Amplimers with 3'-Terminal Phosphorothioate Linkages Resist Degradation by Vent Polymerase and Reduce *Taq* Polymerase Mispriming

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The 3'→5' exonuclease activity of Vent, a thermostable polymerase from *Thermococcus litoralis*, enhances DNA replication fidelity but also diverts PCR primers (amplimers) from targeted amplification by degrading their 3' termini. We demonstrate that amplimers with a 3-base 3'-terminal mismatch can be efficiently truncated by Vent to prime DNA polymerizations that compete with the specific amplification reaction. However, amplimers with phosphorothioate bonds joining their 3'-terminal residues are resistant to degradation and demonstrate greatly enhanced priming specificity. Slight destabilization of base-pairing by phosphorothioate bond-linked residues also diminishes extension of mispaired 3' amplimer termini in *Taq* polymerase-mediated amplifications.

Many applications of the PCR demand that amplifications be performed under conditions most likely to yield faithfully replicated DNA products. Fidelity is most important when individual amplification product molecules become the focus of study. Examples of such applications include characterization of nucleic acid population composition,⁽¹⁻³⁾ site-directed mutagenesis,⁽⁴⁾ and cloning of genetic elements for expression or further characterization.⁽⁵⁾ Vent, a heat-stable polymerase from *Thermococcus litoralis*, with 3'→5' exonuclease activity, has been found in partial heteroduplex gap filling and denaturing gradient gel electrophoresis (DGGE) assays to have higher fidelity than other nonproofreading, heat-stable polymerases,⁽⁶⁻⁸⁾ suggesting that it may be well suited for use in PCR requiring accurate DNA replication. Although the 3'→5' exonuclease activity of Vent appears to contribute to its enhanced fidelity, it may also have the undesired capacity to degrade amplimers.

3'→5' Exonuclease-mediated proofreading relies on removal of mismatched nucleotides from the 3' terminus of the priming strand until polymerization can be initiated from an annealed end (reviewed in ref. 9). Under conditions in which deoxyribonucleotide triphosphates (dNTPs) are depleted or absent, the exonuclease may continue to degrade the primer beyond the mismatch.⁽¹⁰⁾ Thus, exonuclease function can, through enzymatic primer modification, diminish the specificity of primer-directed DNA synthesis in that

the spectrum of putative target sequences is enhanced beyond constraints imposed by sequence identity of the amplimers' 3' termini. The extent to which oligonucleotides can be enzymatically adapted to prime from targets with 3' mismatches may depend on a number of factors, including input DNA complexity, annealing temperature, and amplimer composition, reaction parameters that are often dictated by the nature of the target sequences.

The experiments described here demonstrate that amplimers can bind non-targeted sites and, despite 3' terminal mismatches, efficiently prime DNA synthesis by Vent DNA polymerase due to this enzyme's ability to excise almost quantitatively such unpaired sequences prior to polymerization. This problem can be overcome by using amplimers with 3' terminal phosphorothioate diester linkages, which not only retard amplimer degradation but also appear to diminish mispriming due to extension of 3' mismatched amplimers.

MATERIALS AND METHODS

DNA Amplification Targets

DNA amplification target pCH110RX-FeLV is a modified version of pCH110 (Pharmacia). The pCH110 *EcoRI* site at bp 4487 was cleaved, the 5' overhangs were filled in with the Klenow fragment of *E. coli* polymerase I, and the resulting blunt termini were ligated. The region between the *EcoRI* site at bp 3286 and the *BamHI* site at bp 3736 was replaced

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by sequences from feline leukemia virus (FeLV) proviral clone FeLV-F6A⁽¹¹⁾ from the *Bgl*II site at bp 1106 to an *Eco*RI site introduced by site-directed mutagenesis at bp 3900. pCH110RX-FeLV was linearized and mixed with feline genomic DNA (1 pg of target/ μ g genomic DNA, approximately 1 copy/haploid genome). An additional DNA amplification target was genomic DNA from uninfected and FeLV-F6A-infected feline T cells (FeT cells).⁽¹²⁾

