Nucleic Acids Research, Vol. 20, No. 14 3551–3554

Phosphorothioate primers improve the amplification of DNA sequences by DNA polymerases with proofreading activity

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Received June 15, 1992; Accepted June 25, 1992

ABSTRACT

Two thermostable DNA polymerases with proofreading activity—*Vent* DNA polymerase and *Pfu* DNA polymerase—have attracted recent attention, mainly because of their enhanced fidelities during amplification of DNA sequences by the polymerase chain reaction. A severe disadvantage for their practical application, however, results from the observation that due to their 3′ to 5′ exonuclease activities these enzymes degrade the oligodeoxynucleotides serving as primers for the DNA synthesis. It is demonstrated that this exonucleolytic attack on the primer molecules can be efficiently prevented by the introduction of single phosphorothioate bonds at their 3′ termini. This strategy, which can be easily accomplished using routine DNA synthesis methodology, may open the way to a widespread use of these novel enzymes in the polymerase chain reaction.

INTRODUCTION

Apart from its application in the detection and analysis of novel nucleic acid sequences the polymerase chain reaction (PCR) is gaining extended use during the routine manipulation of already well characterized DNA sequences (for a recent review see ref. 1). In particular in the field of protein engineering PCR frequently replaces previously established techniques for the cloning and subcloning of structural genes, the construction of expression plasmids, and the introduction of site-specific mutations. However, it has been recognized that the occurence of random mutations during DNA amplification caused by the limited fidelity of *Taq* DNA polymerase—up to now the enzyme almost exclusively used for PCR—constitutes a general problem.

Recently, two novel thermostable DNA polymerases were introduced apart from *Thermus aquaticus* DNA polymerase: *Vent* DNA polymerase from *Thermococcus litoralis* manufactured by New England Biolabs (2) and *Pfu* DNA Polymerase from *Pyrococcus furiosus* manufactured by Stratagene (3). Both enzymes have been reported to exhibit 3′ to 5′ exonuclease or proofreading activity resulting in a significantly increased base substitution fidelity compared to *Taq* polymerase, an enzyme not displaying this property. In addition, it was shown that—in contrast to *Taq* DNA polymerase—*Vent* DNA polymerase generates PCR products with blunt ends, thus permitting their direct cloning into a blunt-cut vector without the need for further modification steps (4).

Although these properties make the practical application of both thermostable proofreading DNA polymerases highly desirable, published investigations which make use of these novel molecular biology reagents have been rare so far. This is probably due to the observation that the two enzymes show a marked dependence on the design of the primer oligodeoxynucleotides used for the PCR reaction, both in terms of yield and specificity of the product. This behaviour of *Pfu* DNA polymerase and *Vent* DNA polymerase represents a strong contrast to *Taq* DNA polymerase, an enzyme which leads to consistent amplification results over a wide range of experimental setups, even under non-optimized standard reaction conditions.

Observations made in the author's laboratory during the use of *Pfu* DNA polymerase and *Vent* DNA polymerase with different combinations of template nucleic acids and primer oligodeoxynucleotides lead, together with a technical note on *Vent* DNA polymerase released by the manufacturer (2), to the following hypothesis for the explanation of the experimental observations.

The thermostable proofreading DNA polymerases act on single-stranded primer molecules present in the reaction solution, degrading them from the 3′ terminus and leaving a limit 5′ residual product of approximately 15 nucleotides in length (2). If still complementary to the template, these shortened primer molecules in principle are able to anneal to the template, at least at lower temperatures, though with reduced specificity. If, however, one of the primer oligodeoxynucleotides was designed such that only its 3′ terminus matches to the template but its 5′ terminal sequence does not—which is frequently the case when introducing novel restriction sites for cloning purposes or when even changing the nucleic acid sequences flanking, e. g., the coding region of a gene—the degraded primer does not give rise to a PCR product at all.

Therefore, a much better performance of the thermostable proofreading DNA polymerases was to be expected if a way was found to prevent the oligodeoxynucleotide primers from this exonucleolytic attack. As will be demonstrated here, this protection can be achieved simply by the introduction of a single phosphorothioate bond during synthesis of the oligodeoxynucleotide, leading indeed to the anticipated positive

effect on the specific and efficient amplification of DNA sequences with *Pfu* DNA polymerase and *Vent* DNA polymerase.

MATERIALS AND METHODS

The primer oligodeoxynucleotides were synthesized on a model 392A-05 Applied Biosystems automated DNA synthesizer using standard phosphoamidite solid phase chemistry (5). Phosphorothioate bonds were selectively introduced by oxidizing the intermediary phosphite triester with either tetraethylthiuram disulfide (6) supplied by Applied Biosystems or the Beaucage thiolating reagent (7) purchased from Pharmacia. Both reagents were found to perform similarly well. However, care had to be taken that the thio-oxidation was complete because otherwise a mixture of two reaction products, one with the phosphorothioate bond and one with the usual phosphodiester bond, was obtained. Suitable synthesis cycle programs will be made available from the author upon request. The oligodeoxynucleotides were purified by denaturing polyacrylamide gel electrophoresis and finally quantified by UV absorption at 260 nm (cf. ref. 8).

