An Efficient and Optimized PCR Method with High Fidelity for Site-directed Mutagenesis

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We have developed an efficient method for site-directed mutagenesis using two subsequential rounds of PCR. In this method, PCR conditions are optimized to favor high fidelity of Taq DNA polymerase in the presence of equimolar concentrations of MgCl₂ and dNTP in the reaction mixture (pH 5.5—6.2). This method makes use of a pair of universal primers and the multiple cloning site of pUC/M13 vectors. Only one mutagenic primer is required per target site. In the second round of PCR, the 3’ extension of the wild-type DNA strand is blocked by the presence of a segment of nonhomologous sequence at its 3’ end, and as a consequence, the amplified, full-length DNA fragment is chiefly from the mutant strand. Furthermore, because the mutated DNA fragment has flanking restriction sites different from those of the wild-type DNA fragment, the wild-type DNA fragment is totally excluded in the step involving selective cloning of the mutant DNA fragment. This method was successfully used to introduce four, nonadjacent mutations in the 5’ regulatory region of the cytochrome P450bmt-3 gene. All 20 analyzed clones from these four cases of mutagenesis carried the desired mutations, and no undesired mutations were observed. We observed that the larger the number of mismatched nucleotide residues in the mutagenic primer, the higher the concentration of MgCl₂ was necessary for successful PCR amplification. Our experimental results indicate that this method offers improvements in efficiency, flexibility, and fidelity.

PCR with Taq DNA polymerase has been widely used for both the amplification of specific DNA sequences and site-directed mutagenesis. In the last 6 years, two major methods for site-directed mutagenesis using two subsequential rounds of PCR amplification have been developed. The first method, designated overlap extension, was described by Ho et al. In this method, a pair of overlapping mutagenic primers and two flanking primers are required. With two different combinations of one flanking primer and one mutagenic primer, two DNA fragments having overlapping ends are generated by two separate PCR amplifications. These fragments are combined in a subsequent “fusion” reaction in which the overlapping ends anneal to each other, allowing the 3’ overhang of each strand to serve as a primer for the 3’ extension of the complementary strand. The resulting fusion product is amplified further by PCR. In practice, however, we found that the annealing of overlapping ends often fails to occur, which may be attributable to the complementary reassociation of double-stranded DNA in the fusion reaction and the adoption of secondary structure in single-stranded DNA produced in the subsequent PCR. The second method termed megaprimer, was developed by Landt et al. based on the scheme described by Kammann et al. This method requires just one mutagenic primer and two flanking universal primers for pUC/M13 vectors. In the first round of PCR amplification, the mutagenic primer and the antiparallel universal primer are used to produce a specific DNA fragment carrying the mutation. This DNA fragment is purified and then used as a primer together with the second universal primer for the second round of PCR amplification. However, a frequent drawback of this method, as pointed out by Barettino et al., is inefficient priming by the megaprimer fragment in the second round of PCR amplification, which results in very low yields of the full-length fragment of target DNA. Recently, several different approaches for improving the megaprimer method have been described, but, as the investigators, note, these approaches introduce their own limitations. Moreover, in reviewing the various procedures for PCR-mediated site-directed mutagenesis published to date, we found that little attention had been paid to the optimization of mutagenic PCR with regard to the fidelity of Taq DNA polymerase. Should DNA synthesis by Taq DNA polymerase, used for this purpose, be carried out under low-fidelity reaction conditions, the occurrence of undesired mutations would become a major concern.

On the basis of the megaprimer strategy, we developed an efficient method for site-directed mutagenesis using PCR for the introduction of multiple desired mutations in the 5’ regulatory region of the cytochrome P450bmt-3 gene. There are three major advantages in the method that we present here. First, the extension of the 3’ end of wild-type DNA strand is blocked by the introduction of a segment of nonhomologous DNA sequence
to its 3' end so that the full-length fragment produced by the second round of PCR amplification is mainly from the 3' extension of the mutant strand. Second, the mutated DNA fragment can be selectively cloned into a plasmid vector by using appropriate flanking restriction sites, as the mutated DNA fragment has flanking restriction sites different from those of the wild-type DNA fragment. Finally, the conditions of PCR for site-directed mutagenesis have been optimized with regard to fidelity of Taq DNA polymerase to minimize undesired mutations.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were purchased from either New England Biolabs or GIBCO BRL. Taq DNA polymerase was purchased from Promega. Oligonucleotide primers used in PCR and DNA sequencing were synthesized by Integrated DNA Technologies, Inc. Deoxynucleotide triphosphates (dNTPs) and [α-32P]dTTP were from Amersham Corp., and the Geneclean kit was purchased from BIO 101, Inc., and the Sequenase kit and Taq DNA polymerase were from U.S. Biochemical Corp. The 5' regulatory region of the cytochrome P450BM3 gene in Bacillus megaterium ATCC 14681 was cloned in our laboratory.(6) The DNA sequence of the P450BM3 gene, including the 5' regulatory region, is available under GenBank accession number J04832. Escherichia coli strain JM109 was used as a host for plasmid transformation and preparation. All chemicals used in the experiments were reagent grade or better.

