors, and the presence of 3'-5' exonuclease activity is one of the most critical elements. The presence of 3'-5' exonuclease activity will remove mismatched nucleotides and thereby reduce the error rate. *Taq*, *Pfu*, and Vent DNA polymerases are currently the most widely used thermophilic DNA polymerases.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-thio-β-galactopyranoside; KF, Klenow fragment; SF, Stoffel fragment (*Taq* DNA polymerase deleting 5'-3' exonuclease domain); PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Their fidelities for DNA synthesis vary; the error rates of *Taq, Pfu,* and Vent DNA polymerases were reported at 1.8×10^{-5}, 0.2×10^{-5}, and 1.2×10^{-5}, respectiviely [7]. The disadvantage of the lower 3'-5' exonuclease activity of *Taq* DNA polymerase has been a point of contention for other thermophilic DNA polymerases which share the market.

All DNA polymerases can be grouped into six families—Pol I, Pol α, Pol β, DNA-dependent RNA polymerase, reverse transcriptase, and RNA-dependent RNA polymerase—on the basis of amino acid homology [11]. *Taq* DNA polymerase is classified into the Pol I family, which is represented by *E. coli* DNA polymerase I [3]. A comparison of a Klenow fragment (KF) with the corresponding part of *Taq* DNA polymerase, called a Stoffel fragment (SF) consisting of polymerase and 3'-5' exonuclease domains without the 5'-3' exonuclease domain, indicates that the polymerase domains are nearly identical, whereas the 3'-5' exonuclease domains differ extensively [16]. Unlike the KF, the 3'-5' exonuclease domain of the SF has lost the editing activity of 3'-5' exonuclease [14, 18]. High resolution structural data from crystallographic studies have been published on the polymerase and 3'-5' exonuclease domains of KF and SF, and the reaction mechanism of 3'-5' exonuclease was nearly identified [1, 2, 4, 5, 8, 13, 20, 22].

A study was undertaken to improve the 3'-5' exonuclease activity in the PCR of *Taq* DNA polymerase. The objective was to fuse a functional 3'-5' exonuclease domain from *E. coli* DNA polymerase I exhibiting good proof-reading with the polymerase domain of *Taq* DNA polymerase, which results in a fusion protein corresponding to SF. However, the mesophilic nature of the 3'-5' exonuclease domain would be remaining and its thermostability must be improved for it to be a useful enzyme for PCR. In this report, we only attempted to determine whether a fusion-SF could be made by protein engineering and how it functions.

MATERIALS AND METHODS

Materials

The mutagenesis kit and *Taq* DNA polymerase were obtained from Bioneer Company (Taejon, Korea). All other enzymes were purchased from Promega Company (Promega, U.S.A.). Radioactive compounds of [γ-^{32}P]dCTP for DNA labeling were purchased from Amersham International (Amersham, U.S.A.). The *E. coli* DNA polymerase I gene was obtained from Dr. Cathy M. Joyce at Yale University.

Plasmid Construction for a Fusion-Stoffel Fragment

Site-directed mutagenesis was conducted to construct a fusion-SF. The DNA fragments carrying the polymerase domain from *Taq* DNA polymerase and the 3'-5' exonuclease domain from *E. coli* DNA polymerase I were fused using pDS2 plasmid [12, 15]. The two different domains were connected at residues His509 and Leu510 of the *E. coli* DNA polymerase I and residues Phe413 and Ala414 of the *Taq* DNA polymerase. Since there is no unique restriction site at those positions to connect the two fragments, a unique restriction enzyme site, *Nru*I (TCGCGA), was designed by site-directed mutagenesis. We chose the *Nru*I site because it apparently does not change the primary structure significantly in terms of the amino acid sequence homology of *Taq* DNA polymerase. First, residue Arg398 (CGC) of *E. coli* DNA polymerase I was site-directed mutagenesized to Arg398 (CGG) by silent mutation to eliminate the *Nru*I restriction enzyme site which pre-exists in *E. coli* DNA polymerase I. Inserting a *Nru*I site by site-directed mutagenesis created unique sites for the two DNA polymerases. To create the *Nru*I site (TCGCGA), His509 and Leu510 (CATCTG) were mutagenized to Phe509 and Ala510 (TTCGCG) in *E. coli* DNA polymerase I, and Phe413 and Ala414 (TTCGCC) were mutated to Phe413 and Ala414 (TTCGCG) in *Taq* DNA polymerase. The 3'-5' exonuclease domain of *E. coli* DNA polymerase I, comprised of residues 324 to 508 of *E. coli* DNA polymerase I and the first residue (Met1) to initiate translation, was linked to the polymerase domain of *Taq* DNA polymerase, which includes residues 413 to 832 of the *Taq* DNA polymerase.

