Identification of a premature termination of DNA polymerization *in vitro* by Klenow fragment mutants

GUOJIE ZHAO[1], HUA WEI[2] and YIFU GUAN[1,*]

[1]*Department of Biochemistry and Molecular Biology, Key Laboratory of Medical Cell Biology of Ministry of Education, China Medical University, Shenyang, China*

[2]*Animal Science and Veterinary Medicine College, Shenyang Agricultural University, Shenyang, China*

**Corresponding author (Fax, +86-24-23255240; Email, yfguan55@sina.com)*

DNA polymerization products by Klenow fragment (KF) are blunt-ended. In the present study, we found that the Klenow fragment mutants with partial deletions of thumb subdomain were unable to extend primers to the 5′ terminal of templates, thus creating 5′ overhanging sticky ends 2 nt long. We termed this phenomenon as PmTP (premature termination of polymerization). The KF mutants produced homogenous sticky-ended products only under mild reaction conditions, whereas under vigorous reaction conditions, the sticky ends were prone to be blunt-ended. It was also identified that deletions of more than four residues of KF thumb subdomain could induce PmTP, and two-residue deletion of KF thumb subdomain only induced PmTP in a lower-concentration situation. Structure modelling analysis suggested that shortening or destruction of α helix H_1 at the tip of the thumb subdomain was crucial to PmTP, while the conserved residues in front of α helix was less important. PmTP might be caused by the reduced DNA-binding affinity of the mutants. The sticky ends made by PmTP have potential applications in gene splicing and molecular cloning techniques.

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1. Introduction

DNA polymerases replicate genomes for cell growth and cell division. DNA polymerases have been widely used as sophisticated powerful tools in molecular biology for DNA cloning and genetic diagnosis (Raymond *et al.* 2010). Using parental single-stranded DNA as template and oligonucleotide as primer, these DNA polymerases can extend daughter strands to the 5′ end of the template strands, forming double-stranded DNA with perfect blunt ends (Costa and Weiner 1994; Yang *et al.* 2005) (figure 1A). Some DNA polymerases have terminal deoxynucleotidyl transferase (TdT) activity, and extend primer strands beyond the 5′ end of the templates, adding one more nucleotide to the blunt end in the absence of template, and resulting in a 3′ overhanging sticky end (Clark 1988; Motea and Berdis 2010) (figure 1B). Development of this feature has led to the invention of TA cloning, which has been commonly used in molecular biology until now (Zhou and Gomez-Sanchez 2000). However, to our knowledge, it has never been reported that DNA polymerization is premature at 5′ terminal of template to produce a 5′ overhanging sticky end.

Numerous DNA polymerases have been discovered. Many of them have been sequenced and crystallized. Based

Keywords. Blunt end; DNA polymerase; Klenow fragment; sticky end

Abbreviations used: DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside; KF, Klenow fragment; KPB, potassium phosphate buffer; ODN, oligodeoxyribonucleotide; PAGE, polyacrylamide gel electrophoresis; PmTP, premature termination of polymerization; TdT, terminal deoxynucleotidyl transferase

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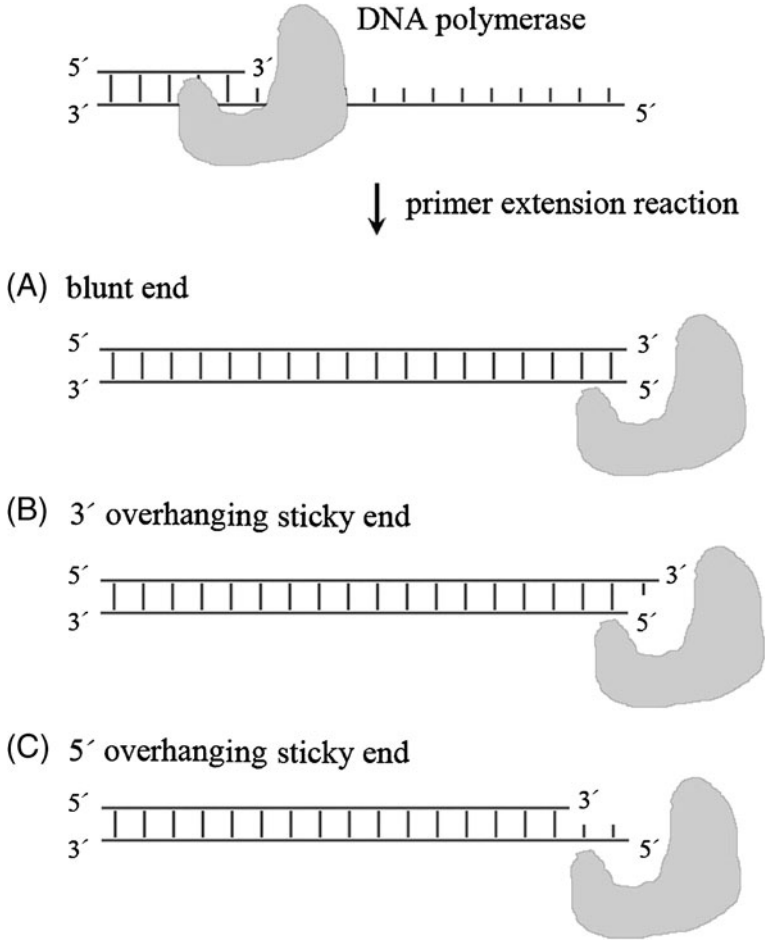

