In vitro Replication Slippage by DNA Polymerases from Thermophilic Organisms

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Replication slippage of DNA polymerases is a potential source of spontaneous genetic rearrangements in prokaryotic and eukaryotic cells. Here we show that different thermostable DNA polymerases undergo replication slippage in vitro, during single-round replication of a single-stranded DNA template carrying a hairpin structure. Low-fidelity polymerases, such as Thermus aquaticus (Taq), high-fidelity polymerases, such as Pyrococcus furiosus (Pfu) and a highly thermostable polymerase from Pyrococcus abyssi (Pyraexo) undergo slippage. Thermococcus litoralis DNA polymerase (Vent) is also able to slip; however, slippage can be inhibited when its strand-displacement activity is induced. Moreover, DNA polymerases that have a constitutive strand-displacement activity, such as Bacillus stearothermophilus DNA polymerase (Bst), do not slip. Polymerases that slip during single-round replication generate hairpin deletions during PCR amplification, with the exception of Vent polymerase because its strand-displacement activity is induced under these conditions. We show that these hairpin deletions occurring during PCR are due to replication slippage, and not to a previously proposed process involving polymerization across the hairpin base.

Keywords: thermophilic DNA polymerases; fidelity; genetic rearrangements; replication slippage; hairpin deletion

Introduction

Arrests of DNA replication machinery are frequently associated with DNA rearrangements (reviewed by Michel). Replication slippage (also known as copy-choice or primer-template misalignment) is one of the mechanisms by which these rearrangements can occur. Originally proposed to account for frameshift mutations at homopolymeric runs, polymerase slippage was also proposed to promote deletions between either short or long directly repeated sequences, in prokaryotes and eukaryotes. Direct evidence for the replication slippage between repeated sequences in Escherichia coli in vivo and of the DNA polymerase III holoenzyme in vitro has been reported. Recently, we have demonstrated that this process involves the following steps: (i) arrest of DNA synthesis within a direct repeat (DR); (ii) dissociation of the polymerase from the template; (iii) unpairing of the tip of the newly synthesized strand, and its annealing with another DR; and (iv) resumption of DNA synthesis. This leads to the formation of a heteroduplex molecule composed of a parental strand and a newly synthesized strand, which lacks one DR and the region situated between the DR. A homoduplex deletant molecule results from replication of the heteroduplex molecule. Studies of six polymerases from mesophilic eubacteria replicating a single-stranded DNA (ssDNA) template that carries a hairpin structure flanked by two DR revealed that the strand-displacement activity of a polymerase determines its capacity to slip. Polymerases that possess high strand-displacement activity can enter the
hairpin and do not slip, whereas polymerases that have no strand-displacement activity cannot enter the hairpin and thus slip.

To determine whether the above conclusions can be generalized to evolutionary distant enzymes we studied DNA polymerases of thermophilic eubacteria and archaea (reviewed by Perler et al.15). We focused on these enzymes for several reasons. First, there is a growing interest in understanding of thermophilic organisms at the molecular level, and in comparison of thermostable and thermolabile enzymes. Second, thermostable DNA polymerases are used widely for a number of applications, including DNA cloning, sequencing, labeling, mutagenesis and PCR amplification. Fidelity of these enzymes is thus a critical point that needs to be understood in terms of nucleotide mis-insertion and frameshifting, as well as in terms of their ability to promote larger rearrangements, such as deletions. It was proposed that deletions observed during PCR amplification are formed by a process termed “polymerization across”16,17 the hairpin, which is different from the replication slippage,16,17 suggesting that thermostable polymerases may have properties intrinsically different from the mesophilic ones.

Here, we studied six thermostable DNA polymerases found in thermophilic eubacteria and archaea, using two different assays that detect formation of deletions during replication. One consists of a single round of replication on a ssDNA template that carries a long hairpin flanked by two short DR. The other involves multiple rounds of replication of a similar template under PCR amplification conditions. The first assay revealed that four polymerases slip while two do not, the capacity to slip being correlated with the absence of the strand-displacement activity. The polymerases that slipped in the first assay were found to slip in the second, with the exception of the polymerase possessing the strand-displacement activity that is induced under PCR amplification conditions. Furthermore, we present data showing that formation of deletions during PCR amplification involves replication slippage and not polymerization across the hairpin as previously proposed.