The PCR reaction proceeded as follows to insert a *Nru*I restriction site into the Taq DNA polymerase gene using four primers. In the first step, the mutagenic fragment was substituted for the wild-type DNA fragment at the *Kpn*I and *Bam*HI sites on the pDS2 plasmid so that the *Taq* DNA polymerase gene could carry the unique *Nru*I site. The second step was to insert the 3'-5'exonuclease DNA fragment of *E. coli* DNA polymerase I obtained from PCR into the pDS2 plasmid carrying the *Taq* DNA polymerase gene. Residue Arg398 (CGC) was first site-directed mutagenesized to Arg398 (CGG) by silent mutation to eliminate the *Nru*I restriction site which pre-exists in *E. coli* DNA polymerase I. Sequencially, the PCR reaction was carried out to obtain the 3'-5' exonuclease DNA fragment from the *E. coli* DNA polymerase I gene. The PCR fragment carried unique *Eco*RI and *Nru*I sites at the 5' and 3' ends, which were used for fusing with the DNA fragment of the polymerase domain of *Taq* DNA polymerase on the pDS2 plasmid. The *Eco*RI site was positioned at the start site of the translation in the resulting fusion-SF. The overall scheme for cloning the fusion-polymerase is shown in Fig. 1. The final construction of the pDS2 plasmid carrying the fusion-SF gene was transformed into competent *E. coli* DH5α cells. The restriction digests were conducted to confirm the proper construction

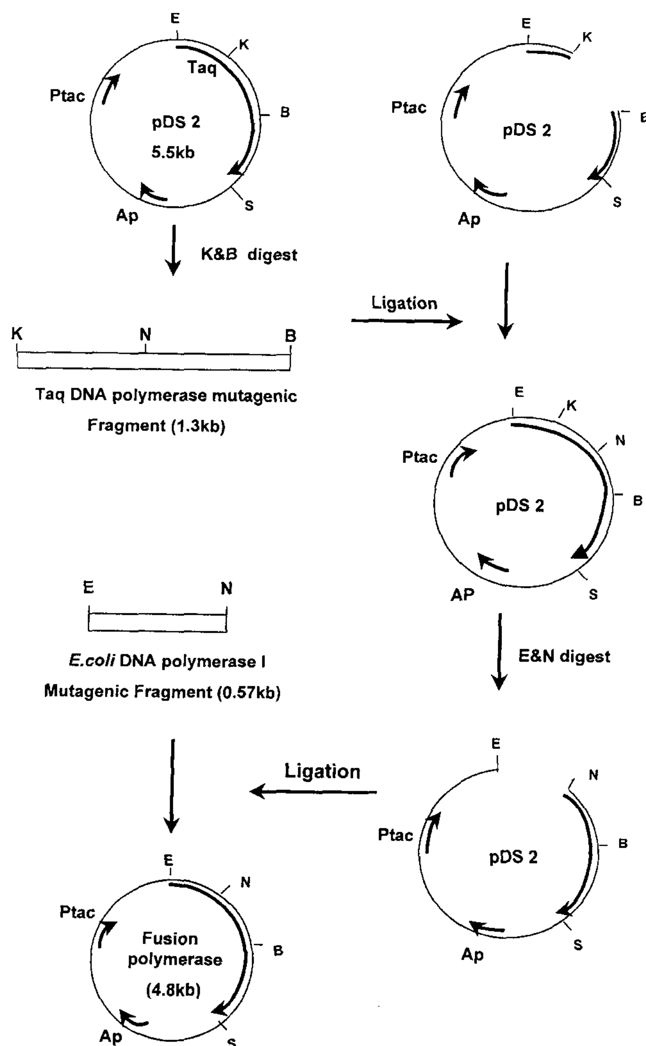

Fig. 1. Schematic drawing for the construction of a fusion-Stoffel fragment.