Figure 1. Three types of polymerization products. (A) Blunt end generated by common DNA polymerases. (B) 3′ overhanging sticky end created by TdT activity of some DNA polymerases. (C) 5′ overhanging sticky end observed in our recent study.

on their sequence comparison and crystal structure analysis, they are classified into seven families (Filee *et al.* 2002). Although the primary structures vary a lot, they show similar tertiary structures like 'right hand', containing three subdomains: 'thumb', 'palm', and 'fingers' in general (Kohlstaedt *et al.* 1992). The 3′ end of DNA primer enters catalytic centre that is located in the 'palm' subdomain. The 'fingers' subdomain delivers an incoming dNTP into the 3′ end of the primer. The 'thumb' subdomain holds DNA duplex. Interaction between DNA polymerase and DNA duplex stem is important for high processive polymerization. HIV-1 reverse transcriptase, mutated at sites responsible for binding primers, shows reduced DNA binding affinity and processivity (Bebenek *et al.* 1995). T7 DNA polymerase binds thioredoxin at the tip of its thumb subdomain to hold DNA duplex. In the absence of thioredoxin, processivity of T7 DNA polymerase is decreased (Kroutil *et al.* 1996). A deletion mutant at the tip of Klenow fragment (KF) 'thumb' subdomain also shows reduced DNA binding affinity and processivity (Minnick *et al.* 1996). These shows DNA duplex binding by thumb subdomain plays an important role in polymerization.

In polA family, six members have been crystallized. KF, *Taq*, *Bst* and T7 DNA polymerase are the most studied among them (Li *et al.* 1998, 2004; Hsu *et al.* 2004). We analysed structures of the tip of their thumb subdomains, and found the similarity except thioredoxin binding domain of T7 polymerase. Their structures have two small α helices and conserved residues in front of the helices. Cocrystal structures with DNA also suggest interactions between these residues and DNA duplex (Beese *et al.* 1993). However, studies on detailed functions of these structures are rare.

KF has been used as a model to study DNA polymerase. The polymerization activity and the 3′-5′ exonuclease activity qualify KF a favourable tool enzyme for making blunt-ended DNA products. In a recent study, we prepared a KF mutant partially deleting at the tip of the thumb subdomain, and found this mutant was able to produce polymerization product with a 5′ overhanging sticky end. Because the exonuclease activity of the mutant was diminished by D424A

site-directed mutation, this property must be due to its polymerization activity. The polymerization process ceased several nucleotides ahead of the 5′ end of the template strand (figure 1C). We termed this phenomenon 'premature termination of polymerization' (PmTP). In order to investigate this phenomenon further, we prepared a serial of mutations at the tip of the thumb subdomain, and found that they had this behavior in different extents. The effects of reaction conditions were also discussed. The application of this phenomenon is possible in developing new gene splicing techniques.

2. Materials and methods

2.1 *Materials*

Plasmid pET28a carrying KF was constructed and kept in our lab. Oligonucleotides were synthesized from Genscript Corporation (Nanjing, China). The stock solutions of oligonucleotides were prepared in concentration of 100 μM and were stored at −20°C.

2.2 *Reagents*

Pfu DNA polymerase, DNA Gel Extraction Kit and Plasmid Miniprep Kit were purchased from Tiangen Biotech Corporation (Beijing, China). Restriction endonucleases *Eco*RI and *Hind*III, DNA ligation kit and Klenow fragment were obtained from TaKaRa Biotechnology Company (Dalian, China). Ni-NTA column was from GE Healthcare.

2.3 *In vitro site-directed mutagenesis*

Mutations were made from pET28a carrying KF gene fragment by overlapping extension PCR mutagenesis as described (Horton *et al.* 1990). D424A was made by site-directed PCR mutation from pET28a-KF and was cloned into pET28a plasmid. Other mutations in this study were constructed by PCR mutation from pET28a-D424A. Consequently, all mutants in this study carried D424A site-directed mutations. D424A mutant was used as a 'wild type' control.

2.4 *Expression and purification of mutant proteins*

Recombinant plasmids of KF mutants were transformed into BL21 competent cells. Cell cultures in LB containing kanamycin was induced by 5 mM IPTG when OD600 of cell culture solution reached 0.6. After continuous incubation for 4 h at 37°C, cells were collected by centrifugation. BL21 cells were resuspended in lysis buffer and were lysed by ultrasonication. Cell lysates were centrifuged. The supernatant was purified by Ni-NTA column according to GE product manual. The eluent was dialysed, and the protein solution was prepared in buffer containing 50 mM KPB pH6.5, 10 mM β-mercaptoethanol, 50% glycerol and was stored at −20°C.

2.5 *Polymerization reaction and gel shift assay*

Oligonucleotides of 0.5 μM were prepared in 10 μL reaction buffer containing 10 mM Tris pH7.5, $MgCl_2$ 7 mM, DTT 0.1 mM, and 1 mM dNTP. Polymerization reaction was started by adding 0.3 μM DNA polymerase at 20°C for 10 min. The reaction was stopped by adding 6× loading buffer containing 30 mM EDTA, 36% glycerol, 0.035% xylene cyanol FF, and 0.05% Bromophenol Blue. The products were analysed by 18% nondenaturing PAGE and fast silver stain method as described (Zhao and Guan 2010).

2.6 *Exonuclease reaction and gel shift assay*

Oligonucleotides ODN7 (5′-TTCGCCA-3′) 0.5 μM were prepared in 10 μL reaction buffer containing 10 mM Tris pH7.5, $MgCl_2$ 7 mM, and DTT 0.1 mM. Cleavage reaction was started by adding 0.3 μM DNA polymerase at 24°C for 10 min. The reaction was stopped by adding 6× loading buffer containing 30 mM EDTA, 36% glycerol, 0.035% xylene cyanol FF, and 0.05% Bromophenol Blue. The products were analysed by 18% nondenaturing PAGE and fast silver stain method as described (Zhao and Guan 2010).