Results

Single-round replication assay

To determine whether thermostable DNA polymerases undergo replication slippage, we used a primer-extension assay on a ssDNA template, where only a single round of replication takes place with most polymerases.12–14 The template contained two 27 bp DR flanking two 300 bp inverted repeats (IR) able to form a hairpin structure, and a 1.4 kb insert (Figure 1(a)). DNA synthesis was carried out in the presence of labeled dNTP and the products were analyzed by agarose gel electrophoresis followed by autoradiography. Faithful copy of the template generates a double-stranded parental molecule that migrates slowly in the gel. A slippage event generates a heteroduplex molecule, composed of a parental and a recombinant strand lacking one of the DR and the 2 kb region between them, which migrates ahead of parental molecules. Slippage efficiency is estimated by comparing the proportions of parental and heteroduplex products. Replication stalled at the base of the hairpin generates a molecule that migrates further than parental and heteroduplex molecules. Precise characterization of reaction products was done by restriction analysis, and determination of the size of different DNA fragments in sequencing gels as described.12–14

Slippage of Thermus aquaticus DNA polymerase

The thermostable polymerase from T. aquaticus, designated Taq pol herein, is widely used in molecular biology for PCR amplification. It is active at high temperatures and several parameters, such as the concentration of magnesium ions, dNTPs concentration, pH and temperature affect its fidelity.15,16 Taq pol generated three types of products in the assay described above (Figure 1(b)), the presence of the heteroduplex molecules indicating that it can slip. The proportion of heteroduplex molecules, and thus the slippage efficiency, decreased with increasing polymerase amounts (Figure 1(b), lanes 2-5). This indicates that Taq pol can progress within the hairpin when present at a high concentration, as previously observed with E. coli DNA polymerase I and III and DNA polymerase from phage T7.12,14

Magnesium concentration affects Taq pol fidelity18, which prompted us to analyze its effect on replication slippage. Two effects were detected, one on the total DNA synthesis, the other on slippage (Figure 1(c)). At very low magnesium concentrations (0.1 mM) there was essentially no synthesis (lane 2) while at the highest concentration (20 mM) the synthesis was inhibited significantly (lane 10). In contrast, slippage was inhibited at low, and stimulated at high, magnesium concentrations (lanes 3-10), which could be due to the stabilization of the hairpin structure by high magnesium concentrations.

We have shown previously that the ssDNA-binding protein (SSB) from E. coli affects slippage of different DNA polymerases on the same DNA template in a different way, indicating that it acts not only by binding to the template, but also by specific and direct interactions with polymerases.12–14 We found that SSB inhibits slippage of Taq pol (Figure 1(d); magnesium concentration that promotes the slippage was used). A similar inhibitory effect of SSB was observed for E. coli DNA polymerase I and DNA polymerase from phage T7, and was shown to result from the stimulation of the polymerase strand-displacement activity.14 We suggest, by analogy, that SSB stimulates the strand-displacement activity of Taq pol.
Slippage of Pyrococcus furiosus DNA polymerase

Fidelity, in terms of nucleotide mis-incorporation, varies significantly among thermostable DNA polymerases. *P. furiosus* polymerase (Pfu pol) has the lowest error rate in PCR-based forward-mutation assays (1.3 × 10^{-6}), compared with other commonly used thermostable polymerases, and is thus a polymerase of choice for high-fidelity amplification. We therefore decided to test the propensity of this polymerase to slip, using different magnesium, polymerase and SSB concentrations; results are shown in Figure 2. Total DNA synthesis was inhibited at very low (0.1 mM; Figure 2(a), lane 1) or high magnesium concentrations (10-15 mM; Figure 2(a), lanes 8-9), but only the heteroduplex product was detected whenever the synthesis was efficient (Figure 2(a), lanes 2-7). Formation of the heteroduplex was not prevented either by the SSB protein or the high polymerase concentrations (Figure 2(b)). We conclude that, despite its high fidelity, Pfu pol is very prone to slip on ssDNA templates upon encounter with a secondary structure.