The mutagenic PCR fragment (1.3 kb) was designed for generating an *Nru*I site on *Taq* DNA polymerase. The mutagenic PCR fragment (0.57 kb) was designed to generate *Eco*RI and *Kpn*I sites at the 3' and 5' ends of the polymerase domain on the *E. coli* DNA polymerase I gene, deleting one pre-existing *Nru*I site. Finally, a 4.8-kb plasmid was constructed carrying the fusion-Stoffel fragment gene. The Ptac, E, B, K, S, N, and Ap represent tac promotor, *Eco*RI, *Bam*HI, *Kpn*I, *Sal*I, *Nru*I, and ampicillin resistance sites, respectively.

of the plasmid by monitoring the sizes of inserted DNAs, as shown in Fig. 2. The 0.57-kb fragment of the 3'-5' exonuclease domain from *E. coli* DNA polymerase I, the 1.3-kb DNA of the polymerase domain from *Taq* DNA polymerase, and the 1.9-kb DNA of the constructed fusion-SF are shown in the correct sizes in Fig. 2, indicating that the vectors are properly ligated. The constructed vector DNAs were subjected to thermal DNA sequencing to identify the correct DNA sequence [10]. The constructed clone was saved for the next overexpression experiment.

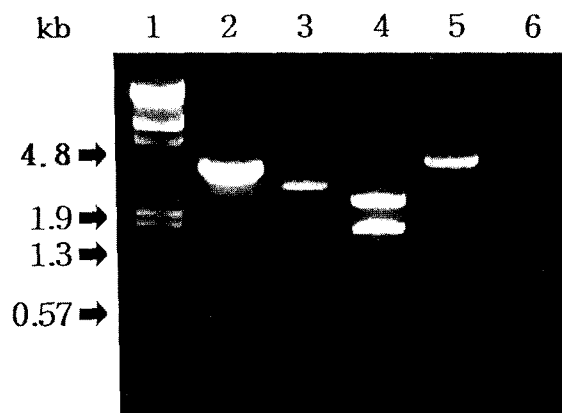

Fig. 2. The agarose gel electrophoresis for the restriction digests of the constructed plasmid.

The arrows indicate the right sizes of DNA inserts in the plasmid construction from lanes 2 to 4. Lane 1: DNA marker, λDNA *Hin*dIII digest (125 to 23,130 bases), Lane 2: *Eco*RI/*Nru*I digest from *E. coli* DNA polymerase I, 0.57 kb, Lane 3: *Nru*I/*Sal*I digest from *Taq* DNA polymerase, 1.3 kb, Lane 4: *Eco*RI/*Sal*I digest of fusion-Stoffel fragment, 1.9 kb, Lane 5: *Eco*RI digest of full plasmid, 4.8 kb, Lane 6: DNA marker, KB II of Bioneer Inc. (140 to 1,510 bases).

Expression of Fusion-Stoffel Fragment

The overexpression of fusion-SF was accomplished using the pET-28a expression system (Novagen, U.S.A.). The DNA fragment of fusion-SF on the pDS2 vector was restriction-digested at two sites of the *Eco*RI and *Hin*dIII and the DNA fragment was inserted into the pET-28a expression system. The pET-28a expression vector was transformed into *E. coli* BL21(DE3). The *E. coli* BL21 (DE3) cells carrying the expression plasmid were incubated at 37°C and 250 rpm. The *E. coli* BL21(DE3) cells in an LB medium were cultured with 30 μg/ml kanamycin. The induction of gene expression and the purification procedure using His-tag resin progressed basically as described in the Novagen expression system manual.