PCR

The 5' regulatory region (1.63 kb) of the cytochrome P450BM3 gene was cloned into plasmid vector pTZ19R to yield a recombinant plasmid designated pFL3-1. To introduce site-directed mutations in the regulatory region, the first round of PCR was set up in a total volume of 100 μl containing 1 μl of pFL3-1 (~5 ng), 20 pmol of each primer, 10 μl of 10× reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0% Triton X-100], and 2.5 units of Taq DNA polymerase; optimized amount of equimolar concentrations of MgCl2 and dNTP (see Optimization of Reaction Conditions for Mutagenic PCR, below) were added. The reaction mixtures were overlayed with 50 μl of light mineral oil, and the reaction was carried out in an automatic thermal cycler for four cycles of 2 min at 94°C, 1 min at 48°C, and 2 min at 72°C, and then for 20 cycles of 1 min at 92°C, 1 min at 58°C, and 2 min at 72°C. The 72°C incubation of the last cycle was extended for an extra 5 min before the reaction samples were cooled to room temperature. The second round of PCR amplification was set up as described previously, except 2 mM MgCl2 and 0.5 mM each of the four dNTPs were used here. The PCR was performed for 20 cycles, each cycle consisting of 92°C for 1 min, 57°C for 1 min, and 72°C for 2.5 min. The last cycle was followed by 72°C for 5 min before cooling to room temperature.

RESULTS

Optimization of Reaction Conditions for Mutagenic PCR

Minimizing the frequency of undesired mutations in PCR-mediated site-directed mutagenesis can be achieved by maximizing the fidelity of the DNA polymerase. The error rate of Taq DNA polymerase used in the PCR can be influenced by many factors in the reaction; major factors include MgCl2 level, dNTP concentration, and pH conditions. Eckert and Kunkel evaluated these factors extensively in PCR and reported that high fidelity of Taq DNA polymerase could be reached when MgCl2 and dNTP were present at equimolar concentration or at a pH between 5 and 6 (70°C) at 1.5 mM MgCl2. Because the PCR mixtures, when set up with the 10× reaction buffer supplied with Taq DNA polymerase from Promega, gave a pH of 5.5–6.2 at 70°C, our efforts to optimize the mutagenic PCR conditions with regard to the fidelity of Taq DNA polymerase were focused on the adjustment of the MgCl2 and dNTP concentrations. Mutagenic primers and pUCM13 universal primers used for the introduction of mutations in three different locations of the 5' regulatory region of the P450BM3 gene are shown in Table 1. The mutagenic primers carry one to nine mutant nucleotide residues. Because the error rate of DNA synthesis by Taq DNA polymerase in the PCR has been shown to increase with increasing MgCl2 concentration (dNTP at 1 mM), the minimum concentration of MgCl2 required for efficient mutagenic PCR amplification is the optimum level of MgCl2 in the reaction mixture for the purpose of minimizing undesired mutations. PCR was set up with 1–4.5 mM MgCl2 for each pair of primers, with the results shown in Figure 1. In PCR with a pair of universal primers, forward primer (pUCM13-F) and reverse primer (pUCM13-R), the tar-

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence</th>
<th>Mismatches (bp)</th>
<th>Product size (bp)</th>
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<tr>
<td>1 pUCM13-F</td>
<td>5'-CGCCAGGGTTTTCGACCAGCAC-3'</td>
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<td>2 pUCM13-R</td>
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<tr>
<td>Otm2</td>
<td>5'-CTTTTCATACATGCTTCCAGCTTACATTAC-3'</td>
<td>9</td>
<td>1025</td>
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</tbody>
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TABLE 1 Two Universal and Three Mutagenic Primers Used for Site-directed Mutagenesis by PCR in the 5'-Regulatory Region of the Cytochrome P450BM3 Gene of Bacillus megaterium

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FIGURE 1 The minimal requirement of MgCl₂ for each mutagenic PCR is determined by monitoring the PCR-amplified target DNA by electrophoresis on an agarose gel. Each primer combination and its corresponding amplified DNA fragment, as analyzed by agarose gel electrophoresis, are placed side by side. PCR samples on all four gels have the same lane alignments. For each PCR reaction (100 μl total), 10 μl of sample was loaded onto the gel. (Lane 1) DNA molecular weight standards; (lanes 2–9) results of eight different PCR samples with a range of 1–4.5 mM MgCl₂ in the reaction mixtures (see the plot, top). The sizes of the standards (left) and those of specific amplified DNA fragments (right) are shown on the sides of the electrophoretographs.