**Strand-displacement activity of thermostable polymerases prevents slippage**

Strand-displacement activity was previously shown to determine the propensity of a polymerase to slip. Thermococcus litoralis DNA polymerase (Vent pol) possesses a temperature-inducible strand-displacement activity, absent at 50 °C but unmasked at 65 °C. We therefore tested the capacity of this polymerase to slip at the two temperatures (Figure 3(a)). Heteroduplex molecules were largely predominant at 50 °C, irrespective of magnesium concentration (lanes 1-4), which shows that Vent pol is very prone to slippage. In contrast, heteroduplex molecules were no longer present at 65 °C and low magnesium concentrations, being replaced by DNA forms that migrated more slowly than the parental molecules (lanes 9-10). This material corresponds to σ-type molecules, as deduced from restriction-fragment analysis on a sequencing gel (data not shown). These were presumably formed by rolling-circle replication, which ensues after a single round of replication of a circular template when a polymerase possesses a high enough strand-displacement activity. We conclude that Vent pol does not slip when its strand-displacement activity is unmasked. However, high magnesium concentrations suppressed formation of the σ-type molecules and restored the synthesis of heteroduplex molecules (lanes 9 and 12), indicating that the strand-displacement activity was inhibited and that slippage became possible.

Interestingly, Kong et al. described that Vent pol exon mutant enzyme has some strand-displacement activity at 65 °C, as well as at 50 °C. We therefore tested its capacity to slip at both tempera-
The above results indicate that strand-displacement activity prevents slippage of thermostable polymerases, as found previously for mesophilic polymerases. To further test this conclusion, we examined 

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To further generalize our observations, we studied two other thermostable DNA polymerases. *T. fumicolans* DNA polymerase (Tfu pol), which is active at 72°C, has a high fidelity and a very efficient 3'-5' exonuclease proofreading activity. Similarly to Bst pol, it generated very high molecular mass products and no heteroduplex molecules (Figure 4(a), lane 1), which shows that it does not slip, and suggests that it has a high strand-displacement activity. Ammonium sulfate enhances PCR efficiency of the Tfu pol and reduces its 3' exonuclease proofreading activity. Similary to Bst pol, it generated very high molecular mass products and no heteroduplex molecules (Figure 4(a), lane 1), which shows that it does not slip, and suggests that it has a high strand-displacement activity. Ammonium sulfate enhances PCR efficiency of the Tfu pol and reduces its 3' exonuclease proofreading activity.
cleave activity, and thus the fidelity, according to the commercial supplier of the enzyme. However, (NH₄)₂SO₄ did not affect the ability of the Tfu pol to generate very high molecular mass products (Figure 4(a), lanes 2-4), suggesting that it does not interfere with its strand-displacement activity.

P. abyssi DNA polymerase (Pyra® exo− pol) is one of the most thermostable DNA polymerases, active at 92 °C and devoid of proofreading activity. At 65 °C it formed heteroduplex molecules almost exclusively at all magnesium concentrations tested (Figure 4(b), lanes 1-4), which indicates that it is very prone to slip, and suggests that it has low or no strand-displacement activity; this behavior is similar to that of Pfu pol.

Hairpin deletions occur during PCR amplification

Among the six thermostable polymerases tested, four (Taq pol, Vent® pol, Pfu pol, and Pyra® exo− pol) slip and cause deletions during a single-round replication of the ssDNA template. To determine whether the thermostable polymerases also cause deletions under the PCR amplification conditions, we used the following assay: plasmid FXc (Figure 1(a)) was digested with MfeI and Stul, and the 2.6 kb DNA fragment, which carries the 27 bp DR involved in slippage and 300 bp IR that can form the hairpin structure (Figure 5(a)), was purified and used as template for PCR reactions. Two oligonucleotides (designated no. 66 and no. 243, Figure 5(a)), which flank the DR/IR region, were used as primers. Amplification without slippage should generate the full-length fragment, of 2.3 kb (the distance between the primers), whereas slippage should generate a short fragment, of 272 bp, lacking all the material between the two DR and one DR (Figure 5(a)). The two products can easily be distinguished by agarose gel electrophoresis.

