wax for several reasons. First, wax layers of <20 μL often leak while 30 μL of wax results in approximately a 2 mm space between the upper and lower aqueous reagents. Second, the temperature at which mixing occurs depends on both the melting point of the wax and the amount of wax present. We have found that the upper aqueous layer does not enter the bottom layer until the tube has reached >80°C. We determined when the upper aqueous layer mixed with the bottom aqueous layer by withdrawing tubes from the PCR machine at various temperatures and visually determining when the bright red upper aqueous phase entered and mixed with the bottom aqueous layer.

Thus, while higher melting point paraffin waxes are available, their use is not necessary to hot-start RT-PCR at temperatures above a reasonable annealing temperature (generally 55°–65°C). In addition, higher melting point waxes are more difficult to apply to tubes and are much harder to puncture than the 54°–61°C paraffin wax used in this study. We found that the method described here reduces false priming (primer dimers and laddering) with several primer sets used by our group.

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Robert J. Klebe, Sylvia A. Rodriguez, Melissa L. VerBeek and Troy A. Giambernardi
*University of Texas
Health Science Center
San Antonio, TX, USA*

Prevention of Unexpectedly Long PCR Products Primed at Short Inverted Repeats

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Linear amplification (primer extension) is widely used for direct cycle sequencing of PCR-amplified DNA fragments (9), structural studies of DNA, its susceptibility to chemicals and UV light and protein-DNA interactions (2, 5, 7). A number of problems are associated with the formation of secondary structures in polymerization reactions. Hairpin formation in DNA templates often causes premature termination of DNA synthesis, which gives rise to shorter than expected products (3, 8).

Here, we report that in certain cases, the linear amplification of DNA fragments results in a correct full-length product and another product with a length that implies a longer than expected template DNA. We identified the long PCR by-products and the way to inhibit their formation to simplify the data interpretation for DNA heteroduplex and triple-stranded structures (which may give rise to DNA polymerization products of variable length, depending on the heteroduplex strand composition or premature chain termination within triple-stranded structures). Understanding the nature of the secondary structure involved is significant because short inverted repeats that initiate PCR by-products occur once in several hundred base pairs in prokaryotic and eukaryotic genomes (6).

We have routinely used primer extension on short chemically modified DNA templates obtained by cutting pUC derivative plasmids with *PvuII* (2). With a standard M13 Reverse (M13R) 24-mer primer, we consistently obtained polynucleotides with the size longer than the template length, in addition to the correct full-length products. The unexpectedly long products were obtained in both sequencing and primer extension reactions, using either pUC8 or pUC19 as parental plasmids, and with different DNA polymerases (*Taq*, *KlenTaq1*, *Pfu* and a Stoffel fragment of *Taq* DNA polymerase). Figure 1A

Benchmarks

Table 1. Relative Amounts of Full-Length Products as a Function of the 3' Terminal Hairpin Length and Thermodynamic Stability^a

Hairpin Length	Free Energy (kcal/mol)	Long Product	Correct Product
4 bp	0.9	12.4%	87.6%
5 bp	0.4	17.9%	82.1%
6 bp	-0.5	50.4%	49.6%
7 bp	-1.9	57.9%	42.1%

^aPrimer extension products were synthesized and quantified as indicated in the Figure 1 legend. Free energies of the hairpins at 50 mM monovalent salt, 4 mM Mg²⁺ and 55°C were calculated using the MFOLD program, which is freely accessible at the DNA mfold server at Washington University School of Medicine (St. Louis, MO, USA) (<http://mfold2.wustl.edu/~mfold/dna/form1.cgi>), and published thermodynamic parameters (4).

shows that sequencing the *PvuII*-*PvuII* fragment of pUC8 resulted in two full-length (major termination) products. The 122 nucleotide (nt) sequence be-

yond the expected termination site contains a partial copy of the template DNA strand and spans from 43 nt 5' of the right-hand *PvuII* site to the M13R

hybridization site (Figure 1B). Synthesis of the longer sequence is a result of the presence of an inverted repeat (IR), with one-half at the six terminal bases at the *PvuII* site and the other half at the complementary sequence 43 nt away from the fragment end.

During PCR amplification with only one primer, the nascent strands are not used in subsequent rounds of reaction, and they can fold into an intramolecular stem/loop or hairpin structure. The hairpin then primes the DNA synthesis on the remaining 5' part of the nascent strand that becomes the template (Figure 1C). A stable hairpin recruits the DNA polymerase that additionally stabilizes the priming structure and elongates the M13R-*PvuII* fragment as far as the template permits, that is, to the 5' end of the M13R primer. By varying

