Acta Biochim Biophys Sin 2010, 42: 722–728 | © The Author 2010. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmq082.

Original Article

Polymerization behavior of Klenow fragment and *Taq* DNA polymerase in short primer extension reactions

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DNA polymerases amplify DNA fragments through primer extension reactions. However, polymerization behavior of short primers in the primer extension process has not been systematically explored. In this study, we examined the minimal primer length required for primer extension, and the effect of primer length, mismatches and other conditions on DNA polymerization using a non-radioactive method. Under the condition we conducted, the shortest primers polymerized by Klenow fragment (KF) and *Taq* DNA polymerase in our experiments were respectively heptamer and octamer. The extension efficiency was also affected by the up-stream overhanging structure of the primer–template complex. We hypothesized a simple model to interpret these observations based on the polymerase structures. Furthermore, it was found that the longer the primer, the more efficient is the primer extension. These polymerization behavior of short primers lay foundation about DNA polymerization mechanism and development of novel nucleic acid detection assays.

Keywords DNA polymerase; short oligonucleotide; activity analysis; non-radioactive method

Received: May 9, 2010 Accepted: July 14, 2010

Introduction

DNA polymerase plays a central role in transferring the genetic information from generation to generation of any kind of organism. Many DNA polymerases have been discovered and well characterized. Based on the amino acid sequence comparison and crystal structure analyses, they have been classified into six families [1]. Although their amino acid sequences vary from each other, they share a structural similarity to some extent. In general, their global architectures resemble a shape of 'right hand' with three distinct subdomains of 'palm', 'thumb', and 'fingers' [2,3]. Experimental evidence also showed that catalytic mechanisms of these DNA polymerases are identical: two-metal ion mechanisms [3,4]. But more details of the catalytic mechanisms still remain to be explored, especially the interaction of polymerase residues with DNA substrate and their roles in catalytic reaction.

DNA polymerases have been widely used as tool enzymes, such as in PCR, nucleotide labeling, and DNA sequencing. Considering the expensive and delicate PCR instruments required in PCR, as an alternative method, isothermal DNA amplification processes are preferred over the thermal cycling PCR in certain circumstances such as on-site biological warfare detection in the battlefield or instant inspection in the customs. In the process of developing a novel label-free, easy-operation, cost-effective DNA analysis method, it is realized the necessity to characterize these polymerases in terms of the extension efficiency, and polymerization fidelity for short primers. In addition, short oligonucleotides do play an important role in many polymerization reactions, such as genetic bit analysis [5] and mini-sequencing [6], and their polymerizations are also side reactions in PCR or other reaction systems involved polymerases and oligonucleotides. Unfortunately, little has been reported about short primer extension catalyzed by different polymerases under different conditions and the effect of the mismatches on the primer extension efficiency.

To obtain an understanding about the effectiveness of polymerases for short oligonucleotide primers, we conducted a study to examine the effects of the primer length, mismatches, and other factors on the primer extension reactions of KF, and compared with that of *Taq* DNA polymerase. We found that primer length affects short primer extension reactions; other factors such as mismatch and temperature also affect the short primer extension efficiency. These could enhance our understanding about the polymerization properties from other aspects, and assist the development of a novel isothermal DNA amplification-based platform for nucleic acid analysis and detection.

Materials and Methods

Oligonucleotide preparation

All oligonucleotides in HPLC purity were synthesized from Genscript Corporation (Nanjing, China), and their stock solutions were prepared with sterilized ddH_2O to the final concentration of 100 μM and stored at −20°C. The nucleotide sequences are listed in **Table 1**.

Primer extension reaction

DNA polymerase KF and *Taq* DNA polymerase examined in this experiment were purchased from TaKaRa Biotechnology Company (Dalian, China) with stock concentration of 0.6 μg/μl and enzyme activity of 4 U/μl for KF, and stock concentration of 0.08 μg/μl and enzyme activity of 5 U/μl for *Taq* DNA polymerase. The total volume of the primer extension reaction system was 10 μl, containing 1 μM oligonucleotides, 1 mM dNTP, 10 mM Tris (pH 7.5), 7 mM $MgCl_2$, 0.1 mM DTT, and 0.25 μl of stock polymerase. After incubation at room temperature for 10 min, the primer extension reaction was stopped by adding 6× loading buffer (30 mM EDTA, 36% glycerol, 0.06% xylene, and 0.06% bromophenol blue), and followed by boiling for 5 min and cooling down slowly to room temperature.