PCR amplification was carried out under standard conditions with different thermostable DNA polymerases (Figure 5(b)). Bst pol was not tested in this assay (although its high strand-displacement activity would render it interesting to test in PCR), because it is not stable enough to withstand the high temperatures used during amplification. Taq pol formed only the short product (lane 3). Changes in PCR cycling program or addition of chemicals or proteins such as DMSO, bovine serum albumin, dithiothreitol, glycerol or SSB, known to enhance PCR DNA synthesis,²² did not lead to formation of the long product (not shown). As dNTP and MgCl₂ concentration affect the fidelity of Taq pol, we carried out PCR reactions at different concentration of dNTP (0.2-1.5 mM) or Mg²⁺ (1-10 mM), and obtained the short product only (data not shown). The short product was also obtained with Pfu pol, Pyra® exo− pol or a mixture of Taq pol and Pwo pol commercialized under the name of Expand High Fidelity (Figure 5(b), lanes 4-6). In contrast, Vent® pol, which possesses a high strand-displacement activity and does not slip at temperatures above 65 °C and at low magnesium concentration (see above, Figure 3(a), lanes 9-10), generated the long product rather than the short one during PCR (Figure 5(b), lane 7). Similarly, the main product generated by Tfu pol was the full-length one (Figure 5(b), lane 8), which correlates with the strong strand-displacement activity observed for this polymerase during primer extension (Figure 4(a)). Changing either the salt concentration (NH₄SO₄), or the annealing temperature led to similar results (data not shown).

We conclude that deletion of a hairpin structure can occur very efficiently during PCR amplification, except if a polymerase with high strand-displacement activity is used.

Deletions do not occur by polymerization across the hairpin during PCR

It was reported that Taq pol is able to bypass a hairpin structure during PCR by polymerization across its basis,¹⁶,¹⁷ and thus generate a deletion by a mechanism unrelated to replication slippage. Two lines of evidence argue against this possibility. First, sequence analysis of the short product obtained with Taq pol, Pfu pol, Pyra® exo− pol and Expand High Fidelity (samples from Figure 5, lanes 3-6) revealed deletion of the intervening sequence and of one DR, yielding a slippage product of 272 bp. As depicted in Figure 6, polymerization across would generate a different product, which would contain both DR. Second, polymeriz-
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Figure 5. Hairpin deletion during PCR amplification. (a) Schematic representation of the PCR reaction. The 2616 bp MfeI-Stul restriction fragment of plasmid Fx
with indication of sizes in bp) and the expected PCR products are represented. Black arrows represent the DR flanking the IR (gray arrows). Position of primers no. 66 and no. 243 (semi-arrows) is indicated. (b) Analysis of the PCR products obtained by PCR amplification with Taq pol, Pfu pol, Pyraexo pol, Expand, Vent pol and Tfu pol. Reactions were done as described in Materials and Methods in the presence of unlabeled primers (briefly: 45 seconds at 94°C; one minute at 50°C; two minutes at 72°C; 30 cycles) and analyzed on an agarose gel stained with ethidium bromide. Lane 1, size marker (1 kb DNA ladder); lane 2, 2626 bp MfeI-Stul restriction fragment of plasmid Fx used for PCR amplification; lanes 3-8, PCR products obtained on MfeI-Stul restriction fragment of plasmid Fx, with Taq pol, Pfu pol, Pyraexo pol, Expand, Vent pol and Tfu pol, respectively; lane 9, size marker (100 bp DNA ladder); lane 10, PCR product obtained with Taq pol, on MfeI-Stul restriction fragments of plasmid Fx0 that do not contain DR at the base of the hairpin. Position and size of the different products are indicated by arrows on the right of the gel.