Amplimers

All amplimers used in these experiments were synthesized on an Applied Biosystems Model 940 DNA synthesizer using version 1.01 software and reagents purchased from Applied Biosystems. Standard, nonsulfurized oligonucleotides were generated in 0.2 μ M syntheses. Sulfurized oligonucleotides were synthesized as follows: The sulfurized portions of the oligonucleotides were generated using a 0.2 μ M sulfurization synthesis program with tetraethylthiuram disulfide in acetonitrile replacing the standard iodine-based oxidant. The dimethoxytrityl was left in place and the ammonium hydroxide cleavage was omitted. The tetraethylthiuram disulfide oxidant was then replaced with the iodine solution and a new synthesis begun with the last addition of the previous synthesis designated as the column-bound nucleotide in the synthesis instructions. The amplimers used were: Pol-1, 5'-GGAATTCGAAAGGACATTGGGTCGCGATTGC-3'; Z-lac-1, 5'-GATAACGAGCTCCTGCACTGGATG-3'; Int-1, 5'-GATGAGAATTCAGGATTTGCGAAGG-3'; Pol-2A, 5'-GTAGGGTAGGGTAGACATCGGTTAAGG-3'.

Amplimers Pol-1 and Z-lac-1 bind to opposite strands within the FeLV and lacZ regions, respectively, of pCH110RX-FeLV and amplify a 2.5-kb segment composed of 1 kb of lacZ sequence and 1.5 kb of FeLV sequence (Fig. 1A). Z-lac-1 is also internally homologous to an 11-bp segment of the pCH110RX-FeLV FeLV sequence antisense strand that excludes the amplimer's 3'-most 3 nucleotides as well as its 5'-most 10 (Fig. 1B). Amplimers Int-1 and Pol-2A are designed to specifically amplify a 2.2-kb region of exogenous FeLV spanning sequences in both the polymerase and envelope genes (bp 3900–6130). Amplimers synthesized with phosphorothioate linkages joining

FIGURE 1 Alteration of amplimer specificity by Vent 3'→5' exonuclease activity. (A) Amplimers Pol-1(0S) and Z-lac-1(0S) were used to amplify pCH110RX-FeLV target sequences in the presence of feline genomic DNA. The same amplification was mediated by *Taq* polymerase (lane 1) or by Vent polymerase (lane 2). A 1-kb ladder (BRL, Inc.) was electrophoresed in lane M as a molecular-weight standard. (B) The 771-bp anomalous Vent product was cloned into pUC19 and its sequence was characterized. Analysis revealed that the 771-bp fragment is the product of mispriming within FeLV sequences, as indicated in the schematic diagram of pCH110RX-FeLV. The alignment indicates, within the boxed region, identity between the Z-lac-1 primer and the corresponding strands of pCH110RX-FeLV and cloned anomalous amplification products. Limited sequence divergence between pCH110RX-FeLV and the second and third PCR clones suggests that they may have been amplified from endogenous proviruses present in the feline genomic DNA.

3'-terminal residues are designated with the number of these linkages and the letter S in parentheses following the designation given above [e.g., Pol-1(2S)].

DNA Amplification

Both *Taq* polymerase and Vent polymerase DNA amplifications, unless otherwise specified, were carried out in 50- μ l reactions with buffer composed of 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM magnesium chloride, and 0.1% Triton X-100 with 200 μ M concentrations of each dNTP, 140 nM concentrations of each amplimer, and 50 ng of DNA. *Taq* reactions were carried out with 1.25 units of enzyme while Vent reactions used 1.0 unit and were supplemented with 5 μ g of acetylated bovine serum albumin. The reactions were set up on ice, transferred to a Perkin-Elmer Cetus DNA Thermal Cycler preheated to 94°C, incubated for 3 min, and cycled 35 times as follows: 94°C for 45 sec, 56°C for 1 min, and 72°C for 2 min. The last cycle was followed by a 10-min extension at 72°C.

DNA Sequence Analysis

All sequences analyzed were cloned as *Eco*RI–*Sac*I fragments into the corresponding sites of the pUC-19 polylinker.