Assay of 3'-5' Exonuclease Activity

The substrate of pUC18 DNA was digested with *Xma*I restriction enzyme and labeled at the 3'-terminus with [γ-^{32}P]dCTP by *Taq* DNA polymerase to make two mismatched base pairs of C-C [21]. The labeled DNA was purified by a Gene Clean kit (Bio101 Company, U.S.A.) and the unbound radioactive nucleotides were discarded. The 3'-5' exonuclease reaction followed at 37°C for one hour in a 50 μl reaction mixture containing 25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.5 μg DNA substrate, and 7 μl of enzyme solution (about 0.5 μg enzyme added depending on 3'-5' exonuclease activity). After the reaction stopped, the mixture was cooled on ice with the addition of 2 μl of 60 mM EDTA. The 52 μl aliquot was spotted on a 2.3 cm-diameter DE-81 Whatman filter paper and dried in a heat

block for 10 min. The dried filter was washed twice with 0.5 M Na_2HPO_4, pH 7.0, for 15 min and then washed with 70% ethanol for 5 min. The radioactivity was counted in a Beckmann liquid scintillation counter model LS 6500.

RESULTS

Structural Comparison of Polymerase and 3'-5' Exonuclease Domains Between *Taq* DNA Polymerase and *E. coli* DNA polymerase I

Taq DNA polymerase is generally homologous to *E. coli* DNA polymerase I in both its primary and tertiary structures. The two polymerase domains are very similar in terms of general sequence homology, so they were superimposed in a Cα carbon backbone within a 1.2 Å deviation, as shown in Fig. 3. The polymerase structures appear to be nearly identical in conformational folding [13].

The comparison indicated that the two polymerase domains are very similar in primary and tertiary conformations. It was conceivable that the two polymerase domains could be interchangeable between *Taq* DNA polymerase and *E. coli* DNA polymerase I. Therefore, the 3'-5'exonuclease domain from *E. coli* DNA polymerase I was separated and pasted into the polymerase domain of *Taq* DNA polymerase, resulting in a fusion-SF consisting of a thermophilic polymerase domain and a mesophilic 3'-5' exonuclease domain. It was important to determine which residues were selected in order to link two domains from two different sources of DNA polymerases. In addition, there must be common unique restriction enzyme sites to connect two domains. Unfortunately, no convenient unique enzyme sites existed where the polymerase and 3'-5' exonuclease domains could be linked. Therefore, we had to select one or two amino acid residues to link the sites and be site-directed mutagenized to create unique *Nru*I restriction sites in both the *Taq* DNA polymerase and *E. coli* DNA

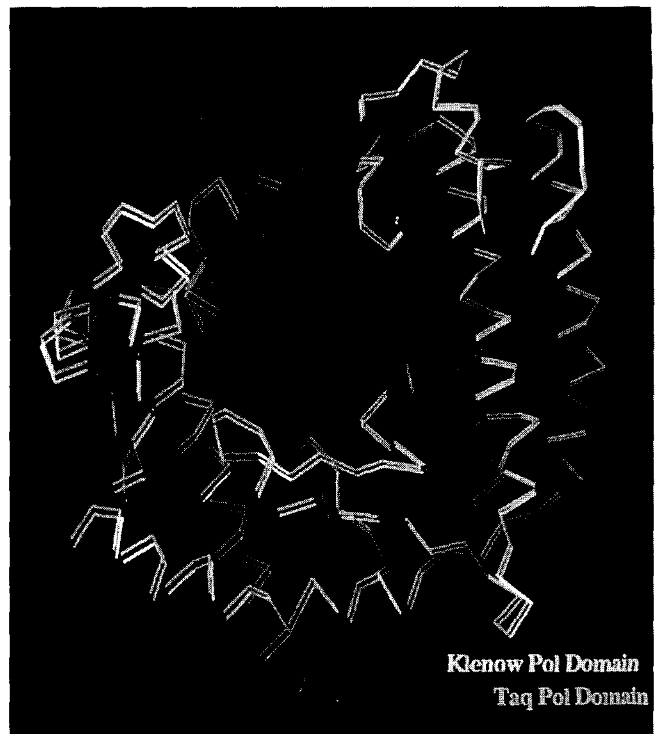

Fig. 3. Superposition of Klenow fragment and *Taq* DNA polymerase where Klenow fragment and *Taq* DNA polymerase are shown at the residues from 520 to 928 and from 424 to 832, respectively [13].