The polymerase chain reactions were carried out in a total volume of 50 µl with 5 µl 10× reaction buffer, 4 µl dNTP solution containing 2.5 mM of each dATP, dCTP, dGTP, dTTP (Pharmacia), 2.5 µl of each primer at a concentration of 10 µM, and approximately 100 pg pASK30 supercoiled plasmid DNA (9) as the template. The reagents were mixed, overlaid with paraffin oil, and heated for approximately 2 min at 94°C. Thermocycling was started after addition of the DNA polymerase. The following reaction buffers and amounts of the different enzymes were used:

i) 0.5 µl *Taq* DNA polymerase (5 u/µl; Perkin Elmer Cetus) with a standard 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 0.1% gelatin, and 1% Triton X-100);

ii) 1 µl *Pfu* DNA Polymerase (2.5 u/µl; Stratagene) with the 10× reaction buffer # 1 (200 mM Tris-HCl pH 8.2, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1% Triton X-100, 100 ng/µl BSA) supplied by the manufacturer of the DNA polymerase;

iii) 1 µl *Vent* DNA Polymerase (1 u/µl; New England Biolabs) with the 10× standard reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton X-100) supplied by the manufacturer of the DNA polymerase.

Irrespective of the DNA polymerase, the PCR amplification was carried out using 25 thermocycles with 60 s at 94°C (strand separation), 60 s at 55°C (annealing), and 90 s at 72°C (second strand synthesis), followed by a final incubation at 60°C for 5 min. In the case of *Vent* DNA polymerase, which was used at lower activity, 1 ng template DNA was amplified with 20 thermocycles and an annealing temperature of 65°C. After addition of loading buffer, 10 µl of each of the resulting solutions was directly applied to a 1% agarose gel containing 1µg/ml ethidium bromide in TBE buffer (8).

RESULTS AND DISCUSSION

Starting from the assumptions outlined above it was sought to prevent the 3′ terminal hydrolysis of the PCR primers by introducing a single phosphorothioate bond at the first 3′ terminal internucleotide linkage during synthesis of the oligodeoxynucleotide. The phosphorothioate bond was described to be a much less favoured substrate to nuclease activity than the naturally occuring phosphodiester bond (10). In order to test whether this chemical modification can improve the PCR reaction with thermostable proofreading DNA polymerases the following experimental setup was used.

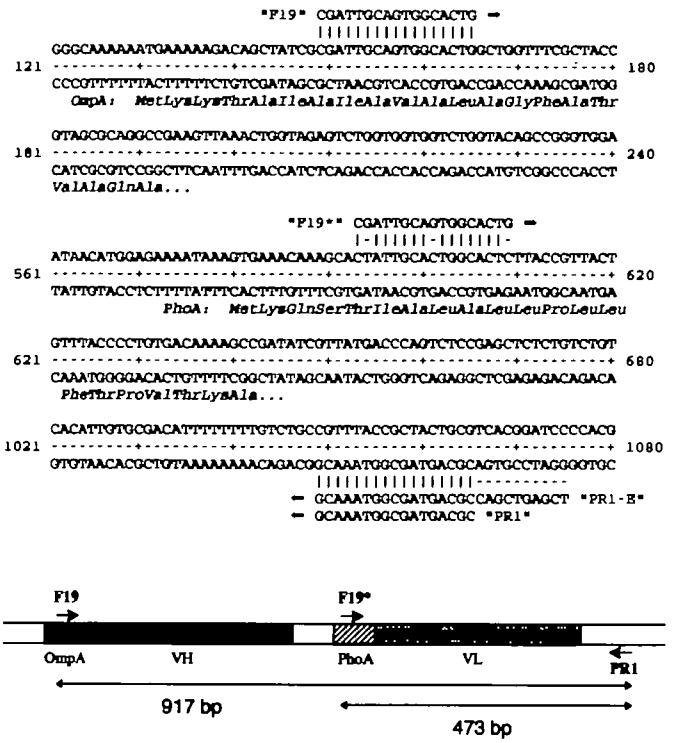

Figure 1. Nucleotide sequences from plasmid pASK30 with matching regions for the primers 'F19', 'PR1', and 'PR1-E' (numbering according to ref. 9). The original priming site for 'F19' lies within the coding sequence for the *Omp*A signal peptide giving rise to a PCR product of 917 base pairs in length with 'PR1' and of 927 base pairs in length with 'PR1-E', respectively. The secondary priming site for 'F19' (marked by a *) with three mismatches is situated in the coding region of the *Pho*A signal peptide leading to a PCR product of 473 base pairs in length with 'PR1'. The formation of the two differently sized amplification products is schematically shown below.