2.7 *Gene splicing using PmTP sticky ends*

Polymerization PmTP product of 2.5 pmol by Δ(577-589) was treated by 'phenol:chloroform:isoamyl alcohol (25:24:1)' to remove proteins, and purified by ethanol precipitation. Then the pure product and synthesized 2.5 pmol of ligation substrate were ligated by 175 units of T4 DNA ligase in 10 μL reaction buffer containing 45 mM Tris pH 7.6, $MgCl_2$ 7 mM, DTT 5 mM, and ATP 2.5 mM. After 10 min at 24°C, ligation reaction was stopped by adding 0.1% SDS. The ligation products were analysed by 18% nondenaturing PAGE and fast silver stain method as described (Zhao and Guan 2010).

2.8 *Biosoftware analysis*

Structures of KF mutants were analyzed by homology modelling using SWISS-MODEL (Arnold *et al.* 2006). Electrophoresis bands were analysed by Tanon GIS 1D software version 4.12 (Shanghai, China).

3. Results

3.1 *Plasmids construction and protein expression of KF mutants*

We constructed seven recombinant plasmids including one control D424A, one site-directed mutant N579A/S581A/S582A, and five deletion mutants: Δ(581-582), Δ(579-582), Δ(577-582), Δ(583-589), Δ(577-589), respectively. The last six mutants located on α helix H_1 and conserved residues in front of the helix. The deletion length ranged between 2 residues and 13 residues. All recombinant plasmids were transformed into BL21 for expression. The expressed mutants were purified by Ni-NTA column. The recombinant plasmids were confirmed by DNA sequencing, and the purified proteins were confirmed by SDS-PAGE (figure 2).

3.2 *Polymerase activity and 3′-5′ exonuclease activity assay*

KF has both the polymerization activity and the 3′-5′ exonuclease activity. To eliminate the effect of exonuclease on the polymerization, D424A mutation was prepared, and both of its polymerase activity and exonuclease activity were tested.

We used oligonucleotide substrate ODN7 and s/s8 for the activity assay. ODN7 was a single-stranded oligonucleotide, and s/s8 was an oligonucleotide duplex having 5′ overhang tails at both sides due to the partial self-hybridization (figure 3A). In exonuclease activity assay, ODN7 was digested by KF to a lower place in gel shift assay, indicating the 3′-5′ exonuclease activity of KF (figure 3, lane 2). However, incubated with D424A, ODN7 did not shift band position, indicating the eliminated 3′-5′ exonuclease activity of D424A mutant (figure 3, lane 3). In polymerase activity assay, after s/s8 was incubated with KF or D424A, respectively, their products demonstrated the same gel shift mobility (figure 3, lanes 5 and 6), indicating that both D424A and KF had polymerase activity (Zhao and Guan 2010). Other mutants were also tested using the same assay. Consequently, reaction products obtained in this study must be due to the polymerization activity rather than the 3′-5′ exonuclease activity.

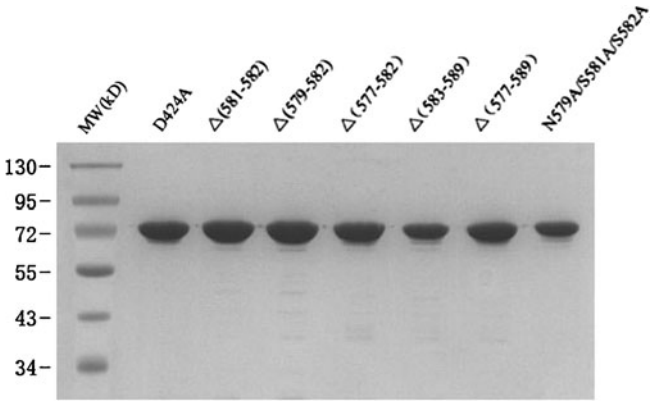

Figure 2. SDS-PAGE of purified KF mutants. Proteins were loaded 5 μg each lane and were separated in 10% SDS-PAGE.

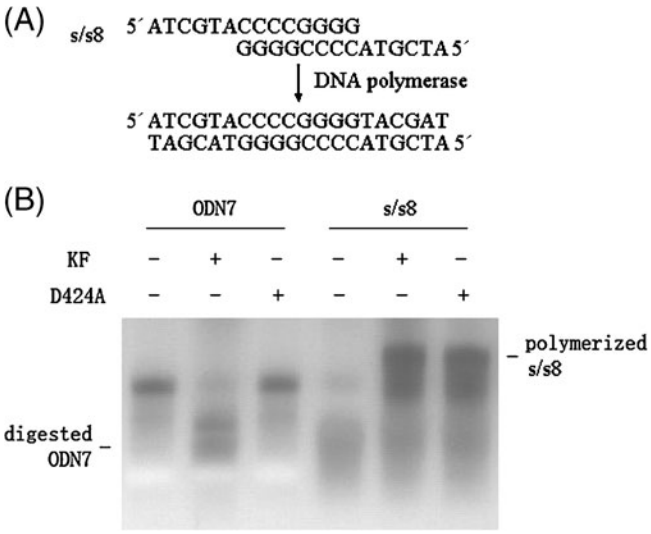

Figure 3. Gel shift assay of 3′-5′ exonuclease activity and polymerase activity of KF and D424A. (**A**) Illustration of polymerase activity assay. (**B**) Substrate ODN7 was for exonuclease activity assay, and substrate s/s8 was for polymerase activity assay. Substrate concentration was 0.5 μM. Enzyme concentration was 0.3 μM.