The extension products were analyzed using the native PAGE and fast silver stain method [7]. Briefly, 18% acrylamide gel was produced in Tris–borate–EDTA (TBE) buffer. Electrophoresis was carried out at the constant voltage of 100 V in TBE buffer. After fixation, the native PAGE gel was incubated with silver nitrate for 15 min, and visualized with development solution for 20 min. The band intensities were quantified in terms of the logarithm of the darkness using software Glyko BandScan 5.0 (Glyko, Inc., Madison, USA).

Table 1 Oligonucleotides used in experiments

Oligonucleotide	Sequences
T14[a]	3′-TAAGCGGTGCTGCT-5′
P14	5′-ATTCGCCACGACGA-3′
P9	5′-ATTCGCCAC-3′
P8	5′-ATTCGCCA-3′
P7	5′-ATTCGCC-3′
P6	5′-ATTCGC-3′
P8m1[b]	5′-ATTCGCC**C**-3′
P8m5[b]	5′-ATT**A**GCCA-3′
P8m8[b]	5′-**C**TTCGCCA-3′
S18	5′-ATCGTACCCGGGTACGAT-3′
S14	5′-ATCGTACCCCGGGG-3′
S12	5′-ATCGTACCCGGG-3′
S11	5′-ATCGTAGCCGG-3′

[a]For convenience, the sequence of T14 is written from the 3′-terminal to the 5′-terminal.

[b]For P8m1, P8m5, and P8m8, the mismatches are labeled in bold and underlined.

Data analysis

Each number was the average of at least three independent experimental data, and the experimental errors were specified in terms of standard deviation (SD). The polymerization efficiency was presented in percentage by dividing each result by a number obtained under a standard condition.

Results

Short primer extension reactions

Two groups of oligonucleotide primers were examined. Primers in the first group, named as Pn ($n = 9$, 8, 7, and 6), have 9, 8, 7, and 6 nucleotides long, respectively. They can hybridize with a template T14 (14 nucleotides long) to form template–primer complexes (T/P complexes). Since these complexes have an overhanging tail at the 5′-terminal of the template, these primers can be extended only in one direction. Primers in the second group, named as Sn ($n = 18$, 14, 12, and 11), can be self-dimerized, forming palindrome structures with two overhanging tails at both the 5′-terminal (S/n complexes). Thus, these primers can be extended in two directions. The symbol S12/6 is designated to the S/n complex composed of the primer S12 through six pairs of nucleobases (**Fig. 1**).

Figure 1 shows the primer extension results. Synthetic complementary oligonucleotide duplexes (T14/P14 and S18/18) were used as positive controls. The T14/P8 and S12/6 complexes did not extend these primers when polymerases were not added. When the polymerases were mixed with these reaction systems, the reactions showed the expected products, which were identical to the positive controls. These results proved the validity of the native PAGE analysis for the primer extension reactions.

Effects of primer length on short primer extension reactions

The minimal primer length required for the primer extension reaction by polymerases was examined. Four complexes (T14/P7, T14/P6, S12/6, and S11/4) were examined for KF polymerase, and four complexes (T14/P8, T14/P7, S14/8, and S12/6) were examined for *Taq* DNA polymerase. The reactions were performed at 16°C to ensure the formation of stable duplexes.

Figure 2 shows that when the primer length of the T/P complex is shorter than seven nucleotides, KF polymerase could not generate any extension products, meaning that these primers could not be extended [**Fig. 2(A)**, lane 4].