The plasmid pASK30 (9), a vector designed for the functional expression of an antibody F_v fragment in *E. coli*, was chosen as the template nucleic acid. This plasmid codes for the V_H domain and the V_L domain of the myeloma protein McPC603 on a single operon, both fused to bacterial signal sequences, *Omp*A in the first and *Pho*A in the latter case. The primers used in the PCR were originally designed as primers for dideoxy sequencing (11), 'PR1' as an upstream primer matching in the region of the transcription terminator and 'F19' as a downstream primer matching within the coding region for the *Omp*A signal peptide (figure 1).

These primer molecules represent oligodeoxynucleotides perfectly complementary to the template and with lengths close to the lower limit as a substrate for the 3′ to 5′ exonuclease activity expected. Furthermore, 'PR1-E' was used with a ten nucleotide 5′ terminal extension in comparison to 'PR1', which includes a *Pvu*II restriction site and provides no additional complementarity to the template (see figure 1). This oligodeoxynucleotide ought to serve as an example of a primer with the practical properties discussed in the introduction.

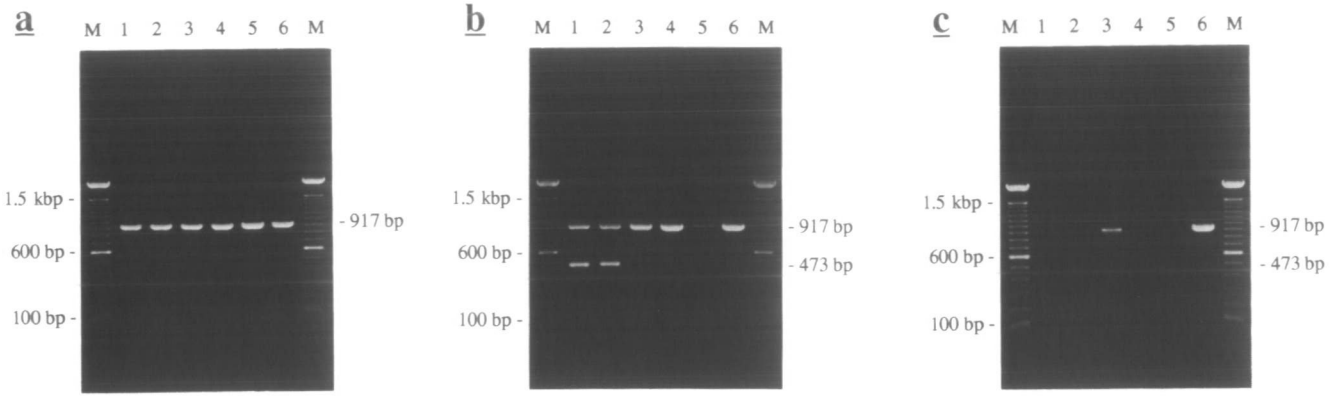

Figure 2. a) PCR reactions were performed using *Taq* DNA polymerase with pASK30 plasmid DNA as the template and the following primer combinations: 'PR1' and 'F19' (lane 1); 'PR1-T' and 'F19' (lane 2); 'PR1-T' and 'F19-T' (lane 3); 'PR1' and 'F19-T' (lane 4); 'PR1-E' and 'F19-T' (lane 5); 'PR1-ET' and 'F19-T' (lane 6). Lane M shows a 100 base pair ladder (Gibco BRL) as the size standard. b) Same as in a) except that *Pfu* DNA Polymerase was used for each PCR reaction. c) Same as in a) except that *Vent* DNA Polymerase was used for each PCR reaction.

Figure 1 also shows a secondary priming site for 'F19' situated in the coding region for the *Pho*A signal peptide with three mismatches, one involving the 3′ terminal base. Priming at this site was neither observed during dideoxy sequencing with T7 DNA polymerase (not shown) nor during PCR with *Taq* polymerase (see below). All three primers were synthesized according to standard phosphoamidite solid phase chemistry (5). In addition, analogues of these oligodeoxynucleotides with identical nucleotide sequences were synthesized, carrying single phosphorothioate bonds at their 3′ termini and named 'PR1-T', 'F19-T', and 'PR1-ET', respectively (cf. Materials and Methods).

The results from polymerase chain reactions performed with these six primers, pASK30 as the template, and *Pfu* DNA polymerase or *Vent* DNA polymerase are shown in figure 2. In addition, the PCR reactions carried out with *Taq* DNA polymerase instead of the thermostable proofreading DNA polymerases are shown as a control (fig. 2a). In this case, for each primer combination only the expected full length product (917 bp or 927 bp) is observed in a homogenous form and with reproducible yield, thus demonstrating that the effects described below are not due to intrinsic properties of the primer molecules themselves.

