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On-off Regulation of 3' Exonuclease Excision to DNA Polymerization by Exo+ Polymerase

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The role of 3' exonuclease excision in DNA polymerization was evaluated in primer extensions using 3' allele-specific primers that had exonuclease-digestible and exonuclease-resistant 3' termini. With exonuclease-digestible unmodified 3' mismatched primers, the exo+ polymerase yielded template-dependent products. Using exonuclease-resistant 3' mismatched primers, no primer-extended product resulted from exo+ polymerase. As a control, polymerase without proofreading activity yielded primer-dependent products from 3' mismatched primers. These data indicated that a successful removal of the mismatch is required for DNA polymerization from the 3' mismatched primers by exo+ polymerase. In addition to the well-known proofreading from this mismatch removal, the premature termination in DNA polymerization, due to the failure of the efficient removal of the mismatched nucleotides, worked as an off-switch in maintaining the high fidelity in DNA replication from exo+ polymerase.

Keywords: DNA polymerase, 3' Exonuclease, Primer extension, Proofreading, SNP

Introduction

The discovery of antimutagenic T4 polymerase mutants initiated a remarkable fertile area of research into DNA replication fidelity (Drake *et al*., 1969). Polymerases with 3' exonuclease have a proofreading function by which they maintain a high fidelity in newly-synthesized DNA. However, in a single nucleotide polymorphism (SNP) analysis, the polymerase that is used in practice is exclusively exo- polymerase instead of exo+ polymerase (Nedelcheva Kristensen *et al*., 2001; Bowtell and Sambrook 2002).

We recently suggested the potential of exo+ polymerase in a SNP analysis (Zhang and Li, 2001). In a broad range of annealing temperatures, exo+ polymerase yielded template-dependent products while exo- polymerase yielded primer-dependent products in extending the 3' terminal mismatched primers (Zhang *et al*., 2003a). In this study, we further evaluated the role of 3' exonuclease excision in DNA polymerization from 3' terminal mismatched primers by comparing the extension from mismatched primers with exonuclease-digestible and exonuclease-resistant 3' termini.

The application of exonuclease-resistant primers turned off DNA polymerization from 3' mismatched primers by exo+ polymerase, which strongly supports the crucial role of a successful mismatch removal in DNA polymerization from 3' mismatched primers by exo+ polymerase. In addition to the well-known proofreading from the mismatch removal, the premature termination in DNA polymerization, due to the failure of the efficient removal of mismatched nucleotides, worked as an off-switch to maintain the high fidelity in DNA replication from exo+ polymerase. Furthermore, our data also suggested that the interventions that interfere with the 3' exonuclease excision could be used in the development of assays for single base discrimination.

Materials and Methods

The amplicon set that was used in this study was from Genomapping Inc. (Tianjin, China), which included one template, two sense primers, and one antisense primer. The template of this amplicon had the following sequences: 5'atcccaagatatctgagaattgagc agccttccatttagaagggtgttgttgtctctgaggcaaaaccacatttcttaccgcacaactaga gactgagaccagtttctctcattgtcattgctgctcagagccagcagaaaagcactcatgacaca cacttagaataatagtgcatctgagccaggactgcccttggggtccattcagctgtttc3'. The two sense primers had the identical sequence of 5'-atcccaagatatctga gaattc-3', which is 3' terminal mismatched to the template

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Table 1. Components of the amplicon and products amplified by polymerases with or without 3' exonuclease activity

	Amplicon	by exo- pol	by exo+ pol
SNP template	3'…cttaac…5'		
Unmodified primer	5'…gaattc3'	Extended *Eco*RI digestible	Extended *Eco*RI resistant
PTO-modified primer	5'…gaattc3'	Extended	No products

PTO, phosphorothioate. The "off switch" on DNA polymerization was due to the failure of 3' exonuclease excision to the mismatched nucleotides.

(…gaattg…). One of the sense primers was unmodified and another sense primers had a 3' terminal phosphorothioate modification (Table 1). The antisense primer had the sequences of 5'-cagtctctagttgtgcggtaa gaaat-3' without a specific modification. Two types of DNA-dependent DNA polymerases, Deep vent and Deep Vent-, were purchased from New England Biolab Inc. (Beverly, USA). Deep Vent is the wild type that contains a strong 3' to 5' exonuclease activity. Deep Vent- is the form with a point mutation that resulted in the loss of proofreading function.