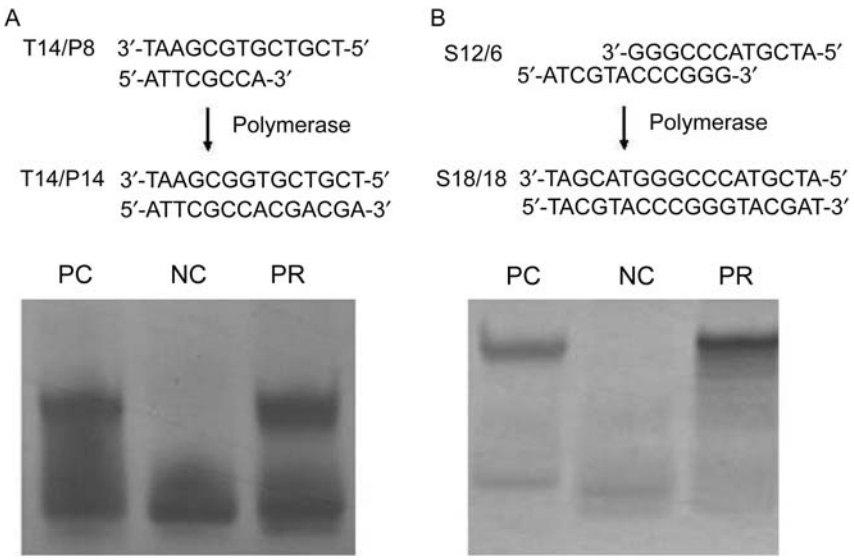

Figure 1 Native PAGE analysis of primer extension products (A) PC, positive control (synthetic T14/P14); NC, negative control (T14/P8 without polymerase); PR, extension product (T14/P8 complex plus KF). (B) PC, positive control (synthetic S18/18); NC, negative control (S12/6 without polymerase); PR, extension product (S12/6 complex plus KF). These reactions were carried out at room temperature for 10 min with 12 ng/μl KF and 1.0 μM oligonucleotides.

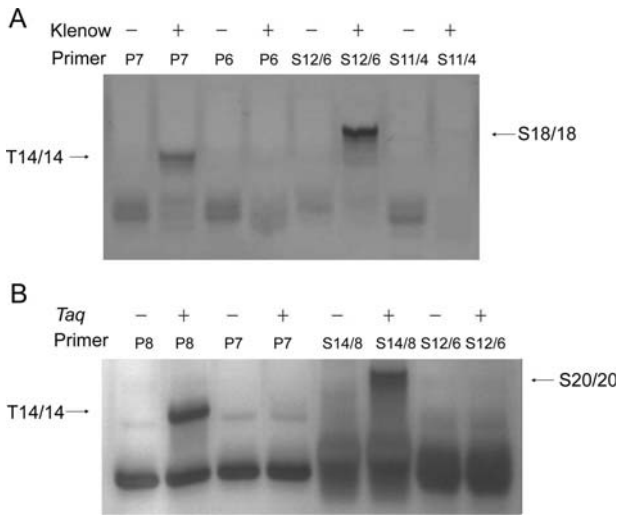

Figure 2 The minimal primer length required by KF and *Taq* DNA polymerase for the primer extension reactions The primer extension was carried out at 16°C overnight with the enzyme concentration of 12 ng/μl for KF and 1.6 ng/μl for *Taq* polymerase. (A) Reaction products catalyzed by KF. (B) Reaction products catalyzed by *Taq* DNA polymerase.

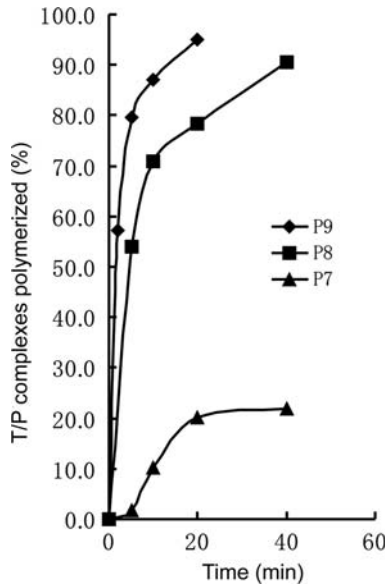

Figure 3 Effect of the primer length on the primer extension efficiency Three primers P9, P8, and P7 were hybridized with T14, respectively, and then extended at 37°C for different periods with the KF concentration of 0.18 ng/μl. The experimental data are average values of three-independent measurements, and the experimental errors (SD) are <1%.