Stereo of the Cα backbone of the Klenow fragment polymerase domain (bottom) superimposed on the corresponding atoms (top) of the *Taq* DNA polymerase. The graphics program "O" was used to generate the superposition.

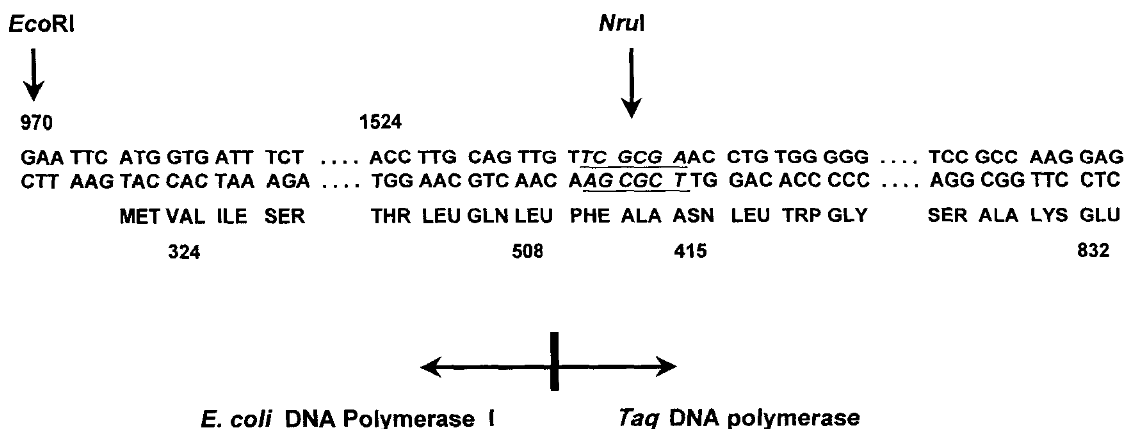

Fig. 4. Primary structure of fusion-Stoffel fragment.

The N-terminal region of residues 324 to 508 originated from the 3'-5' exonuclease domain of the *E. coli* DNA polymerase I, and the C-terminal polymerase domain of residues 413 to 832 originated from the *Taq* DNA polymerase. The residues of Phe and Ala in the middle of the sequence, which are underlined, were the linking region between the two domains, carrying the inserted *Nru*I restriction site. The first amino acid, Met1, was inserted for translation initiation. The numbering represents the original sequences of the two DNA polymerases.

polymerase I. After carefully inspecting the residues in the linking regions of both DNA polymerases, Ala414 (GCC) was mutated to Ala (GCG) in the *Taq* DNA polymerase by silent mutation, and His509 and Leu510 (CATCTG) were mutated to Phe509 and Ala (TTCGCG) in the *E. coli* DNA polymerase I, resulting in unique *Nru*I sites in both polymerases. An *Nru*I restriction site was deleted by site-directed mutagenesis for *E. coli* DNA polymerase I beforehand, as described previously. The polymerase domain of *Taq* DNA polymerase (residues 413 to 832) could be linked to the 3'-5' exonuclease domain (residues 324-508 of *E. coli* DNA polymerase I) using the unique *Nru*I sites. The primary structure of the fusion-SF near the linking region is shown in Fig. 4. The 3'-5'exonuclease domain from *E. coli* DNA polymerase I, the inserted *Nru*I restriction site, and the polymerase domain from *Taq* DNA polymerase are depicted in the sequence in Fig. 4.

