PCR-Mediated Mutagenesis in Sequences Recalcitrant to Homogeneous Amplification

The incorporation of mutagenic oligomers during DNA amplification by the polymerase chain reaction (PCR) has become a widely used method for introducing site-specific mutations into target DNA (6,9). In practice, we have found that DNA segments, which contain repeated elements, may remain very difficult to mutate due to in vitro recombination events that can result in very heterogeneous populations of products. For example, using standard protocols such as the “megaprimer” method (7), attempts to introduce deletion mutations (50–100 bp) into repetitive regions in the 5′ external transcribed spacer of Schizosaccharomyces pombe ribosomal DNA (Figure 1A), always resulted in broad streaks (Figure 1B). All standard modifications to increase the specificity of the reaction by alterations in annealing temperature (37°–65°C, 5° increments), annealing times (1-, 15- and 2-min increments), amount of primer (10–100 ng), amount of template (1–50 ng), magnesium chloride concentration (0.25–3 mM, 0.25 increments) and cycle number (20–35) provided little improvement. Hot start PCR (1) and touchdown PCR (2), methods that have been designed to improve the specificity of the reaction, also gave the same unsatisfactory results. We also examined overlap extension (3), a method which like the megaprimer method was initially designed to embed mutations within a DNA sequence by PCR amplification. This method has been shown to be a more efficient technique for generating large deletion mutations (>200 bp) (8). However, as found with the megaprimer method, the first PCR amplifications of the overlapping fragments were successful, but the extension step again yielded an extremely heterogeneous mixture of products (Figure 1C). Apparently, alternate pairing between the complementary strands continued to provide heterogeneous initiations for DNA replication, which resulted in highly recombined populations of replicated molecules.

Because we believed that the observed product heterogeneity was likely the result of recombination between complementary sequences in the opposite strand, we decided to eliminate these competing factors using an overlap extension strategy with only one set of complementary single strands (Figure 2A). Using methods previously developed for DNA sequencing by chemical degradation (5), the products of the first PCR amplifications were strand-separated and gel-purified (Figure 2B). Alternative methods of strand separation such as the application of biotin/streptavidin (4) can be substituted if desirable. The isolated complementary strands were then annealed, extended by Taq DNA polymerase and then the template was PCR-amplified with normal primers to yield the desired 500-bp product. As shown by the example in Figure 2C, this approach has been successfully applied to a number of difficult mutations, yielding very satisfactory results with only a single major mutant product. In each case, the muta-
Benchmarks

Figure 2. Homogeneous DNA amplification using PCR-mediated mutagenesis by single-strand overlap extension (SSOE). (A) Schematic of the SSOE method. Step I: Two separate reactions initially are performed with oligomers 1 and 2 or 3 and 4. Step II: The products from Step I are strand-separated, fractionated by gel electrophoresis and eluted by gel homogenization (B) before the complementary strands are annealed and extended at 72°C for 5 min using Taq DNA polymerase. To form the complementary hybrid, one strand from the first reaction is hybridized separately with each strand from the other reaction overnight at 37°C in 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, containing 0.1% Triton® X-100. Step III: The template generated in Step II is PCR-amplified. Since complementary strands are not identified in advance, both hybrid reactions are amplified, with only one resulting in product of the desired size. Normal and mutagenic primers are indicated by open and closed arrows, respectively. All PCRs were performed using a 50°C annealing temperature; Figure 1 describes the primers and cycle conditions. When autoradiography was used to detect the products, 5 µCi of [α-32P]dCTP were included in the reaction mixture. (B) Autoradiograph of strand-separated products produced during Step I. Double-stranded PCR products using primers 1 and 2 (Lane a) or 3 and 4 (Lane b) were denatured in 30% dimethyl sulfoxide (DMSO) (vol/vol) and separated on an 8% non-denaturing polyacrylamide gel overnight at 400 V at 4°C. (C) Site-directed deletion of a 52-bp hairpin structure in the 5′ ETS of the Sc. pombe rDNA. Products of Step III were fractionated on a 1% agarose gel and stained with ethidium bromide (Lane a). A HindIII digest of pBR322 is included as a size marker (m).

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