In the case of *Taq* DNA polymerase, it needs a primer of at least eight nucleotides long for extension [**Fig. 2(B)**, lanes 2 and 6]. When these two polymerases were applied to the S/n complexes of two overhanging tails, the minimal length of the paired segment showed a slight difference. KF requires a hexamer, whereas *Taq* DNA polymerase needs an octamer. These data indicated that KF and *Taq* DNA polymerases have different requirements for minimal lengths of the paired segments in the T/P and S/n complexes to extend the primers. Furthermore, as the primer length is longer than the minimal requirement, the extension efficiency becomes increased in accord with the increase of the length of primers (**Fig. 3**). In the T/P complexes, P8 is extended more efficient than P7, and the extension efficiency of P9 is higher than that of P8. The S/n complexes have similar extension behavior (data not shown).

Effect of mismatches on short primer extension efficiency

To examine the effect of primer mismatches on the extension efficiency, we made three mismatches in T14/P8 complexes once a time. C:T mismatch was made once a time at two different positions: the 3′-terminal (P8m1), the 5′-terminal (P8m8), and one A:G mismatch was made at the middle region of the P8 primer (P8m5). The P8 primer was used as a positive control. **Figure 4** shows the trend of the extension efficiency of KF influenced by mismatches: P8＞P8m8＞P8m5＞P8m1.

Effects of pH, Mg^{2+}, and temperature on short primer extension efficiency

To examine the effect of the pH on the short primer extension efficiency, the pH of the reaction system was adjusted to 6.0, 7.0, 8.0, 9.0, and 10.0, respectively. Then the primer extension was performed with KF when other conditions remained unchanged. **Figure 5(A)** shows that the favorable pH for KF extension activity is ∼7.0−8.0.

The primer extension reaction was also carried out at the Mg^{2+} concentrations of 1.25, 2.50, 5.00, 10.00, and 20.00 mM, respectively, when other reaction conditions were kept unchanged. **Figure 5(B)** shows that the favorable Mg^{2+} concentration for KF extension activity is between 5 and 10 mM.

The temperature effect on the primer extension efficiency presents a bell-shaped curve for both polymerases. **Figure 5(C)** shows that the optimal temperature is around 30 and 45°C for KF and *Taq* polymerase, respectively.

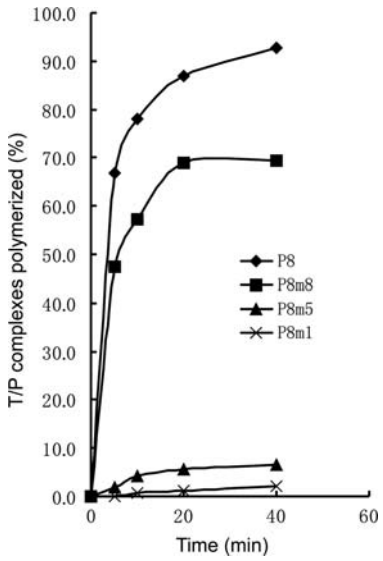

Figure 4 Effect of mismatches on the primer extension efficiency
P8m8, P8m5, and P8m1 were hybridized with T14 to form T/P complexes having mismatches at different positions, and then primers were extended with KF at the polymerase concentration of 0.7 ng/μl. The experimental data are average values of three-independent measurements, and the experimental errors (SD) are <0.5% for complementary and <1.0% for mismatch T/P complex, respectively.

Discussion

Non-radioactive method for DNA polymerase product analysis

DNA polymerases are responsible for replicating DNA molecules *in vivo* as well as for creating a variety of useful technical formats for nucleic acid analysis. The traditional method for polymerization product analysis is the radioactive-labeled primer extension assay that is still commonly used. Though this method offers an extremely high sensitivity, it could bring radioactive contamination and be harmful to operators. The fluorescence-labeled method also has high detection sensitivity similar to the radioactive-labeled method, but it is expensive and needs the delicate equipment to acquire fluorescence signals [8]. In the current study, we used the silver staining method to analyze the polymerization products in native PAGE. By setting positive and negative controls, whether the polymerization reaction occurs can be verified. In comparison with traditional analytical methods of nucleic acids, the non-radioactive method is simple, efficient, cost-effective, and user-friendly, which is suitable for our analysis.