The results obtained with *Pfu* DNA polymerase are quite different (fig. 2b). If the 'thio' primer 'F19-T' is used in combination with the 'thio' primers 'PR1-T' or 'PR1-ET' (lanes 3 and 6) a single PCR product with the expected length of 917 or 927 base pairs, respectively, (cf. fig. 1) and with optimum yield is observed. With the 'non-thio' primer 'PR1' instead of 'PR1-T' a comparable result is obtained (lane 4). If, however, 'PR1-ET' is replaced by 'PR1-E', the amount of the PCR product considerably drops (lane 5), thus demonstrating an effect of the protection against exonucleolytic attack on the yield of the polymerase chain reaction for this primer.

Another phenomenon is observed if the 'non-thio' primer 'F19' is used instead of 'F19-T', either in combination with 'PR1' or 'PR1-T' (lanes 1 and 2). Under these circumstances the yield of the expected full length PCR product is slightly decreased and a second band of much smaller size appears with strong intensity on the agarose gel. If a derivative of pASK30 was used as the template DNA where the coding region for the *Pho*A signal peptide was missing, a corresponding side product was not observed (data not shown). Therefore, this product most likely corresponds to the PCR product with an expected size of 473 base pairs caused by the second priming site for 'F19' located downstream to the original matching region (fig. 1). Since it is known that primers with a 3′ terminal mismatch provide poor substrates for DNA polymerases (1), the occurance of this non-specific side product can only be explained by the 3′ to 5′ exonuclease activity of the *Pfu* DNA polymerase. It was already described before that *Pfu* DNA polymerase is able to edit the mismatched 3′ end of a primer molecule (3). Sequence analysis revealed that there is no secondary priming site for 'PR1' with comparable quality which explains the absence of a similar phenomenon whether this oligodeoxynucleotide carries a phosphorothioate bond or not.

Figure 2c shows the analogous amplification experiments carried out with *Vent* DNA polymerase. Here the effect observed with the phosphorothioate primers is even more drastic. Only in those cases where both primers are protected against 3′ terminal degradation—either 'F19-T' and 'PR1-T' (lane 3) or 'F19-T' and 'PR1-ET' (lane 6)—the expected full length PCR product is observed at all. If 'PR1-T' is used in conjunction with the 'non-thio' primer 'F19', a trace amount of the shorter PCR product is detected (lane 2), probably caused by the secondary priming site for the latter primer as described above. None of the other primer combinations gives rise to a major amplification product, suggesting that the 3′ to 5′ exonuclease activity for single stranded primer oligodeoxynucleotides exhibited by *Vent* DNA polymerase is significantly more pronounced than it is the case for *Pfu* DNA polymerase.

The results described here clearly demonstrate that the proofreading activity of thermostable DNA polymerases can severely impair with the correct functioning of primers in the amplification of a DNA sequence. This may be the case either by lowering the yield of the PCR product, in some cases even down to no PCR product at all, or by causing non-specific side products resulting from 3′ terminal editing of the primer molecule. Both effects can be completely avoided by the introduction of a single phosphorothioate bond at the very 3′ terminus of the primer which seems to effectively protect the oligodeoxynucleotide from 3′ terminal exonuleolytic attack. This inhibitory effect could not be a priori expected for *Pfu* DNA

polymerase and *Vent* DNA polymerase since earlier studies lead to an inconclusive description regarding the resistance of phosphorothioate linkages towards 3′ to 5′ exonuclease activities (10). So it was reported that the exonuclease activity of *E. coli* DNA polymerase I is inhibited whereas that of T4 DNA polymerase is not.

The strategy outlined in this contribution has already been successfully used during several cloning experiments in the author's laboratory (Skerra, A. et al., to be published), suggesting that the single phosphorothioate bonds introduced into the PCR products neither interfere with restriction enzyme digest (the restriction sites being embedded in the primer sequences) nor with cloning of the nucleic acid itself. In fact, significantly improved amplification efficiencies were obtained routinely both with *Vent* DNA polymerase and with *Pfu* DNA polymerase when using phosphorothioate primer oligodeoxynucleotide analogues, in the case of the latter enzyme essentially matching the performance of *Taq* DNA polymerase. It is therefore believed that this methodology will help the more widespread use of thermostable DNA polymerases with proofreading activity in the amplification of DNA sequences, giving broad access to their enhanced properties compared to *Taq* DNA polymerase.

ACKNOWLEDGEMENTS

The author wishes to thank Christina Wardenberg for technical assistance and Hartmut Michel for continuous encouragement. This work was supported by the Deutsche Forschungsgemeinschaft.

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