Figure 1. (A) Sequence of the *PvuII*-*PvuII* fragment of plasmid pUC8 (lanes GATC) determined with *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA) and M13R primer that reads past the *PvuII* site. Primer extension (lane PE) on the same template results in the two full-length products terminated at the *PvuII* site and at the second stop. For sequencing, 0.25 µg of *PvuII*-digested plasmid was mixed with 2.5 U of *Taq* DNA polymerase in PCR buffer containing 45 nM of ³²P-labeled primer, 10 µM dNTPs and ddNTP. The PCR protocol consisted of 20 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 1 min). The primer extension reaction contained 2.5 U of a Stoffel fragment of AmpliTaq® DNA polymerase, 0.125 pmol DNA template, 16 nM of labeled primer and 0.5 mM dNTPs in 25 µL of 10 mM Tris-HCl, pH 8.3, 50 mM KCl. The PCR protocol consisted of 10 cycles (96°C for 1 min, 55°C for 30 s, 72°C for 3 min). The products of sequencing and primer extension reactions were resolved on a 7% denaturing polyacrylamide gel in TBE buffer and analyzed on a PhosphorImager® using ImageQuant™ 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA). Percentages of correctly and incorrectly terminated primer extension products listed in Table 1 were calculated from intensities of radioactive spots designated “stop at *PvuII*” and “second stop” relative to the total radioactivity in the lane. (B) The sequence of the longer than expected product. Primer M13R at the 5' end is indicated with lowercase letters (5'-agcggataacaatttcacacagga-3'). Two hexanucleotide parts of an inverted repeat separated by 43 nt are indicated by the arrows. The sequence from M13R to the left-hand half of the inverted repeat is complementary to the 122 extra nucleotides which therefore repeat part of the template DNA strand. Primers 5'-GGCGAAAGGGGGATGTGCTGCAAG-3', 5'-TGGCGAAAGGGGGATGTGCTGCAAG-3' and 5'-CCTGGCGAAAGGGGGATGTGCTGCAAG-3' at the *PvuII* site were used to make a series of templates capable of forming 4, 5 and 7 bp hairpins, respectively. (C) In each PCR cycle, DNA polymerase extends the M13R primer until the growing chain is terminated at the *PvuII* site. At the same time, the 3' end of a nascent strand synthesized in a previous PCR cycle may form a different priming structure to produce a longer than expected PCR product as shown in panel B.

Benchmarks

the primer's sequence at the distal half of the IR, we made a series of templates that were capable of forming 4–7 bp terminal hairpins. Our quantification of the longer than expected products (Table 1) shows that 6 and 7 bp hairpins (that gave rise to approximately equal amounts of correct and longer nascent chains) were significantly more effective priming structures than the shorter hairpins. The 4 and 5 bp hairpins initiated 12% and 18% of the long termination products, respectively. Table 1 also shows that the priming efficiency of the hairpins (the amount of the long termination product) grows with the increase of their thermodynamic stability (decreasing free energy).

The intramolecularly templated synthesis of PCR by-products is not unique to the *PvuII* site. In pUC plasmids, the restriction digestion at the *NdeI* site leaves one-half of an imperfect IR region at the 3' terminal 8 nt that may pair with the 5' located sequence 15 bases away. Priming from this hairpin results in an accumulation of longer than expected products (48%). At another site in pUC plasmids where strand folding is possible, there is an imperfect IR with the halves at the *SfoI* and near the *PvuI* sites. In this case, a 6 bp hairpin forms with an internal mismatch. Primer extension past the expected *SfoI* site results in approximately 10% of the longer products. In human, yeast and *Escherichia coli* genomes, IRs with the halves longer than 8 bp and separated by a central spacer of 3–6 bp occur about once in 5000 bp (6). Shorter (6–7 bp) IRs with other spacer lengths may be several times more frequent (i.e., one in several hundred bp). Thus, one may expect several IRs in DNA of a usual plasmid size (3–5 kb). Indeed, in pUC plasmid series, there are at least three repeats in which 6 bp halves are separated by several to several dozen base pairs. To develop new cloning vectors, certain parts, especially polylinkers and surrounding sequences, are often carried over from existing plasmids to the new constructs. At least one of the described IRs at the *PvuII* site is present in other cloning vectors, for example, phagemid vector pBluescript® II SK(+/-) (Stratagene, La Jolla, CA, USA) and shuttle vector pRS303. Because of their frequent occurrence in

the genomes, IRs may also happen at the end of a PCR amplified fragment. If a boundary of an IR coincides with the 5' end of the template (whether it is generated by a restriction digest or by PCR amplification), the 3' end of the nascent strand is likely to initiate synthesis of the by-products.

To inhibit hairpin formation and therefore the synthesis of elongated polynucleotides, we tested elevated primer annealing temperatures and the addition of the secondary structure destabilizing agents, dimethyl sulfoxide (DMSO) and betaine (1). Increasing the PCR annealing temperature from 55°C to 60°C (which is 7°C below the predicted primer's melting point) decreases the amount of long polynucleotides that are synthesized in primer extension reaction at 70°C from 50% to 33%. DMSO at 9% concentration has a relatively weak effect, and 2 M betaine has a significantly stronger destabilizing effect on a hairpin-initiated polymerization. Combining the destabilizing effects of an elevated temperature (60°C) and 2 M betaine reduces the amount of undesired long products to a negligible level (2%).

Hairpin formation in DNA templates often causes premature termination of DNA synthesis, which gives rise to the shorter than expected products (3,8). In our work, another important problem has been the synthesis of longer than expected polynucleotides that are primed by the IR structures at the 3' ends of nascent strands. IRs, with terminal 6 or more nucleotides that frequently occur in prokaryotic and eukaryotic genomes, may form strong priming structures for such an intramolecularly templated DNA synthesis even at elevated temperatures of PCR. If the hairpin forming potential of the DNA fragment cannot be reduced by an alternative restriction digestion or primer design, the formation of reaction by-products may be greatly inhibited by increasing the primer annealing temperatures and the addition of hairpin destabilizing agents.

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Vladimir N. Potaman
Texas A&M University System
Health Science Center
Houston, TX, USA