Minimal primer length required for primer extension

Our results showed that KF polymerase requires a primer of at least seven nucleotides long (heptamer) for the one-direction primer extension of the T/P duplexes, whereas it needs a primer of at least six nucleotides long (hexamer) for the two-direction primer extension of the S/n duplexes (**Fig. 2**), suggesting that the overhanging tail might affect the primer extension. *Taq* DNA polymerase requires a minimal length of primer at least eight nucleotides long (octamer) for both T/P complexes and S/n complexes.

Many DNA polymerase structures have been resolved by X-crystallographic and NMR methods [2,3,9−11]. The structural information increases our understanding about the mechanism of DNA polymerization. However, these structures are either polymerase alone or complexed with a long duplex. There is no structural information regarding how the short primers are complexed with the DNA polymerases so far. By analyzing these existing structural data, we intended to uncover the polymerization mechanism of these short primers. Accumulated structural information has revealed that the tertiary structures of DNA polymerase share a common feature: a ‘right hand’ shape with three distinct subdomains of ‘palm’, ‘thumb’, and ‘fingers’. The center of the palm subdomain is the polymerization catalytic site, the tip of the thumb subdomain holds the upstream of the primer−template duplex, and the ‘O-helix’ of the finger subdomain is responsible for transporting dNTPs to

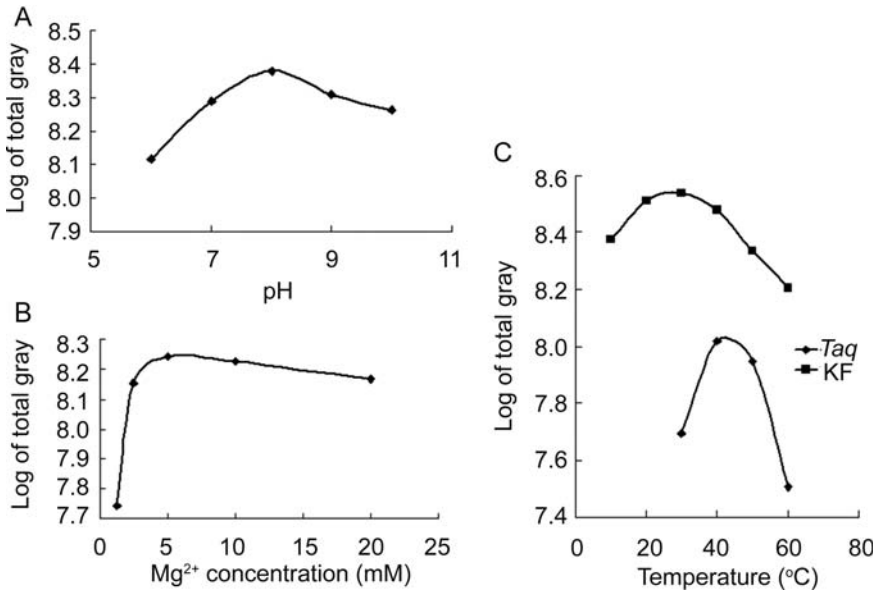

Figure 5 Effects of pH, Mg^{2+}, and temperature on short primer extension efficiency (A) The pH effect on the primer extension efficiency. Palindrome S12/6 was extended by KF. The experimental errors (SD) are <4% for each data point. (B) The Mg^{2+} effect on the primer extension efficiency. Palindrome S12/6 was extended by KF. The experimental errors (SD) are <5% for each data point. (C) Temperature effect on the primer extension efficiency. T14/P8 complex was extended by KF and *Taq* DNA polymerase, respectively. The extension reactions were carried out at different temperatures for 10 min with the KF concentration of 0.18 ng/μl and *Taq* DNA polymerase concentration of 0.24 ng/μl. The experimental errors (SD) are <2% and <4% for KF and *Taq* DNA polymerase, respectively. Log of total gray (a.u.) is an arbitrary unit used to describe log of total signal value in band analyzed by software.