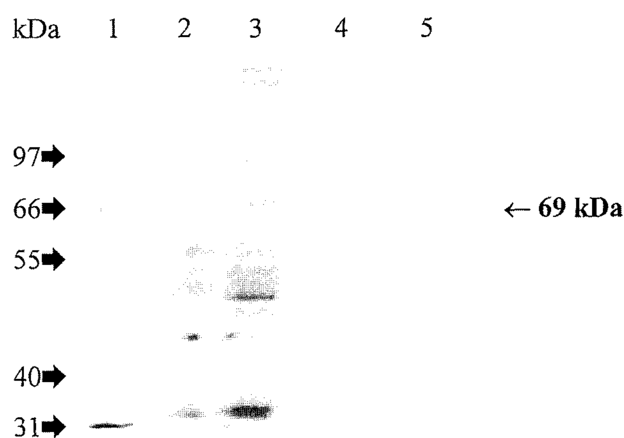

Fig. 5. SDS-PAGE of the purified fusion-Stoffel fragment from *E. coli* BL21 (DE3) cells. The fusion-Stoffel fragment is indicated by an arrow at 69 kDa size.

Lane 1, Molecular weight markers. From the top, 97, 66, 55, 43, 40, and 31 kDa, repectively. Lane 2, Cell extract without induction. Lane 3, Cell extract with induction. Lane 4, Ammonium sulfate fraction. Lane 5, His-tag affinity column fraction.

Overexpression of Fusion-Stoffel Fragment

The fusion-SF consists of the polymerase domain of the residues 413 to 832 in *Taq* DNA polymerase and the 3'-5' exonuclease domain of the residues 324 to 508 from *E. coli* DNA polymerase I. The first amino acid of methionine is positioned in the leader sequence. In addition, the linker residues consisting of a phenlyalanine and an alanine are located between the 3'-5' exonuclease and polymerase domains, as shown in Fig. 4. The resulting fusion-SF contains 606 amino acids and its molecular weight is 68,604 Da. The fusion-SF was overexpressed initially in *E. coli* DH5α using a tac promotor on the pDS2 plasmid, but the level of expression was so low that it was very difficult to obtain enough protein for a biochemical assay. The pET-28a expression system, utilizing His-tag affinity chromatography (Novagen, U.S.A.), enabled us to purify the fusion protein to 95% pure protein as indicated in the arrow of Fig. 5. The fusion-SF was overexpressed in *E. coli* BL21(DE3) by induction with 0.5 mM IPTG at 0.5 O.D. units at 550 nm wavelength. This expression system also produced only a small amount of protein. The cell extract was fractionated with 30% to 70% ammonium sulfate. It was loaded onto the His-tag affinity column. The fusion-SF was bound very weakly to the His tag resin, so they were eluted at 50 mM imidazole gradients. The fusion-SF was identified by SDS-PAGE analysis with molecular weight markers, with the fusion-SF positioned as the band of 69 kDa shown in Fig. 5.

Assay of the 3'-5' Exonuclease Activity for Fusion-Stoffel Fragment and Klenow Fragment

We compared the 3'-5' exonucleolytic activity of fusion-SF to that of *Taq* DNA polymerase as a minimum control, and the 3'-5' exonuclease activity of KF as a maximum control since the fusion-SF consists of polymerase and 3'-5' exonuclease domains like Klenow fragments. The fusion protein consists of two different domains where the polymerase domain originates from the thermophilic microorganism and the 3'-5' exonuclease domain is from the mesophilic organism so that the optimum reaction temperature for the polymerase domain is 74°C and that for the 3'-5' exonuclease domain was 37°C. Therefore, the 3'-5' exonuclease assay was focused on how effectively the 3'-5' exonuclease activity from *E. coli* DNA polymerasae I functions in the fusion-SF. If the 3'-5' exonuclease activity of the fusion-SF is functioning as well as in that of the KF, it would be an ideal. However, in this case, it is not very appropriate to compare polymerase activity since those polymerase domains show their optimum reaction conditions differently. The assays were conducted five times in the previously described conditions and the results are summarized in Table 1. The total amount of about 0.5 μg pure fusion-SF was used per assay so that the radiation count would not exceed 10,000 cpm. While the 3'-5' exonuclease activity of the KF showed $7{,}479\times10^3$ (Δcpm/μmole enzyme), the value of the *Taq* DNA polymerase amounted to 460×10^3 (Δcpm/μmole enzyme), which corresponds to 6. 0% of the KF. The level of 3'-5' exonuclease activity in *Taq* DNA polymerase should be very low in this assay condition. However, the 3'-5' exonuclease activity of the fusion-SF showed $3{,}567\times10^3$ (Δcpm/μmole enzyme) which corresponds to 48% of that of the KF. It might be that the many factors involved in inserting the 3'-5' exonuclease domain into the fusion-SF makes full activity unobtainable. However, the activity of the fusion-SF supports the belief that the 3'-5' exonuclease domain is linked to the polymerase domain of *Taq* DNA polymerase

Table 1. The 3'-5' exonuclease activities of Klenow fragment, fusion-Stoffel fragment, and *Taq* DNA polymerase.

