PCR-based site-directed mutagenesis using primers with mismatched 3′-ends

Michael Nassal* and Andrea Rieger
Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

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The polymerase chain reaction (PCR) with Taq DNA polymerase, now widely used for amplification of specific nucleic acid fragments, is also employed in modifiying the sequence to be amplified by using primers which contain extra nucleotides at their 5′-ends and/or create internal mismatches upon hybridization to the cloned target sequence (1). The polymerase, lacking a 3′-5′-exonuclease activity, is not capable of proof-reading (2). Efficient extension of primers with a mismatched 3′-end would therefore not be expected. We found, however, that 30 thermocycles under our standard PCR conditions with a pair of primers, one of which (28 nt long) contained 2 internal nt exchanges and a mismatched 3′-end, i.e. either a T-, A- or G-residue (primers 1-T, 1-A and 1-G) opposite of the G-residue in the target sequence, still yielded microgram amounts of amplified product. The result of four parallel amplification reactions including a control with an otherwise identical primer having a matching 3′-end (primer 1-C) is shown in Fig. 1. Primers 1-C and 1-T produced similar amounts of amplified product of the expected size (658 bp), which became clearly visible after 20 cycles (lanes 1-C b and 1-T b). Less, but after 30 cycles easily detectable amounts of product were formed by primers 1-G (lane 1-G c) and 1-A (lane 1-A c). After cloning, sequence analaysis of five independent isolates from each amplification reaction showed all isolates to contain the nt specified by the 3′-terminal residue of the corresponding upstream primer in addition to the two internal nt-exchanges, thereby confirming the absence of a 3′-5′-exonuclease activity from Taq polymerase. The efficiency of amplification was clearly dependent on the nature of the mismatch (C-G = T-G > G-G > A-G; see Fig. 1; cf. ref. 3). However, a few molecules faithfully extended beyond the mismatched 3′-end of the primer will become perfect templates for subsequent cycles and, by the very nature of PCR, eventually yield sufficient material for further manipulations. Our data therefore suggest that the versatility of PCR-based site-directed mutagenesis, with DNA-polymerases lacking a proof-reading function, may be generally extended to include the use of primers having a nonmatched 3′-end.

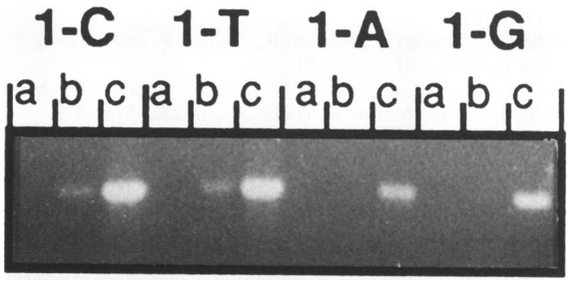

Figure 1. Gel electrophoretic analysis of amplified products obtained from PCR using primers with different 3′-ends. 5 μl aliquots of four parallel reactions with upstream primer 1-C (3′-end matching the target sequence), or primer 1-T, 1-A or 1-G generating the corresponding mismatches with the G-residue in the target sequence were analysed on a 1% agarose gel after 11 (lanes a), 20 (lanes b) or 30 (lanes c) thermocycles. DNA was stained with ethidium bromide.

METHODS

Oligonucleotides were prepared on an Applied Biosystems 380B DNA synthesizer ('trityl off') and used directly for PCR after desalting on Sep-Pak cartridges (4). Plasmid pMH190 (Niepmann,M, and Schaller,H., unpublished results) is a derivative of pMH9/3091 (5), and harbours an overlength hepatitis B virus (HBV) genome (6) in which the nt at positions 3126 and 3127 (7) have been altered to generate a ClaI recognition site within the preC region. The modified primers correspond to nt positions 3115 to 3142 of HBV, and differ only by the nt at their 3′-end:

primer: 5′ GCC TAA TC**A** TCG AT**T** GTT **AA**T GTC CTA N 3′
target sequence: 3′...CGG ATT AGT AGC TAA CAA AAT CAG GAT GAC...5′

The bold-face A residues indicate the two internal nt exchanges (originally T at both positions), and the underlined sequence the ClaI restriction site; N represents either the matching nt C (primer 1-C), or T, A, or G (primers 1-T, 1-A and 1-G). The second primer used for all amplifications was complementary to nt positions 561 to 578 of HBV and carried recognition sites for EcoRI and BamHI at its 5′-end. PCR reactions contained, in a total volume of 100 μl, 10 fmol of plasmid pMH190, 50 pmol each of the two primers, 20 mM Tris/Cl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of total dNTP, 0.1% gelatine, and 5 U of Taq-polymerase (Perkin Elmer/Cetus, Norwalk, CT). Thermocycling parameters employed were 30 s at 94°C, 30 s at 37°C and 60 s at 72°C for 30 cycles. 5 μl aliquots from each reaction were removed after 11, 20 or 30 cycles and analysed by electrophoresis on an agarose gel (Fig. 1). The amplified fragments were purified on low-gelling-temperature agarose (FMC, Rockland, ME), digested with ClaI (within the sequence derived from the upstream

* To whom correspondence should be addressed

primer) and MroI (at nt position 429 in HBV; both enzymes from Boehringer, Mannheim, FRG). After purification on agarose, the ClaI-MroI fragments were cloned by standard techniques into plasmid pMH190 which had been cut with the same enzymes. Sequence analysis was performed on plasmid DNA using Sequenase (USB, Cleveland, Ohio) and a primer complementary to nt positions 85 to 102 of HBV.

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