the 3′-terminal of the primer. Correct localization of the 3′-terminal of the primer (designated as extension-end) to the catalytic site is the prerequisite for the polymerization reaction, and many residues of the polymerase participate in polymerization process, such as R668, Q849, and Y766 of KF [9,12–14]. At the upstream end of the primer–template duplex (designated as upstream-end), several residues on the thumb subdomain interact with the minor groove of the duplex, such as S638, S610, and S581 of KF [9]. The distance from R668 to S638 in the existing polymerase structures was measured to be 2.1–2.6 nm, which is equivalent to the helical distance ~7 bp of a B-form double-stranded DNA [10,15,16]. Previous studies using spectroscopic and footprinting methods have identified that the template–primer duplex binding site with the polymerase extends ~5–8 nucleobases upstream from the polymerase catalytic site of KF [17–19], which is consistent with our results. Based on these analyses, we hypothesized a model to interpret our observations. In order to get extended, the upstream-end of the primer–template duplex interacts with the tip of the thumb subdomain of the polymerase, and at the same time, the extension-end of the duplex has to be positioned properly at the catalytic site on the palm subdomain of KF. The duplex segment of T14/P7 complex is just long enough to locate its upstream-end and its extension-end at the tip of the thumb and the catalytic site of KF, respectively, allowing the primer to be extended smoothly [**Fig. 6(A)**]. In the case of T14/P6, when its

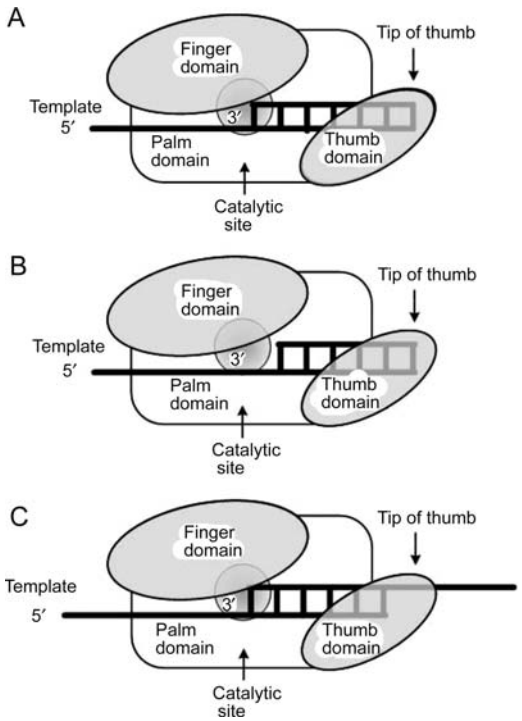

Figure 6 Illustrative picture showing the minimal primer length required for polymerization by KF (A) Nucleotide heptamer can be extended since its length matches the distance between the tip of the thumb subdomain and the catalytic site. (B) Primers shorter than heptamer is not extended since the extension-end of the primer fails to be localized in the catalytic site when its upstream-end interacts with the tip of the thumb subdomain. (C) One of the 5′-terminal overhanging tails is 'held' by the tip of the thumb subdomain, positioning the extension-end of the primer in the catalytic site.

upstream-end is positioned at the tip of the thumb subdomain, its extension-end is out of the catalytic site due to one nucleotide shorter than the required minimal length, leading to no primer extension [**Fig. 6(B)**]. These data showed the interactions between the upstream-end of the duplex and functional groups of polymerase play an important role in stabilizing this duplex–polymerase complex. Therefore, when a palindrome complex of S12/6 is lying in the crevice of the palm subdomain, its 5′-terminal overhanging tail functions as an extra arm to help its extension-end to be positioned correctly at the catalytic point, although it is just six nucleotides long [**Fig. 6(C)**].

The current information could have other implications. In all kinds of polymerase reaction techniques, false duplex formations such as hairpins, false priming, and dimers could occur more or less although sophisticated software has been used to design these primers. Our observations pinpointed that once false priming is longer than octamer, they have possibilities to hybridize with non-target sequences on the templates and to amplify unwanted extension products.