get DNA fragment (1.78 kb) was significantly amplified when the MgCl₂ concentration was ≥1.5 mM. However, with mutagenic primers, the PCR amplification needed higher concentrations of MgCl₂. For example, in the PCR with pUC/M13-F and mutagenic primer JVₐm₂ (which contained one mismatched nucleotide), the minimal concentration of MgCl₂ required for efficiency was 2 mM. For pUC/M13-F and mutagenic primer BB₃m₂ (which contained two mismatched nucleotides) specific PCR amplification was not observed until the MgCl₂ in the reaction mixture reached a concentration of 3 mM. For pUC/M13-F and mutagenic primer O₁₁₁m₂ (nine mismatched nucleotides), the minimal MgCl₂ concentration for significant PCR amplification was 4 mM. Thus, 2, 3, and 4 mM MgCl₂ were used as the optimal MgCl₂ concentrations for the mutagenic PCR (the first round of PCR in the procedures as presented in the next section) with pUC/M13-F and mutagenic primer JVₐm₂, BB₃m₂, and O₁₁₁m₂, respectively. The corresponding equimolar dNTP concentrations in the reactions for these three mutagenic PCR runs were 0.5, 0.75, and 1.0 mM, respectively, of each of the four dNTPs (i.e., 2, 3, and 4 mM total).

Procedures for Site-directed Mutagenesis Using PCR

The strategy of the PCR method for site-directed mutagenesis is outlined in Figure 2. The P450ₐm₃ promoter region of the 1.6-kb DNA fragment was cloned into pTZ19R through the unique SalI and BamHI sites at the multiple cloning site of the vector. The resulting construct contained multiple unique restriction sites at both flanking ends of the insert. The first round of PCR was mutagenic, using flanking primer pUC/M13-F and the mutagenic primer m₂. The reaction was performed under the optimized conditions to favor the high fidelity of Taq DNA polymerase activity as described above. The specifically amplified DNA fragment was purified by low-melting agarose gel electrophoresis and the Geneclean kit. In the interim, the flanking restriction site “A” of the insert in the plasmid was replaced by restriction site “C” (HindIII in our examples) by DNA manipulation. In our experiments, the KpnI site served as the A site. A 12-bp HindIII linker with the 5’ end phosphorylated was used as the C site to replace the KpnI site. Following KpnI restriction enzyme digestion, the linearized plasmid was treated with T₄ DNA polymerase in the presence of dNTP to generate blunt ends and then purified using Geneclean. Next, the linear plasmid was recircularized with the HindIII linker using DNA ligase. The resulting plasmid then was linearized by digestion with restriction enzyme C and purified by using low-melting agarose gel electrophoresis (to remove undigested circular plasmid) and Geneclean. The linearized plasmid and the PCR-amplified DNA fragment containing the desired mutations (~5 ng for each preparation) were then combined and used as templates for the second round of PCR amplification. As shown in Figure 2, after the two DNA fragments were added together in the reaction mixture, the single-stranded DNA from the fragment of the first round of PCR (together with the identical single-
stranded fragment from the first cycle of the second round of PCR as primed by pUC/M13-F) anneals to its complementary strand of the linearized plasmid DNA or to the single-stranded DNA generated by the pUC/M13-R primer in the PCR to form a heteroduplex. In this heteroduplex, the 3' end of the mutant strand DNA could be extended to the pUC/M13-R primer annealing site to generate a full-length mutated single-stranded insert. This single-stranded DNA then became a homoduplex DNA in the second cycle of the PCR primed by pUC/M13-R primer. In contrast, the 3' end of the wild-type strand DNA of the heteroduplex could not be extended because the 3' end (4 nucleotide residues in our examples) was not homologous to its template and the Taq DNA polymerase, which lacks a 3'→5' exonuclease activity, was not capable of proofreading. The homoduplex DNA containing the desired mutation was then selected by digestion with restriction enzymes A and B and recovered by low-melting agarose gel electrophoresis and GeneClean treatment. The resulting mutated DNA fragment was cloned into vector pTZ19R by using the same A and B restriction sites. Because any wild-type DNA fragments that might, by chance, be amplified in the PCR would carry flanking restriction sites B and C, they were excluded in the cloning step because the A and B restriction sites were used for this process. Finally, the desired mutation in the insert may now be confirmed by DNA sequencing analysis.