3.3 *Premature termination of polymerization*

Using substrate s/s8, we carried out polymerization reaction by D424A and Δ(577-589), respectively. We observed that the polymerization product by Δ(577-589) presented a lower band position than product by D424A (figure 4B, lanes 1–3,). Two other synthesized gel shift markers were compared. Markers s/s12 and s/s16 had 4 and 2 nt (nucleotides) of 5′ overhangs, respectively (figure 4A). The band of polymerization product by Δ(577-589) was at the same position of s/s12 band. This indicated polymerization product by Δ(577-589) had 2 nt 5′ overhanging sticky ends. When s/s12 and s/s16 were polymerized as substrates by D424A, fully extended product bands were present. However, by adding Δ(577-589), only bands at the same position of s/s12 band were observed. These data suggested that Δ(577-589) was unable to extend primer to the 5′ end of the template, and it produced sticky ends with about 2 nt of 5′ overhang (figure 4B). In this study, the use of native gel analysis might raise

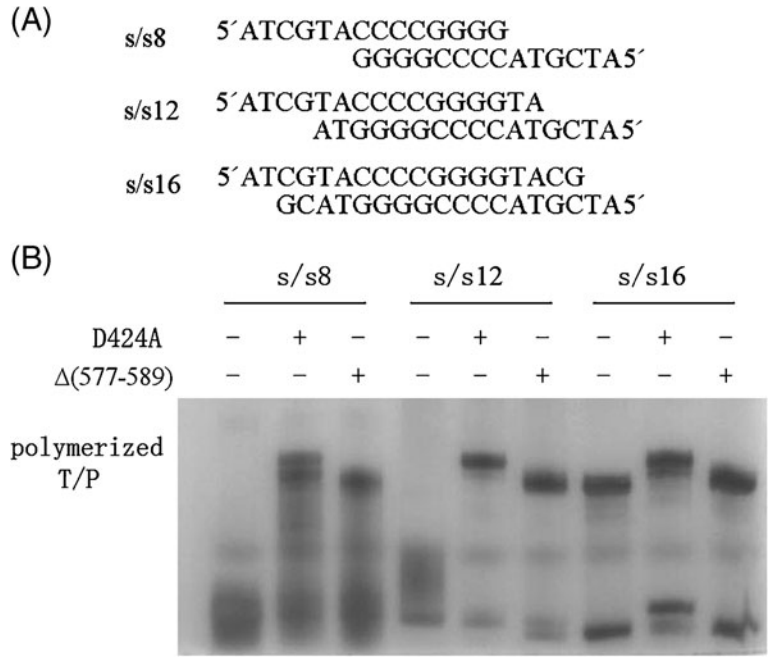

Figure 4. Gel shift assay of polymerization products of three different substrates by D424A and Δ(577-589). (**A**) Illustration of three substrates. s/s8, s/s12, s/s16 have 6-, 4- and 2-nt overhangs at the 5′ ends, respectively. (**B**) Gel shift image of polymerized products. Substrate concentration was 0.5 μM. Enzyme concentration was 0.3 μM.

concerns that protein might bind the polymarization product and affect band shifting. After treating reaction products by SDS, PmTP bands still presented in the same position (data not shown), excluding the possibility of gel shifting caused by protein binding.

3.4 *Effects of reaction conditions on PmTP*

We tested the effects of temperature, polymerase concentration, and dNTP concentration on PmTP. We used D424A as a 'wild type' control. First, as the reaction temperature reduced by 5°C at a time, the reaction products of D424A showed band intensity decrease without shifted band positions. On the contrary, the reaction products of Δ(577-589) shifted to lower positions when the temperature changed from 30°C to 20°C, and completely disappeared below 15°C (figure 5A). Secondly, as polymerase concentration decreased by two-fold dilution, the reaction products of D424A showed only band intensity decrease without band shifting. However, the product bands of Δ(577-589) shifted to lower positions when enzyme concentration decreased from 1.2 μM to 0.3 μM (figure 5B). Finally, as dNTP concentration decreased by ten-fold dilution at a time, the products of D424A showed only band intensity decrease without band position shifts. However, the product bands of Δ(577-589) shifted to lower positions when dNTP concentration decreased from 1 mM to 10 μM, and then bands disappeared below 10 μM concentration of dNTP (figure 5C).

D424A was unable to produce PmTP product under various reaction conditions. However, under appropriate conditions, Δ(577-589) made obvious shifted lower bands of sticky-ended products. When reaction conditions became inappropriate, such as higher temperature or lower enzyme concentration, the sticky ends tended to become blunt-ended.

3.5 *Premature termination of polymerization by different loop mutants*

Polymerization reactions of thumb mutants were also tested. In controlled reactions, neither N579A/S581A/S582A nor Δ(581-582) showed PmTP band, however, other four deletion mutants presented the PmTP activity (figure 6). Although Δ(579-582), Δ(577-582), Δ(583-589) and Δ(577-589) had various deletion length ranging between 4 and 13 residues, their shifting band positions were the same.