Comparison of different polymerases

Both KF and *Taq* DNA polymerases belong to Pol(A) family, showing no obvious difference in global tertiary structures [11]. Our results, however, showed that the minimal primer length is heptamer for KF and octamer for *Taq* DNA polymerase. The difference could be attributed to the intrinsic properties between KF and *Taq* DNA polymerases: KF is a regular enzyme and *Taq* is a thermo-stable enzyme. The activity of *Taq* DNA polymerase showed >10-fold reduction at room temperature in comparison with that at the optimal temperature [20]. The decreased catalytic ability of *Taq* in room temperature could be embodied as difficulty in extending a heptamer primer, which is not a problem for KF at its optimal temperature.

Interestingly, similar phenomenon was also observed for ligases, another important enzyme in the DNA replication process. The minimal length of oligonucleotides that could be linked by ligases was enzyme dependent, it was at least heptamer for *Tth* DNA ligase and hexamer for T7 DNA ligase, respectively [21].

Effect of primer length on short primer extension efficiency

When a longer primer was paired with the template T14, it could be extended more quickly by KF with the order of nonamer>octamer>hepamer (**Fig. 3**). In the case of the S/n complexes, the polymerization efficacy of KF polymerase showed similar results: D8 dimer was polymerized faster than D6 dimer (data not shown). The duplex stability, evaluated in terms of the melting temperature, is determined by its nucleotide composition and its length. A longer primer will be more stable with its template, and consequently, it will be more ready to be extended. The current results are consistent with the report that ligases catalyze the substrates of different duplex lengths with different velocities, and the shorter the duplex , the slower is the reaction [21]. This notified us that it might be a common phenomenon that the lengths of short oligonucleotides affect the activity of the polymerases.

Effect of mismatches on short primer extension efficiency

In the current study, we used the mismatched base pairs C:T and G:A to examine the mismatch effect. The data showed that the mismatch at the 3′-terminal (P8m1) had the lowest primer extension efficiency, whereas the mismatch at the 5′-terminal (P8m8) had almost the same efficiency as that of the complementary primer P8. The primer P8m5 had the primer extension efficiency similar to that of P8m1. Moreover, we have observed that higher KF concentration (12 ng/μl) was still able to extend P8m1 primer (data not shown). Previously reported results of normal PCR [22] and quantitative PCR experiments [23] showed that the mismatches at the 3′-terminal of primers could influence PCR products significantly. Another study using the modified single base extension (SBE) method demonstrated that mismatches at the last 3–4 positions from the 3′-terminal generated no or very little extension products, and the extension efficiency increased as the mismatch position shifted toward the 5′-terminal [24]. Since false priming could create unwanted products due to the non-complementary hybridization, extra caution must be taken when designing the primers to secure a high degree of fidelity of replication and the effective discrimination of mismatches.

Effects of pH, Mg^{2+}, and temperature on short primer extension efficiency

Many factors could affect the polymerase activity, such as pH, Mg^{2+} concentration, and temperature. The data showed that the optimal conditions for KF polymerase activity were pH = 7.0–8.0 and $[Mg^{2+}] = 5–10$ mM, respectively. These results are consistent with previous measurements when using long primers. Experimental evidence has clarified the catalytic mechanisms of DNA polymerases: two magnesium ions near the 3′-terminal of the primer are coordinated with phosphate groups of dNTP and ready to participate in the primer extension [3,4]. In comparison with the optimal temperatures of ~37°C for KF and ~72°C for *Taq* DNA polymerase reported previously, our polymerase activity curves against the temperature are shifted leftward. It could be attributed to the collective effect of the polymerase activity and the primer–template stability. *Taq* DNA polymerase is a thermal stable enzyme, and as the reaction temperature increases, the polymerases approach their optimal catalytic activities, while the

duplexes of primer and template tend to dissociate due to the short primer, resulting in the bell-shaped curve peaked at 30°C for KF and 45°C for *Taq* DNA polymerase, respectively.

As a summary, we employed a simple and effective non-radioactive method to analyze short primer extension reaction. It was observed that different polymerases have different requirements for the minimal primer length to initiate primer extension, and the overhanging tails also affect the polymerization efficiency. A hypothetic model was proposed to interpret our observations. The primer length, the primer mismatch, and other experimental parameters could also influence the primer extension efficiency. This information will enhance our understanding about the nature of polymerization and could be applied to optimize the primer design in isothermal PCR applications.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (No. 30470451).

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