Figure 6. Schematic representation of two mechanisms proposed for hairpin deletions: replication slippage and polymerization across the hairpin. Legend is as in Figure 5. For simplicity, amplification of only one strand of the restriction fragment is represented in the two intermediate steps. The cross indicates that product corresponding to the polymerization across mechanism was not obtained. See the text for details.
Slippage occurs efficiently during the first replication cycle of PCR

The short fragment synthesized by most thermostable polymerases corresponds to that expected from replication slippage. However, this fragment could be formed during PCR by at least two other reactions, involving heat denaturation and annealing of incompletely replicated ("truncated") products, which could arise by the arrest of the polymerase at the base of the hairpin structure (Figure 7(a)). The two possibilities can be distinguished by the analysis of the products formed in the first PCR cycle. Replication slippage would generate products extending between the primer and the end of the template (340 nt), whereas the polymerase arrest would yield products extending only to the base of the hairpin (168 nt, Figure 7(a)). The two types of product can easily be distinguished on a sequencing gel.

Taq pol was used to carry out PCR reactions on the 2.6 kb FXc MfeI-StuI fragment, in the presence of one or two of our standard primers. A single 340 nt product was obtained upon one cycle of amplification in the presence of the labeled primer no. 66 under standard conditions (Figure 7(b), lane 1). This size is expected for a product formed by replication slippage. No product corresponding to the completely replicated fragment (2353 nt) or to the polymerase arrest at the base of the hairpin (168 nt) were detected. The same result was obtained when elongation time at 72°C was increased from one to four minutes (Figure 7(b), lane 2), or when one-cycle amplifications were

![Diagram](image)

Figure 7. Replication slippage during PCR. (a) Schematic representation of the PCR reaction according to three alternative pathways. The 2616 bp MfeI-StuI restriction fragment of plasmid FXc (with indication of sizes in bp) used for amplification with primers no. 66 and no. 243 and the expected products, after the first three PCR cycles, are represented. Black arrows represent the DR flanking the hairpin. Only the primer no. 66 (semi-arrow) is labeled and is indicated by a star. Three different mechanisms leading to the same final deleted products are depicted. At each cycle, the newly synthesized strands are represented by broken lines. Numbers in parenthesis indicate the sizes of the labeled strands only, after each PCR cycle. (b) Analysis of the products after a single PCR cycle. Reactions of one PCR cycle (45 seconds at 94°C; one minute at 50°C; one to four minutes at 72°C) were done with Taq pol in the presence of either one or two primers, and analyzed on a sequencing gel. Lanes 1-2: only one primer (labeled primer no. 66) with one and four minutes of elongation at 72°C, respectively. Lane 3-4: two primers (labeled primer no. 66 and unlabeled primer no. 243) with one and four minutes of elongation at 72°C, respectively.
done with labeled primer no. 66 and non-labeled primer no. 243, using elongation times of either one minute (Figure 7(b), lane 3) or four minutes (Figure 7(b), lane 4). These results show that deletion occurs very efficiently during PCR in just a single cycle by a replication slippage mechanism.

**Discussion**

The aim of this work was to determine whether DNA polymerases from thermophilic organisms generate deletions by replication slippage. We studied slippage ability of two polymerases from thermophilic eubacteria (Taq pol and Bst pol) and four from archaea (Pfu pol, Pyra® exo pol, Vent® pol and Tfu pol), using an assay that involves a single-round replication of a ssDNA template. Among the six polymerases, four (Pfu pol, Pyra® exo pol, Taq pol and Vent® pol) were able to slip, despite very different biochemical properties and different optimal temperatures (ranging from 50 to 72°C). We were able to distinguish three polymerase classes, similar to those previously defined for thermolabile DNA polymerases.14 Pfu pol and Pyra® exo pol slip very efficiently, and thus behave like E. coli DNA polymerase II and DNA polymerase from phage T4. Bst pol and Tfu pol do not slip at all, thus resembling the DNA polymerase from phage Φ29. Finally, the slippage propensity of Taq pol and Vent® pol depends on the reaction conditions, similarly to that of E. coli DNA polymerases I and III and DNA polymerase from phage T7. These results led us to analyze the parameters that modulate slippage.