Considering the effect of reaction conditions on PmTP, we wondered whether the experimental condition we used could conceal PmTP of N579A/S581A/S582A and Δ(581-582). By lowering their enzyme concentration, both of these two mutants presented PmTP bands, although the band of the former is very weak (supplementary materials). For a

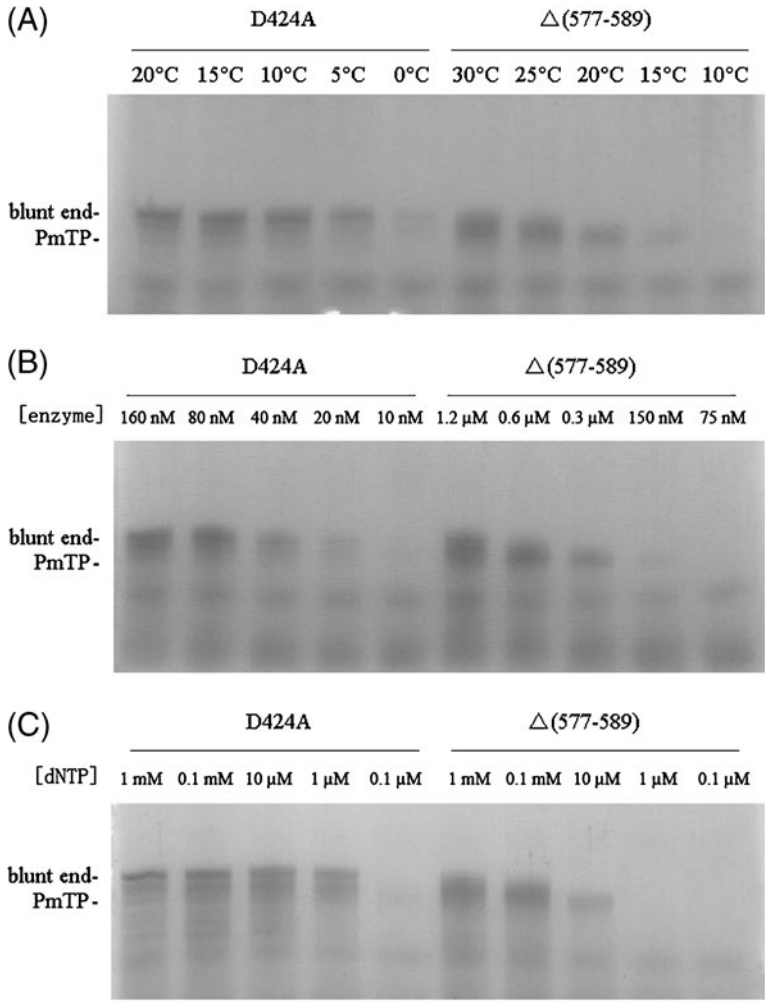

Figure 5. Effects of reaction conditions on PmTP. (**A**) Gel shift assay of primer extension reactions at different temperatures. Substrate s/s8 were polymerized by D424A or Δ(577-589) at different temperatures, respectively. Enzyme concentration was 0.3 μM. Substrate concentration was 0.5 μM. (**B**) Gel shift assay of polymerization for different polymerases concentration. Substrate s/s8 were polymerized by D424A or Δ(577-589) of different concentrations. Substrate concentration was 0.3 μM. (**C**) Gel shift assay of polymerization for different dNTP concentrations. Enzyme concentration was 0.3 μM. Substrate concentration was 0.5 μM.

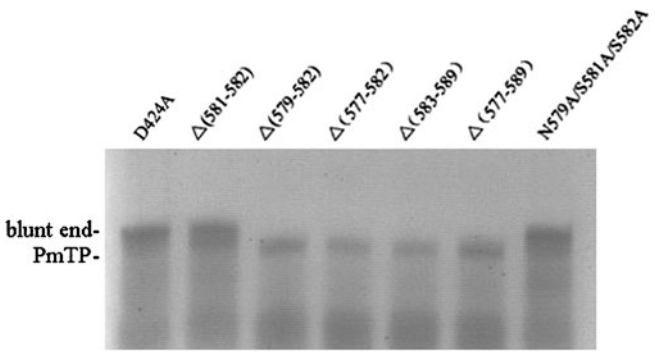

Figure 6. Gel shift assay of polymerization products by seven KF mutants using substrates s/s8. Enzyme concentration was 0.3 μM. Substrate concentration was 0.5 μM.

standard and comprehensive comparison, each of the seven mutants was analysed for PmTP bands in a range of enzyme concentration. The relative amount of PmTP band was calculated for each concentration level and each mutant, and the values were plotted as a function of enzyme concentration. The peak value represented optimal enzyme concentration for PmTP. N579A/S581A/S582A had the lowest optimal concentration of 6 nM. Δ(581-582) had the optimal concentration of 48 nM. The optimal concentration was 384 nM for Δ(579-582), Δ(577-582), Δ(583-589) and Δ(577-589) (figure 7).

3.6 *Gene splicing using sticky ends produced by PmTP*

To identify the precise overhang length of sticky end produced by PmTP and to test the application possibility of

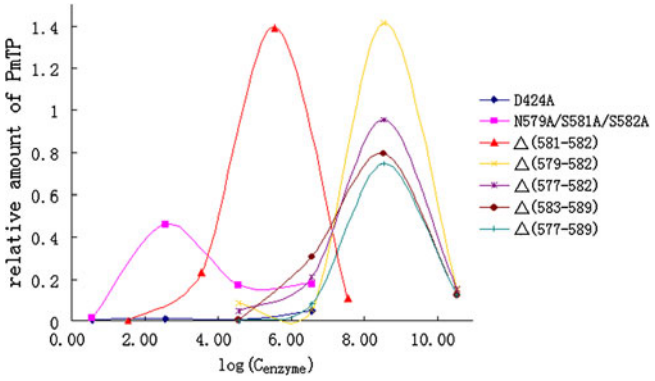

Figure 7. Optimal enzyme concentrations for producing sticky ends were plotted as relative amount of PmTP product versus log (C_{enzyme}). The relative amount of PmTP product was calculated by PmTP products at s/s12 position divided by a standard amout of s/s12 marker using gel band gray. Substrate s/s8 was 0.1 µM. Enzyme concentration range was 1.5 nM–1.5 µM. Enzyme concentration was doubly diluted. For convenience, logarithm-processed enzyme concentrations were used to plot.