Enzymes	3'-5' Exonuclease activity (Δcpm/μmole enzyme) ($\times 10^3$)	Relative activity (%)
Taq DNA polymerase	460±(37) (n=5)	6
Fusion-Stoffel fragment	3,567±(345) (n=5)	48
Klenow fragment	7,479±(600) (n=5)	100

Values are means±(standard deviation), based on n=5.

in functional conformation, and contributes to raising the 3'-5' exonuclease activity. Since the 3'-5' exonuclease activity of the fusion-SF shows a reasonably high value compared to a KF, the two domains from two different DNA polymerases could be linked in the functional conformation, as in a KF.

DISCUSSION

Taq DNA polymerase is homologous to *E. coli* DNA polymerase I in both its primary and tertiary structures. We compared the three-dimensional structural alignment of the 3'-5' exonuclease domain of *Taq* DNA polymerase with that of *E. coli* DNA polymerase I to determine why *Taq* DNA polymerase does not conduct 3'-5' exonuclease activity. We found that there were two very distinct differences in the overall structures. One was the deletion of three loops with lengths between 8 to 27 residues and the replacement of one helix with a random coil, as previously described [13]. The other difference was that all four carboxylic amino acids, which are essential for the two-metal ion mechanism of 3'-5' exonuclease catalysis [1, 2], were replaced by residues incapable of binding the metal ions (L356, G308, V310, and R405) in the vestigial 3'-5' exonuclease domain of *Taq* DNA polymerase.

Since two primary explanations were suggested for the deficiency of 3'-5' exonuclease activity in *Taq* DNA polymerase, we undertook those in two approaches. In the previous report, one attempt was made to introduce 3'-5' exonuclease activity in order to increase the proof-reading by constructing a catalytic module, consisting of four carboxylic amino acids in the active site of the 3'-5' exonuclease domain of *Taq* DNA polymerase [21]. Surprisingly, installing those four carboxylic amino acids into the active site increased the 3'-5' exonuclease activity up to two-folds as high as that of the wild-type. However, a question still remains regarding the roles of the conformational differences, described previously as the deletions of loops and a helix.

In this report, we attempted to replace the 3'-5' exonuclease domain of *Taq* DNA polymerase with that of *E. coli* DNA polymerase I to result in a fusion-SF. The fusion-SF exhibited a value comparable to the 3'-5' exonuclease activity of a KF, which supports the concept that the fusion-SF could be linked in a functional conformation. This fusion-SF is presently not very useful for PCR since the 3'-5' exonuclease domain originated from a mesophilic nature. Experiments to increase the thermostability of the mesophilic part must be conducted for the fusion-SF to be very useful in PCR. Molecular bonds such as ion bonds and hydrogen bonds should be introduced by site-directed mutagenesis to increase the thermal stability, which should be calculated accordingly. Thermal stability experiments could correlate the addition of bond energy to thermal strength in fusion-SF. Studies into the structure of the fusion-SF are presently underway, and will eventually enable the positioning of two domains. The structure could indicate which residues are involved in domain contacts and are eligible for site-direct mutagenesis, in order to increase thermal stability in the mesophilic 3'-5' exonuclease domain.