**High polymerase concentration and strand-displacement activity interfere with slippage**

The previous studies of thermolabile DNA polymerases have shown that strand-displacement activity of the polymerase interferes with slippage.14 The results presented here fit nicely with this proposal. First, Taq pol appears to possess a strand-displacement activity,23 and becomes unable to slip at high polymerase concentration (see below). Second, Vent® pol has an intrinsic temperature-dependent strand-displacement activity,20 and stimulation of this activity by increasing the temperature inhibits slippage. Finally, Bst polymerase, which is endowed with a high strand-displacement activity,21 was totally unable to slip.

We observed that Taq pol at high concentrations slips very little, as we have shown previously for DNA polymerases I and III and for DNA polymerase from phage T7.14 We propose that high concentration of these enzymes facilitates the step by step progression of the polymerase within the hairpin. This proposal is supported by our recent demonstration that the polymerase dissociates after passing at the hairpin, even in conditions where only parental molecules were synthesized.13 This suggests that progression within the hairpin is accompanied by several events of dissociation/re-association of the polymerase. Thus, an elevated concentration of polymerase will favor the probability of polymerase re-association at the 3' end of the newly synthesized strand. However, this phenomenon is limited to the DNA polymerases that possess at least some strand-displacement activity to allow elongation within double-stranded regions of DNA (for instance, slippage was not inhibited, when the amount of Vent® pol at 50°C was increased from 0.5 to two units; data not shown).

**Low magnesium concentration can inhibit slippage**

Precise adjustment of the concentration of divalent cations is a critical point for DNA synthesis. It affects not only primer annealing and duplex denaturation,24,25 but also enzyme activity and fidelity. In our system, Taq pol and Vent® pol slip efficiently at high but not at low Mg2+ concentrations. We propose that this cation affects the stability of the hairpin structure. Consequently, at low Mg2+ concentrations the two polymerases are able to progress within the hairpin, and therefore do not slip, while at high Mg2+ concentrations they are arrested by the hairpin and thus undergo slippage. However, varying magnesium concentration has no effect on the slippage of other polymerases, indicating that the putative effects on hairpin stability are not strong enough to affect them.

**High SSB concentrations can inhibit slippage**

We have previously found that E. coli SSB stimulates strand-displacement activity of DNA polymerase I and DNA polymerase from phage T7 and thus prevents slippage.14 It has been shown also that E. coli SSB increases significantly the efficiency of PCR reactions26,27 and prevents deletion formation during amplification.17 These data, together with the fact that E. coli SSB can be boiled without major loss of activity,28 indicate furthermore that E. coli SSB is functionally stable at temperatures above 60°C. Here, we show that SSB prevents slippage of Taq pol, and propose that SSB may stimulate the strand-displacement activity of this polymerase, possibly by direct protein-protein interactions. In contrast, SSB has no effect on slippage of Pfu pol, as observed for DNA polymerase II and DNA polymerase from phage T4,14 and thus appears unable to elicit any strand-displacement activity in these polymerases.

**Hairpin deletion during PCR amplification**

PCR amplification conditions differ from those used in the assay that involves a single-round replication of a ssDNA template, mainly by denaturation and annealing steps, carried out at 94°C and 50°C, respectively, and the use of a higher temperature for the elongation step, which is 72°C instead.
of 50 or 65 °C. We report that Taq pol and high-fidelity polymerases such as Pyra\textsuperscript{exo−} pol and Pfu pol or the mix Taq/Pwo pol (Expand High Fidelity) generate deletions efficiently under these conditions. Interestingly, Taq pol generates deletions during PCR amplification much more efficiently than during the single-cycle replication, irrespective of the conditions, including the presence of SSB. We speculate that deletion formation is favored during PCR amplification because the high temperature facilitates separation of the 3’ end of the newly synthesized strand from the template, which is a prerequisite for its annealing with another homologous sequence, an obligatory step of the replication slippage process. The mixture Expand High Fidelity was tested here because it has been designed by its supplier for amplification of tough templates and for accurate long-range PCR, because it combines a polymerase with high fidelity (Pwo pol) due to its 3’ → 5’ exonuclease activity, to a polymerase with high processivity (Taq pol). These polymerases are supposed to complement each other in such a way that when a mismatch has been inserted by Taq pol (which is exo\textsuperscript{−}), the polymerase dissociates allowing Pwo pol (which is exo\textsuperscript{+}) to continue, and when Pwo pol dissociates because of its lower processivity, Taq pol takes over. Other authors have used such mixtures of Taq pol (exo−)/other pol (exo+) to achieve high fidelity.\textsuperscript{29} However, these properties did not allow avoidance of the hairpin deletion in our system. On the contrary, Vent\textsuperscript{pol} pol, which forms deletions during single-cycle replication, does not do so during PCR, even at high magnesium concentrations. It is likely that the heat-inducible strand-displacement activity of Vent\textsuperscript{pol} pol and the intrinsic one of Tfu pol interfere with the replication slippage.