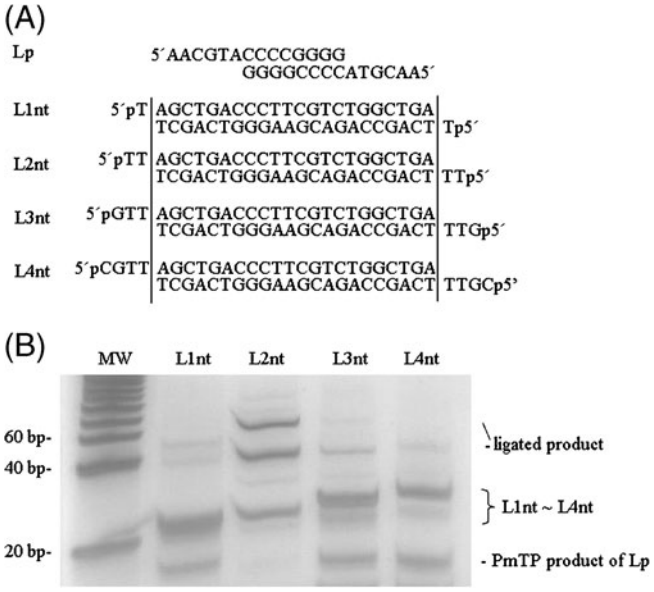

Figure 8. Gel shift assay of gene splicing using PmTP sticky ends. (**A**) Illustration of substrates for ligation. (**B**) Gel shift image of ligation products. Substrates concentration was 0.25 µM. T4 DNA ligase concentration was 17.5 U/µL.

PmTP sticky ends in gene splicing process, a ligation experiment was designed. We polymerized dimer substrate named 'Lp' by KF mutants to make PmTP sticky ends (figure 8A). We also prepared four sticky-ended duplexes, named L1nt, L2nt, L3nt and L4nt, respectively, for ligation reactions. The length of the 5′ overhanging sticky end varied from 1 to 4 nt. These overhangs were complementary to PmTP products sticky ends respectively with 1–4 nt 5′ overhangs (figure 8A). We found only L2nt could be ligated with PmTP products. Since L2nt can be ligated at each end of polymerized Lp, the products are expected to present two bands. Ligation at one end of polymerized Lp would generate a product band of about 40 bp, and ligation at both ends would generate a product band of about 64 bp. Their migrating positions were in perfect agreement of the expected ligation length in gel analysis according to molecular markers. Furthermore, we also observed that the substrates of ligation reaction were decreased, since the band of 'PmTP product of Lp' disappeared completely, and the band of L2nt was weakened (figure 8, lane 3).

4. Discussion

4.1 *Methods to identify sticky ends*

Common products of DNA polymerases are blunt-ended, and therefore methods for DNA duplex end analysis are not imperious demands. Adding dA by the TdT activity of some polymerases had been analysed by radio-labelled gel shift assay, which had been also used in DNA sequencing (Clark 1988). Denatured sequencing gel electrophoresis and radio-labelled substrates render the high resolution but relative tedious and harmful handling. Here, we presented a cost-effective and fast gel shift assay by using short oligonucleotide to analyse duplex ends. Self-hybridized duplex with two 5′ overhangs at each end was analysed in nondenaturing PAGE. Fixing dimer length at 14 bases, the length of 5′ overhang affected electrophoresis migration greatly. The gel band of dimer with 2 nt overhang shifted to a lower position compared with the 14 bp duplex (figure 4B, lane 7). Dimer with 4 nt overhang showed a further low and diffused band (figure 4B, lane 4). Dimer with 6 nt overhang presented much more diffused band in the lowest position (figure 4B, lane 1). Single-stranded overhang is more flexible than duplex. Consequently, the increased overhang length and the decreased duplex length will diffuse bands easily. Because the lengths of single strand and double strand were both several nucleotides, the ratio of single-strand length to double-strand length affected electrophoresis migration obviously. Consequently, sticky ends can be identified easily using nondenaturing PAGE without labelling. Duplex with a overhang at one end can be also identified, but the migration change was less than that of dimer we used (data not shown). Although the method is convenient, we can only acquire the resolution of 2 nt overhang change in conventional electrophoresis equipment. Additional methods are needed to clarify the exact length of sticky end.

4.2 *Characteristics and mechanism of PmTP*

DNA polymerases can extend primers to the 5′ end of the template to create blunt-ended duplex DNA. Some polymerases demonstrate the TdT activity to give a 1 nt 3′ overhang sticky end (Motea and Berdis 2010). However, in our study, the KF mutants with truncated loop bear the ability to create 5′ overhang sticky ends.