Acknowledgments

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REFERENCES

1. Beese, L. S., V. Derbyshire, and T. A. Steitz. 1993. Structure of DNA polymerase I Klenow fragment bound to duplex DNA. *Science* **260:** 352–355.
2. Beese, L. S. and T. A. Steitz. 1991. Structural basis for the 3'-5'exonuclease activity of *Escherichia coli* DNA polymerase I: A two metal ion mechanism. *EMBO J.* **10:** 25–33.
3. Delarue, M., O. Poch, N. Tordo, D. Moras, and P. Argos. 1990. An attempt to unify the structure of polymerase. *Prot. Eng.* **3:** 461–467.
4. Derbyshire, V., N. Grindley, and C. M. Joyce. 1991. The 3'-5'exonuclease of DNA polymerase I of *Escherichia coli*: Contribution of each amino acid at the active site to the reaction. *EMBO J.* **10:** 17–24.
5. Derbyshire, V., P. S. Freemont, M. R. Sanderson, L. Beese, J. M. Friedman, C. M. Joyce, and T. A. Steitz. 1988. Genetic and crystallographic studies of the 3',5'-exonucleolytic site of DNA polymerase I. *Science* **240:** 199–201.
6. Eckert, K. A. and T. A. Kunkel. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucl. Acids Res.* **18:** 3739–3744.
7. Flaman, J.-M. *et al.* 1994. A rapid PCR fidelity assay. *Gene* **112:** 29–35.

8. Freemont, P. S., D. L. Ollis, T. A. Steitz, and C. M. Joyce. 1986. A domain of the Klenow fragment of *Escherichia coli* DNA polymerase I has polymerase but no exonuclease activity. *Proteins* **1:** 66–73.
9. Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3'exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* **88:** 7276–7280.
10. Innis, M. A., K. B. Myambo, D. H. Gelfand, and M. A. D. Brow. 1988, DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. USA* **85:** 9436–9440.
11. Joyce, C. M. and T. A. Steitz. 1994. Functional and structure relationships in DNA polymerases. *Annu. Rev. Biochem.* **63:** 777–822.
12. Kim, Y. 1995. An application of genetic engineering for solving the crystal structure of Taq DNA polymerase. *Mol. Cells* **5:** 452–460.
13. Kim, Y., S. H. Eom, J. Wang, D. S. Lee S. W. Suh, and T. A. Steitz. 1995. Crystal structure of *Thermus aquaticus* DNA polymerase. *Nature* **376:** 612–616.
14. Kornberg, A. and T. Baker. 1992. *DNA Replication*, 2nd ed., Chapter 2. Freeman and Company, San Francisco, CA, U.S.A.
15. Kwon, S. T., J. S. Kim, J. H. Park, S. Koh, and D. S. Lee. 1991. Enhanced expression in *E. coli* of cloned *Thermus aquaticus* DNA polymerase gene by optimized distance between Shine-Dalgarno sequence and ATG codon. *Mol. Cells* **1:** 369–375.
16. Lawyer, F. C., S. Stoffel, R. K. Saiki, K. Myambo, R. Drummond, and D. H. Gelfand. 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus. J. Biol. Chem.* **264:** 6427–6437.
17. Longley, M. J., S. E. Bennett, and D. W. Mosbaugh. 1990. Characterization of the 5'-3' exonuclease associated with *Thermus aquaticus* DNA polymerase. *Nucl. Acids Res.* **18:** 7317–7322.
18. Lyamichev, V., M. A. D. Brow, and S. E. Dahlberg. 1993. Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. *Science* **260:** 778–7835.
19. Myers, T. W. and D. H. Gelfand. 1991. Reverse transcription and DNA amplication by a *Thermophilus* DNA polymerase. *Biochemistry* **30:** 7661–7665.
20. Ollis, D. L., P. Brick, R. Hamlin, N. G. Xuong, and T. A. Steitz. 1985. Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. *Nature* **313:** 762–766.
21. Park, P., C. Choi, D.-S. Lee, Park, and Y. Kim. 1997. Improvement of the 3'-5'exonuclease activity of *Taq* DNA polymerase by protein engineering in the active site. *Mol. Cells* **7:** 419–424.
22. Pelletier, H., M. R. Sawaya, A. Kumar, S. H. Wilson, and J. Kraut. 1994. Structures of ternary complexes of rat DNA polymerase β, a DNA template-primer, and ddCTP. *Science* **264:** 1891–1903.
23. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erich, and N. Arnheim. 1985. Enzymatic amplication of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350–1354.
24. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Prime-directed enzymatic amplication of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.