**Polymerization across versus replication slippage**

Hairpin deletion during PCR has been previously proposed to occur by polymerization across the base of a hairpin (Figure 6).\textsuperscript{16,17} However, our sequence analysis of the deletion products rules out this process, and shows that Taq pol, Pfu pol, Pyra\textsuperscript{exo−} pol and Expand High Fidelity form deletions by replication slippage. We suggest that the previous results can be explained in terms of replication slippage, and propose that the template used in these studies allows formation of a structure (Figure 8(a)) different from that considered in references\textsuperscript{16,17} (Figure 8(b)). The alternative structure consists of a hairpin that possesses a short GC clamp at its basis and is flanked by short DR of 3 bp (CTT). Replication slippage would generate the deletion products that were indeed detected,\textsuperscript{16,17} lacking the hairpin and one DR.

**Although PCR artifacts may arise, they do not occur when slippage is efficient**

Formation of chimeric molecules during PCR amplification has been previously observed, and several mechanisms accounting for their generation have been proposed. Incompletely extended strands produced during the elongation phase of a PCR cycle could re-anneal out of register, after the denaturation step of a subsequent PCR cycle\textsuperscript{30–32} (Figure 7(a), middle). An alternative model is the “annealing between truncated products”, also called “template switching between the two nascent strands”, which also requires a denaturation step in a second PCR cycle\textsuperscript{33} (Figure 7(a), right). In this work, we demonstrate that deletion formation is explained fully by replication slippage (Figure 7(a), left). Indeed, the deletion is generated in only one amplification cycle, and thus in the absence of a second denaturation step, which is absolutely required in the model of “re-anneal out of register”. Furthermore, formation of this deleted product did not require the presence of a second primer, contrary to the annealing between truncated products model.

In conclusion, we propose that replication slippage may occur at high temperatures, provided that the DNA sequence has a potential to form secondary structures and contains repeated sequences.
Several thermostable DNA polymerases having very different biochemical properties, and in particular very different levels of fidelity, can undergo slippage. Only the polymerases endowed with a high strand-displacement activity do not slip. Use of such polymerases should be recommended for amplification of DNA templates with potential secondary structures. These results strengthen our earlier proposal that slippage efficiency of a polymerase is inversely proportional to its strand-displacement activity, and extend the slippage reaction to the very important class of thermostable DNA polymerases.

Materials and Methods

Proteins

Taq pol was purchased from Roche Molecular Biochemicals and Promega Corporation. Pfu pol was obtained from Stratagene. Bst pol, Vent® pol and Vent® pol exo- were purchased from New England Biolabs. Pyra® exo- pol and Tfu pol were obtained from Appligene. Expand® High Fidelity (a mixture of Taq pol and Pwo pol) was purchased from Roche Molecular Biochemicals. E. coli SSB was purchased from US Biochemical Corp. Bovine serum albumin was from New England Biolabs. Proteinase K was from Roche Molecular Biochemicals. DNA polymerase exo- from phage T7 (Sequenase® Version2) was from US Biochemical Corp. Sequencing on ssDNA or double-stranded DNA templates was carried out according to the protocol of the Sequenase® Version2 Sequencing Kit (US Biochem. Corp.). For all polymerases, one unit is defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of dNTP into acid insoluble material in 30 minutes at 65°C (Bst pol), 72°C (Pfu pol), 74°C (Pyra® exo- pol and Tfu pol) or 75°C (Taq pol, Vent® pol and Vent® pol exo-).