Two-directional primer extensions were carried out at three different annealing temperatures, 49.2, 54.4, and 60.6ºC. Following denaturation at 95ºC for 2 min, the primer extension was cycled 30 sec for denaturation at 95ºC, 30 sec for annealing, and 30 sec for extension at 72ºC for 30 cycles. After the 30 cycles, an extra extension cycle of 2 min was done before the reactions were cooled down to 4ºC. The primer extension condition is detailed elsewhere (Zhang *et al*., 2003a). The PCR products were purified using a Qiagen (Hiden, Germany) PCR purification column according to the manufacturers protocol. A 2.5% agarose EtBr gel electrophoresis, running under 10 V/cm in a 0.5x TBE buffer, was used to check whether the primer extended products were *Eco*RI digestible. The primer-extended products were further confirmed by a sequencing analysis.

Results

Template-dependent products yielded from successful mismatch removal As shown in Fig. 1, there were different products from the same primers, depending on the polymerases that were used. With amplicon having a mismatch between the template and 3' terminal of the unmodified primers, exo- polymerase yielded primer-dependent products at the three annealing temperatures, keeping the wild-type sequences of the *Eco*RI site from primers. When amplified with exo+ polymerase, there was a yield of template-dependent products. The *Eco*RI restrictive enzyme digestion and sequencing analysis confirmed that the wild-type sequences of the *Eco*RI site from primers were integrated into the primer-extended products by exo- polymerase; whereas the products by exo+ polymerase had sequences that were identical to the template.

DNA polymerization turned off due to the failure of the mismatch removal Phosphorothioate-modification renders exonuclease-resistant property to oligonucleotide, which is widely employed in antisense technologies (Li and Zhang, 2001). In this study, the 3' phosphorothioate-modified primer was applied to inversely determine the role of the successful mismatch excision by 3' exonuclease in DNA polymerization from mismatched primers.

Similar to the results with unmodified primers, exo- polymerase yielded primer-dependent products from the 3' phosphorothioate-modified mismatched primers at the three annealing temperatures. A breakthrough phenomenon occurred in the proofreading phosphorothioate-modified primer-3'-termini when there was a mismatch between primer-3'-termini and the templates. There were no primer-extended products from the mismatched primers by exo+ polymerase (Fig. 2). Exo+ polymerase, together with 3' phosphorothioate-modified mismatched primers, worked as an off-switch in the DNA polymerization. The result of the off-switch was largely due to the exonuclease-resistant property of the phosphorothioate-modification that blocked the mismatch excision process.

Discussion

In evaluating the potential of exo+ polymerase in the SNP analysis, we identified the crucial role of the successful 3' exonuclease excision in DNA polymerization from the mismatched primers using exo+ polymerase. In addition to the well-known proofreading function, 3' exonuclease actually plays a dual role in maintaining fidelity of DNA polymerization. A successful mismatch excision is required for polymerization from the mismatched primers; whereas a failure in the mismatch excision turns off DNA polymerization. The biological implication of a premature termination from the failure of the mismatch excision is that this mechanism might be attributed to fidelity maintenance in matured products in DNA replication.

For a DNA dependent polymerase with a proofreading function, whether DNA polymerization occurs mainly depends on the process of mismatch excision by 3' exonuclease. When the mismatch is removed by 3' exonuclease, then DNA polymerization turns to the "on status" with the yield of template-dependent products. On the other hand, if the mismatched nucleotide is not excised, then the DNA polymerization stays at the "off status", leading to a premature termination. Premature termination works as a way