The PmTP activity was affected by reaction conditions. With an excessive amount of polymerase, mutants were prone to extend duplex to blunt-ended product. At lower reaction temperatures, sticky end can be finely made. To decrease dNTP concentration, PmTP can also occur for loop mutants. However, neither of these reaction conditions can make PmTP occur for D424A (wild type). This implies an inherent characteristic of these loop mutants. Under the controlled reaction condition, a homogenous 2 nt sticky end majority can be produced, which was verified by ligation experiment. This was unaffected by the length of overhang, as we saw in figure 4. Using other oligonucleotide sequence, gel shift results were the same (data not shown), which showed a sequence-independent behaviour of PmTP. The exact overhang length produced under the controlled reaction conditions was clarified by ligation experiment to be 2 nt (figure 8B). This was in accordance with polymerization gel shift assay (figure 4B).

DNA polymerase contains three subdomains: thumb, palm and fingers. The thumb subdomain is responsible for binding DNA duplex backbone and is related to the processivity of polymerase (Bedford *et al.* 1997; Yang and Richardson 1997). Minnick *et al.* (1996) have compared the loop deletion mutant Δ(590-613) and the 'wild type' D424A. They found duplex-binding affinity and processivity of this mutant are lower than D424A. However, they did not report whether the products polymerized by the mutant are blunt or not.

The decreased binding affinity of loop mutants may contribute to PmTP. A previous study has reported that when the length of the template 5′ overhang decreases from 4 to 2 nt, the binding affinity decreases by three-fold, and as the length of 5′ overhang is shortened to 1 nt, the binding affinity decreases by six-fold. Thus, at least 4 nt on the template are required to interact with KF (Turner *et al.* 2003). A similar phenomenon has also been observed for T4 polymerase (Delagoutte 2003). Consequently, as DNA polymerase approaches to the end of DNA substrate, the shrinking 5′ overhang of template decreases substrate-binding affinity of polymerase rapidly. In the case of wild-type KF, high duplex-binding affinity ensures polymerization to the last template base to be paired. However, in the case of KF mutant, the low duplex-binding affinity will aggravate affinity reduction in terminal polymerization, resulting in an earlier dissociation of the polymerase from substrates and a 5′ overhanging sticky end of 2 nt long. In our study, we observed the decreased affinity of loop mutant to duplex DNA (data not shown), which was also supported by mutant Δ(590-613) in the study of Minnick. We think mutant Δ(590-613) should also behave PmTP if appropriate methods and reaction condition is prepared.

4.3 *Loop structure analysis*

The 'thumb' subdomain of KF contains two parallel long α helices H and I, and 55 residues (570-625) region at the tip of the 'thumb' subdomain (Ollis *et al.* 1985). In cocrystalized structure, the region is a 'W' loop formed by two small loops. For simplicity, we terms loop H_1 and loop H_2. Half of loop H_1 or loop H_2 is α helix H_1 (583-593) or H_2 (612-619) (Beese *et al.* 1993). The N terminal of helix H_1 or H_2, in the middle of each small loop, contains conserved residues N579~S582 (H_1) or S608~E611 (H_2). These residues interact with DNA duplex backbone in polymerization reaction (Minnick *et al.* 1999).

In Minnick's study, deletion includes C terminal of H_1, N terminal of H_2 helices, and random coil between them. Their deletions disrupted both loop H_1 and loop H_2, and the deletion of the conserved residues in front of H_2 helix may contribute to the interaction of enzyme–substrate complex. Our experiment focused on the function of the loop H_1 without changing loop H_2. Structure modelling confirmed an unchanged loop H_2 of our loop mutants. Loop deletion will delete the conserved residues and shorten loop length as well. The reduced affinities of loop mutants might be due to either the deletion of the conserved residues or the disrupted interactions between polymerase and duplex that resulted from the shortened loop length. To distinguish their functions, we prepared a site-directed mutation N579A/S581A/S582A for comparison.

Under the controlled reaction condition, N579A/S581A/S582A and Δ(581-582) showed no PmTP activity, while Δ(579-582), Δ(577-582), Δ(583-589) and Δ(577-589) demonstrated the PmTP behaviour (figure 6). However, in a range of enzyme concentration, both of these mutants behaved like PmTP, although the PmTP band of N579A/S581A/S582A was very weak. For D424A, no sticky band was observed in any enzyme concentration level. Optimal concentration for PmTP was only 6 nM for mutant N579A/S581A/S582A, 48 nM for Δ(581-582), and 384 nM for Δ(579-582), Δ(577-582), Δ(583-589) and Δ(577-589) (figure 7). Therefore, the effects of reaction conditions on PmTP are similar for the four mutants deleting more than four residues. We also compared the affinity of these mutants binding to duplex DNA to be D424A > N579A/S581A/S582A > Δ(581-582) > Δ(579-582) (data not shown). These data are consistent with the optimal enzyme concentration of

these mutants, and support the conclusion that the PmTP behaviour is affinity-related.

Using protein structure modelling, we analysed structure changes of these mutants below. (1) Site-directed mutation N579A/S581A/S582A alters no loop structure (data not shown). Three conserved residues forming hydrogen bond are changed; however, alternative bond donors of peptide backbone or nearby residues may bind DNA. Unchanged loop length is enough for this adapted binding, although possibly at a cost of free energy. (2) When the deletion area expands, loop H_1 shrinks. Δ(581-582) deletes two residues, maintains three circle of α helix, and results in a puckered loop H_1 (figure 9B). However, K584 moves into the place of the deleted S582, and an interaction between K584 and phosphate backbone may compensate the lost hydrogen bond by S582. (3) Δ(579-582) deletes four residues, shortens α helix H_1 by one circle, resulting in a widened loop H_1 (figure 9C). Although T583 and K584 move into the place where N579 and S582 have been located, and one of their interactions with DNA phosphate backbone may compensate the lost hydrogen bonds, the shortened α helix may taut the interaction between loop H_1 and DNA, and reduce the affinity. (4) Δ(577-582), Δ(583-589) and Δ(577-589) delete 6, 7 and 13 residues respectively. Loops H_1 are both shortened for Δ(577-582) and Δ(577-589) obviously, and are hard to catch DNA duplex without forming an unfavorable bending of DNA backbone (figure 9D and 9F). However, loop H_1 of Δ(583-589) is not shortened for a completely destroyed α helix H_1 (figure 9E). Consequently, its loop H_1 is formed only by random coil, and the binding of DNA is weakened by the flexibility of loop H_1.