Chemicals

\ ([x-32P]dATP (3000 Ci/mmol), [x-32P]dCTP (3000 Ci/mmol) and [γ-32P]ATP (6000 Ci/mmol) were purchased from Dupont-NEN. Unlabeled nucleotides were from Amersham Pharmacia Biotec.

ssDNA templates and primer-extension reactions

Plasmid pHP727FXc (FXc) is schematized in Figure 1(a) and has been described previously.12 Plasmid pHP727FX0 is similar to FXc, except that it lacks one DR at the base of the hairpin (20 nt were deleted at the end of the first DR of FXc and 5 nt were deleted before the second DR).

Preparation of the ssDNA templates, and the primer extension reaction have been described (see Figure 1(a) and references12–14). Briefly, a primer designated no. 1233 (24mer) was annealed 1288 bases upstream from the basis of the hairpin. All primer extension reactions contained in 10 µl: 25 ng of primed ssDNA, 200 µM dGTP and dTTP (each) and 40 µM (2.5 µCi) [x-32P]dATP and [γ-32P]dCTP. Where indicated, SSB was added to the reaction mixture and preincubated five minutes at 60°C, before DNA polymerase addition. The reaction buffers were those provided by the suppliers except that MgCl₂ or MgSO₄ concentrations were as indicated in the Figure legends. They contained, in addition to 30 mM NaCl introduced by the primed ssDNA, the following ingredients. For Taq pol: 10 mM Tris-HCl (pH 8.3) (20°C), 50 mM KCl. For Pfu pol: 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin. For Vent® pol and Vent® pol exo-: 10 mM Tris-HCl (pH 8.8; 25°C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100. For Bst pol: 20 mM Tris-HCl (pH 8.8; 25°C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100. For Pyra® exo- pol: 10 mM Tris-HCl (pH 9.0; 25°C), 50 mM KCl, 0.1% Triton X-100, 0.2 mg/ml bovine serum albumin. Reactions were carried out at 50-65°C (as indicated in the legend of the Figures) for 25 minutes. To prevent evaporation, 15 µl of mineral oil (Sigma) were added on the top of the reaction mixture. Synthesis was arrested by the addition of 25 mM EDTA and 500 µg/ml protease K and further incubation for 15 minutes at 55°C. When the reaction products were to be cleaved with restriction enzymes, proteinase K was inactivated by addition of 2 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) (ICN Biomedicals) for ten minutes at room temperature. The reaction mixtures were then dialyzed on a micromembrane (Millipore VS 0.025 µm) for 20 minutes before restriction cleavage.12 Reaction products were analyzed by electrophoresis in 0.8% agarose gels (Seakem GTG) under native conditions, run in TAE buffer (pH 8.3) at 2 V/cm for 16 hours, or through 6% acrylamide-urea sequencing gels (National Diagnostics) run in TBE buffer (90 mM Tris borate, 2 mM EDTA (pH 8.3)), at 60 W, 45 mA for two to three hours. The dried gels were exposed to a Fluor screen and analyzed on a STORM (Molecular Dynamics).

PCR amplifications

PCR amplifications were performed in 20 µl of the buffers supplied by the manufacturers (see above, with magnesium concentrations for Taq pol, Pfu pol, Pyra® exo- pol, Expand, Vent® pol and Tfu pol of 1.5, 2, 3, 1.5, 2 and 1.5 mM, respectively), in the presence of 0.5-5 units of polymerase, 200 µM each dNTP, 1 µM (20 pmol) of each primer and 1 ng (0.56 fmol) of the DNA template. For PCR with 32P-labeled primer, only 0.16 pmol of primer were used.

PCR reactions were heated five minutes at 94°C, followed by 30 cycles under the following conditions: 30 seconds at 94°C; one minute at 50°C; one to three minutes at 65-72°C. PCR reactions were performed in a Perkin-Elmer GeneAmp PCR System 9600.

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