Double-stranded dideoxy sequencing, using the reverse sequencing primer and [³⁵S]dATP, was employed.^(13,14)

RESULTS

Generation of an Anomalous Amplification Product with Vent but Not *Taq* Polymerase

Amplification of template pCH110RX-FeLV with *Taq* polymerase and amplimers Pol-1 and Z-lac-1 yielded a 2.5-kb product composed of 1 kb of lacZ sequence and 1.5 kb of FeLV sequence (Fig. 1A). The same amplimer set when used with Vent polymerase yielded a small quantity of the targeted 2.5-kb product and a large quantity of a 0.8-kb product (Fig. 1A). This anomalous species was also observed when the annealing temperature was raised from 56°C to 62°C in an attempt to enhance amplimer annealing specificity (data not shown). The same product was present in amplifications from a plasmid bearing only proviral FeLV DNA and in amplifications from feline genomic DNA (which contains endogenous FeLV sequences) (data not shown), suggesting priming within FeLV sequences as a source of the 0.8-kb species.

The 0.8-kb products were cloned, and sequence analysis of several clones re-

vealed that their 3' termini were complementary to the 5' terminal 21 nucleotides, but not the 3' terminal 3 nucleotides of the 24 nucleotide Z-lac-1 ampimer (Fig. 1B). The sequence of this product indicated that mispriming was made possible because of removal of the 3' terminal 3 nucleotides of the ampimers, most likely due to the 3'→5' exonuclease activity of Vent. Limited divergence among the clones sequenced and the ability to amplify the anomalous product from feline genomic DNA suggests that both endogenous proviruses and pCH110RX-FeLV served as amplification templates for the anomalous product (Fig. 1B).

Reduction of 3'→5' Exonuclease Truncation of Ampimers by 3' Sulfurization

The large quantity of anomalous product synthesized in the Pol-1/Z-lac-1-directed Vent amplification from pCH110RX-FeLV indicated that 3'→5' exonuclease excision is a highly efficient reaction that can almost quantitatively remove stretches of at least 3 3'-terminal mismatched nucleotides and thereby facilitate alternative ampimer extensions that compete with the specific amplification reaction. To inhibit 3'→5' exonuclease excision of ampimer termini and yet retain the option of cleaving within ampimer encoded restriction sites, 3'-sulfurized oligonucleotides were employed as ampimers. Ampimers Z-lac1(0S), (1S), (2S), (3S), (4S), and Pol-1(0S) were used in a series of amplification reactions to determine how many consecutive phosphorothioate linkages are required at the 3' end to inhibit the observed exonuclease activity and to determine the effect of using these modified oligonucleotides on the overall reaction yield. The results shown in Figure 2 indicate that, for the conditions used, one phosphorothioate linkage only slightly inhibits generation of the anomalous product whereas two phosphorothioate linkages limit its production to levels that are not detectable on an ethidium bromide-stained gel (at least 150- to 300-fold). Incorporation of additional phosphorothioate linkages beyond two depressed specific product yield. Otherwise, as the amount of anomalous product diminished, the specific yield increased.

FIGURE 2 Resistance of sulfurized ampimers to degradation by Vent 3'→5' exonuclease. Ampimer Pol-1(0S) was used in combination with ampimer Z-lac-1(0S), Z-lac-1(1S), Z-lac-1(2S), Z-lac-1(3S), or Z-lac-1(4S) to amplify the pCH110RX-FeLV template in the presence of feline genomic DNA. The products were electrophoresed in sample lanes 1–5, respectively.

Maintenance of Sequence Identity across Partially Sulfurized Ampimers

To determine the utility of sulfurized ampimers for protocols in which sequence identity must be maintained across the ampimers, their function as polymerase templates was evaluated. DNA amplified with ampimers Pol-1(0S) and Z-lac-1(2S) was cloned into pUC-19 and the 3' termini of 20 inserts were sequenced to determine whether the ampimers' phosphorothioate linkages resulted in base misincorporation. No changes were observed in the ampimer region (data not shown).

Effect of dNTP Concentration on Ampimer Degradation

The equilibrium between polymerization and exonuclease activity is regulated by the available dNTP concentration and may influence ampimer degradation as well as proofreading.⁽¹⁵⁾ To investigate the relationship between ampimer degradation and dNTP concentration, oligonucleotide sets Pol-1(4S)/Z-lac-1(0S), Pol-1(4S)/Z-lac-1(1S), and Pol-1(4S)/Z-lac-1(2S) were used to prime amplification reactions with 100, 200, 300, 400, 500, 600, 700, and 800 μ M concentrations of each dNTP in reaction

buffer with 2.5 mM magnesium chloride. The results indicate that degradation of the Z-lac-1(0S) and Z-lac-1(1S) ampimers can be reduced as dNTP concentrations are increased to a level at which the dNTPs reduce free magnesium to concentrations at which the enzyme can no longer function (Fig. 3A, 3B). Ampimer Z-lac-1(2S) showed no evidence of degradation at the dNTP concentrations tested, although here again the enzyme activity was diminished at elevated dNTP concentrations (Fig. 3C).