As a summary, the conserved residues in front of α helix H_1 were not so important for PmTP as expected, and PmTP was generally caused by deletion mutations. When the loop residues were deleted, the length of α helix H_1 was affected. One circle reduce is sufficient to greatly decrease the interaction of loop H_1 and DNA. Mutants with a helix lengths less than two circles showed no difference in PmTP behavior. All these data pinpointed the importance of the α helix in the tip of thumb subdomain in the PmTP generation.

4.4 *Application in gene splicing*

Sticky ends are extremely important in gene splicing process. Traditionally, 3′ overhanging or 5′ overhanging sticky ends can be prepared using restriction endonulceases (RE), which is an essential step for recombinant DNA construction (Carroll 1993; Schamhart and Westerhof 1999). In practice, different REs will create different sticky ends for designed ligation. Another commonly used method for making sticky end is TA cloning, which makes a 3′ overhanging sticky end of one nucleotide using the TdT activity of some polymerases. Our results suggest that sticky ends can also be prepared by PmTP activity of mutated KF. Polymerization products by loop mutants can form a homogenous 2 nt sticky-end band in gel shift assay (figure 4). Using the PmTP sticky end, the PmTP product can be ligated only to its 2 nt

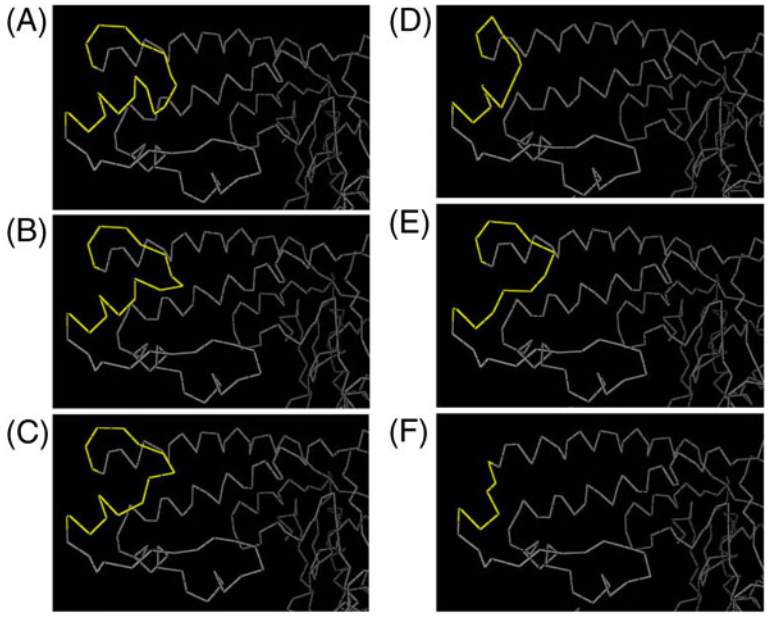

Figure 9. Thumb subdomain structures of KF mutants by structure modelling. (**A**) D424A; (**B**) Δ(581-582); (**C**) Δ(579-582); (**D**) Δ(577-582); (**E**) Δ(583-589); (**F**) Δ(577-589). Loops H_1 were marked with yellow. Loops H_2 were below loops H_1. Helices H and I were horizontally paralleled in upper half of the pictures.

complementary sticky end of synthesized oligonucleotide (figure 8). This shows a good ligation behaviour of the PmTP sticky end and its potential for applications in gene splicing techniques. Considering the tertiary structure similarity of polymerases, PmTP is possible for other DNA polymerases. Studies on the of PmTP of thermostable polymerases will make it possible to apply PmTP sticky end in PCR cloning. However, to implement the potential applications, more fundamental research is required. The mechanism of PmTP should be confirmed. Moreover, in Minnick's study, the frameshift fidelity and processivity are reduced for Δ(590-613) without losing other fidelity. If this is true for loop mutants bearing PmTP activity, in an acceptable extent of compromised frameshift fidelity, it is still possible to use PmTP property in gene cloning and other gene splicing methods.

In summary, KF deletion mutants at the tip of the thumb subdomain generate the prematured termination of the polymerization at the 5′ terminal of template and produce a 5′ overhanging sticky end. The sticky-ended products can be identified conveniently in nondenaturing PAGE. Under the condition of high enzyme concentration or dNTP concentration, or high temperature, sticky ends tend to be blunt-ended. Under the controlled moderate reaction conditions, the homogenous sticky-ended product of 2 nt 5′ overhang is produced. Mutants deleting of more than four residues have similar PmTP activity. However, a two-residue deletion and site-directed mutation of the conserved residues of KF can only induce the PmTP behaviour at a very low enzyme concentration. Structure modelling analysis suggests that shorter α helix affects the PmTP activity more than the conserved residues located in front of the α helix. We hypothesize that PmTP is possibly related to the reduced binding affinity of KF mutants with DNA duplexes. The sticky ends produced by PmTP could find potential applications in gene splicing techniques.

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