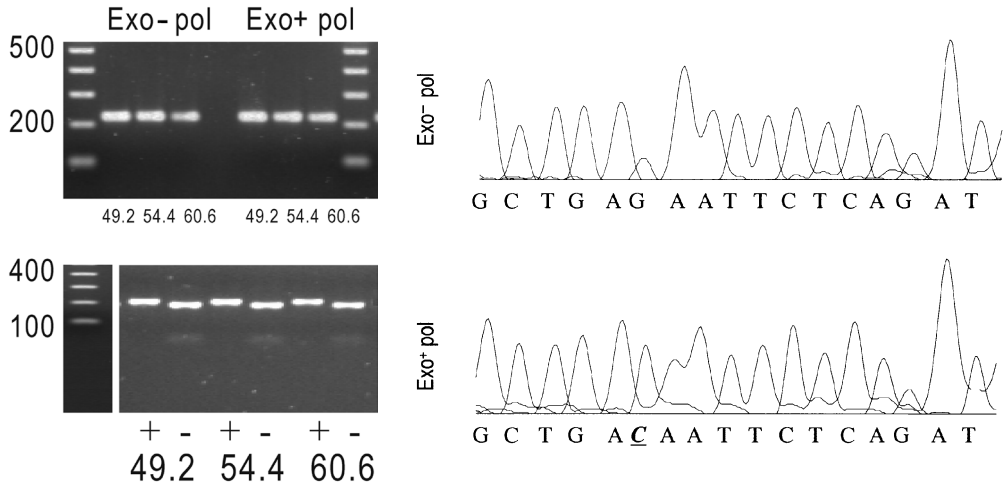

Fig. 1. Efficient extension of 3' mismatched primers by polymerases with and without 3' exonuclease activity. Left upper panel: Exo+ and exo- polymerases both yielded products from 3' mismatched primers at three different annealing temperatures. Left bottom panel: Primer-extended products were then digested with *Eco*RI, where + represents products from exo+ polymerase and - represents products from exo- polymerase. Sequencing analysis showed that primer-extended products by exo- polymerase inherited the 3' terminal nucleotide from primers, whereas products amplified by exo+ polymerase edited the 3' terminal nucleotides from primers.

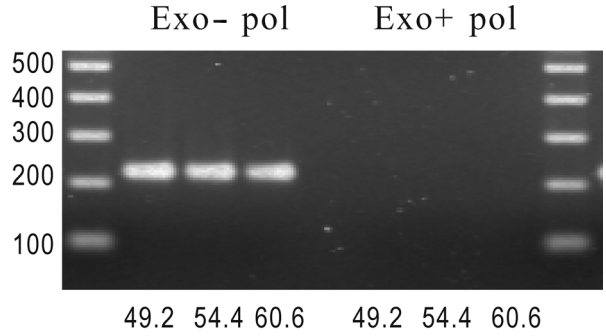

Fig. 2. Representatives showing the effect of the off-switch operated by base-pairing status between phosphorothioate-modified primer-3'-termini and their templates. Without the proofreading function, exo- polymerase yielded products from 3' phosphorothioate-modified 3' mismatched primers.

to minimize mutagenesis in matured polymerization products.

The application of 3' phosphorothioate-modified primers further confirmed the role of the successful excision of the mismatched nucleotide at the exo site by 3' exonuclease in DNA polymerization from the mismatched primers. No products resulted from the mismatched primer with the 3' phosphorothioate modification by exo+ polymerase; this was reasonable as the 3' phosphorothioate-modification blocked the exonuclease function. For exo- polymerase, since no proofreading process was involved in the DNA polymerization, then whether the mismatched primers were extended was largely dependent on the mismatchs influence on the thermodynamics of annealing/melting of the primers (Goodman and Fygenson, 1998). The ability to yield products from the mismatched primers by exo- polymerase could be the molecular basis for false positives in the SNP assay with the conventional allele-specific primer extension (Kwok *et al*., 1990; Huang *et al*., 1992; Zhang *et al*., 2003a).

SNP is the most frequent genetic marker. Efficient screening of SNP will facilitate the individualized medicine as well as fundamental research in biomedicine including a better understanding of both monogenetic and polygenetic diseases (Salisbury *et al*., 2003; Zhang *et al*., 2003b). For example, genetic polymorphism analysis has revealed more molecular machnisms related to the development of hypertension (Morshed *et al*., 2002).

In conclusion, the present data illustrated that 3' exonuclease plays an important role in maintaining the high fidelity of exo+ polymerization by executing the process of mismatch excision. When the 3' exonuclease excision is initiated, then fidelity is ensured as removal of the mismatch that yielded template-dependent products and a failure in the mismatch excision caused a premature termination in DNA polymerization. From a technical point of view, we believe that the “on/off mechanism” renders exo+ polymerase as an enzyme with great potential for the development of SNP assays, especially with the combination of exonuclease-resistant modifications on the primer-3'-termini.

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