Enhanced Specificity of *Taq* polymerase-mediated Amplification Using Sulfurized Ampimers

PCR with *Taq* polymerase amplified the desired 2.5-kb target as well as small amounts of an approximately 800-bp species (Fig. 4A). Because this amplification by-product was close in molecular weight to the anomalous Vent product and was absent from *Taq* amplifications primed by the Pol-1(4S)/Z-lac-1(2S) and Pol-1(0S)/Z-lac-1(2S) ampimer sets, its sequence was characterized to determine whether ampimer specificity had also been altered by exonuclease cleavage. The DNA from this band was cloned and sequenced. Analysis of seven products revealed that each was primed at a unique position and that the ampimers' 3' termini remained intact (Fig. 4B). None of the sequenced amplification products was homologous to pCH110RX-FeLV, and the products were therefore probably amplified from the feline genomic DNA that was also included in the reactions. These results indicate that the 800-bp products resulted from extension of intact, mismatched ampimer termini, and that the mispriming by *Taq* is decreased when 3'-sulfurized ampimers are used.

Utilization of Sulfurized Ampimers by *Taq* and Vent

To examine the benefits of using 3'→5' exonuclease-resistant sulfurized ampimers in standard Vent polymerase-mediated DNA amplifications, dually sulfurized and nonsulfurized versions of ampimers Int-1 and Pol-2A were synthesized. The nonsulfurized ampimer set Int-1(0S)/Pol-2A(0S), unlike Pol-1(0S)/Z-lac-1(0S), primarily directs amplification of targeted sequences (Fig. 5A). Ampimer titration using Vent and both sul-

FIGURE 3 Regulation of 3'→5' exonuclease activity by changes in dNTP concentration. The pCH110RX-FeLV template was amplified in the presence of feline genomic DNA using amplimer Pol-1(4S) in conjunction with either Z-lac-1(0S) (A), Z-lac-1(1S) (B), or Z-lac-1(2S) (C) in the presence of 100, 200, 300, 400, 500, 600, 700, or 800 μ M dNTPs (lanes 1–8, respectively) in buffer containing 2.5 mM magnesium.

furized and nonsulfurized Int-1 and Pol-2A sets at 7, 14, 21, 28, 35, or 42 nM concentrations revealed that lower quantities of the sulfurized than nonsulfurized amplimers are required for detection of amplification product (Fig. 5A). The converse was true for *Taq* polymerase-mediated amplifications in which lower concentrations of Int-1(0S)/Pol-2A(0S) than Int-1(2S)/Pol-2A(2S) were

required to generate detectable yields (Fig. 5B).

DISCUSSION

Applications of the PCR in which individual product molecules are the focus of study require that amplifications be performed under conditions most likely to yield faithfully replicated DNA. Such

applications include investigation of nucleic acid population composition,^(1–3) site-directed mutagenesis,⁽⁴⁾ and the cloning and analysis of genetic elements.⁽⁵⁾ The utility of PCR in such applications has been limited by the relatively lower fidelity of commonly used thermostable polymerases. The advent of heat-stable polymerases with proof-reading activity, such as Vent, has expanded the means by which to enhance PCR fidelity. Comparison of error rates for Vent⁽⁶⁾ and *Taq*⁽¹⁶⁾ indicates that the former has approximately five-fold better fidelity than the latter. Yet, the same 3'→5' exonuclease activity that contributes to the superior fidelity of Vent polymerase can, as illustrated here, greatly diminish the enzyme's utility under many amplification conditions by altering amplimer specificity to allow amplification of nontargeted sequences. This feature of Vent would be especially detrimental in applications that rely upon the necessary match of an amplimer's 3'-terminus with the targeted template to amplify selectively subsets of closely related sequences.^(17, 18) Exonuclease modification of amplimers could, on the other hand, be exploited if inclusion of degenerate sequences is desired.

The extent to which amplimers are degraded by Vent 3'→5' exonuclease ac-

FIGURE 4 Reduction of *Taq* polymerase mispriming by 3' sulfurized amplimers. (A) *Taq* polymerase was used to amplify the pCH110RX-FeLV template in the presence of feline genomic DNA using amplimers Pol-1(0S) and Z-lac-1(0S) (lane 1) or amplimers Pol-1(0S) and Z-lac-1(2S) (lane 2). (B) The band in lane 1, migrating at approximately 800 bp, was cloned into pUC-19, and the sequence directly adjacent to the Z-lac-1 amplimer was characterized. Seven clones, designated MP-1 through MP-7, were characterized.

FIGURE 5 Titration of sulfurized and nonsulfurized amplimers in Vent- and *Taq*-mediated DNA amplifications. Amplification of a 2.2-kb FeLV sequence was carried out with either Vent (A) or *Taq* (B) with 7, 14, 21, 28, 35, or 42 nM concentrations of amplimers Int-1 and Pol-2A (lanes 1–6, respectively). Amplifications were directed by either Int-1(0S) and Pol-2A(0S) (*top*) or Int-1(2S) and Pol-2A(2S) (*bottom*).

tivity is at least in part influenced by template complexity and identity, which will determine the number of potential illegitimate amplimer binding sites, and by dNTP concentrations, which will determine the equilibrium between exonuclease and polymerase activity. In PCRs differential temperature optima for exonuclease and polymerase activity⁽¹⁹⁾ may also contribute to amplimer degradation.

Partial sulfurization of amplimers can, as shown here, inhibit their degradation. A single phosphorothioate linkage between the ultimate and penultimate 3' terminal residues, while significantly retarding degradation, does not abolish it as effectively as phosphorothioate linkages 3' of both the antepenultimate and penultimate residues. Additional sulfurizations beyond two reduce amplification product yield, most likely because phosphorothioate linkages tend to destabilize base-pairing.^(20,21) Destabilization by 3'-terminal phosphorothioate linkages may also enhance binding specificity of the amplimers' 3' termini as suggested by the abolition of misprimed amplification products in the *Taq*-mediated reaction shown in Figure 4.

While elevated dNTP concentrations shift the reaction equilibrium slightly to-

ward polymerization at the expense of exonuclease activity (Fig. 3), this is not an adequate solution to the amplimer degradation problem. First, because dNTP concentration and free magnesium concentration are directly linked, dNTP concentrations that can be achieved in a given reaction are limited by the amount of free magnesium necessary for enzyme function, as evidenced by the decreased amplification product yield at increased dNTP levels in Figure 3. Second, degradation of nonsulfurized amplimer Z-lac-1(0S) was never completely ablated over the dNTP concentration range that permitted polymerase function. The third and most important consideration in attempting to abolish amplimer degradation by manipulating dNTP levels is that the 3'-terminal excision is a function of the 3'→5' exonuclease-mediated proofreading activity that enhances Vent polymerase fidelity. Diminution of this activity may impair proofreading. Finally, high dNTP concentrations may also decrease polymerase fidelity by facilitating extension of mismatched termini.

Partial rather than complete sulfurization of amplimers is advantageous because restriction sites within the phosphodiester bond-linked residues can be recognized efficiently and cleaved by

corresponding endonucleases. Enzymatic recognition also extends to the polymerase for which these oligonucleotides appear to serve well as templates for accurate DNA replication.

The differential amplimer concentration requirements for *Taq* and Vent indicated in Figure 5 suggest that Vent may be diverting quantities of unsulfurized amplimer from the specific amplification reaction. The fate of diverted amplimers is difficult to determine because they can either be truncated to a length where they will no longer efficiently anneal to potential templates, be truncated and extended, or extended without truncation in conventional mispriming events.

The data presented here demonstrate that while 3'→5' exonuclease activity can be a hindrance to efficient specific DNA amplification, its activity can be diverted from amplimer degradation and restricted to proofreading through the use of 3'-sulfurized amplification amplimers.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Awards AI25273 and AI85007. We thank T.A. Reinhart for helpful discussion.

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Received July 15, 1992; accepted in revised form September 10, 1992.

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Genome Res. 1992 2: 131-136

Access the most recent version at doi:[10.1101/gr.2.2.131](https://doi.org/10.1101/gr.2.2.131)

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