**Mutagenesis of the P450BM·3 Promoter Region Using PCR**

Using PCR, we successfully introduced desired mutations into four different locations on the 5' regulatory region of the P450BM·3 gene. Figure 3 shows the results of PCR with two different primer combinations for site-directed mutagenesis at the JV site. In the first case, the mutagenic PCR was carried out with mutagenic primer JV_m1 and pUC/M13-R (Fig. 3, lane 1). The flanking restriction site B was replaced by C in the plasmid. After the combination of the mutated DNA fragment and the plasmid linearized by the digestion with restriction enzyme C, the full-length target DNA fragment containing the desired mutation was amplified successfully in the second round of PCR (Fig. 3, lane 3). In the second case, the first round of PCR was carried out with mutagenic primer JV_m2 and pUC/M13-F (Fig. 3, lane 2). The flanking restriction site A was replaced by C in the plasmid, and the second round of PCR also significantly amplified the target DNA fragment (Fig. 3, lane 4). Our overall evaluation of these two alternative ways for introducing mutations at the JV site, and the successful use of these procedures for the introduction of four non-adjacent mutations into the 5' regulatory region of the P450BM·3 gene, indicates that our PCR method is very flexible in practice.
here has been used successfully in our laboratory for site-directed mutagenesis on the 5' regulatory region of the cytochrome P450BM_3 gene in B. megaterium. Our PCR method offers improvements over other PCR mutagenesis techniques in efficiency, flexibility, and fidelity.

In this method wild-type parental DNA is prevented from extension at the 3' end (Fig. 2) and is thus essentially excluded from amplification during the second round of PCR. Moreover, the mutant DNA fragment generated by the second round of PCR amplification has flanking restriction sites different from those of the wild-type parental DNA fragment; this allows for the selective cloning of the mutant DNA fragment and, hence, exclusion of the wild-type DNA fragment in the subsequent cloning step. When we compared this method with the megaprimer and overlap extension methods for the introduction of site-directed mutations at four different locations on the 5' regulatory region of the P450BM_3 gene, our procedure proved superior. For example, for mutagenesis at the Omu site using the megaprimer method, a very low yield in the second round of PCR amplification was obtained and only one of eight clones analyzed carried the desired mutation. With the overlap extension method, only the desired mutations at the BB3 site were amplified in the second round of PCR; the other three cases of mutagenesis were unsuccessful.

The basic strategy employed in our method involves the use of a pair of universal primers, pUCM13 forward (F) and reverse (R) primers, which flank the inserts in the pUCM13 vectors. Only one mutagenic primer is required per target site. As a consequence, this method, as demonstrated by our experiments presented here, is suitable for introducing multiple, nonadjacent mutations sequentially on the same DNA molecule with high efficiency. Furthermore, primer combinations can be changed at a mutation target site (Fig. 3), a flexibility in methodology that is especially important in cases where a specific primer pair fails to work in PCR for undetermined reasons, as has been reported. However, if used in our PCR method, the 3'→5' proofreading activity of Vent DNA polymerase would remove the 3' nonhomologous sequence of the wild-type DNA strand in the heteroduplex and lead to amplification of parental DNA. An alternative approach would employ Vent DNA polymerase (New England Biolabs), which has been genetically engineered to eliminate the 3'→5' proofreading exonuclease activity. However, because the fidelity of Vent DNA polymerase is only twofold higher than that of Taq DNA polymerase, we would still recommend optimization of the reaction conditions to maximize the fidelity of Vent DNA polymerase in PCR for site-directed mutagenesis. With respect to optimization, the reason for the requirement of higher concentrations of MgCl₂ for PCR amplification with primers containing multiple mismatched nucleotide residues (Fig. 1) is not clear. It seems likely, however, that the mutagenic primer/template hybrids need the presence of higher Mg²⁺ concentrations to maintain their stability at high annealing temperatures.

It should also be noted that Taq DNA polymerase has been reported to catalyze the untemplated addition of an A (deoxyadenosine) residue at the 3' end of the amplified DNA fragment.

**DISCUSSION**

The PCR method that we have described
Mutagenic primers should therefore be designed as described so that the first 5' nucleotide of a mutagenic primer follows a T residue in the same strand of template sequence. The amplified DNA fragment from the first round of PCR will thus have the correct sequence. When a T residue is not available before the first 5' nucleotide of a mutagenic primer, the DNA fragment amplified by the first round of PCR must be treated with T4 DNA polymerase to remove the additional 3' residue before carrying out the second round of PCR amplification.

ACKNOWLEDGMENTS

We thank Keynes Tong from this laboratory for his excellent technical assistance in several of the experiments reported here. The research reported in this paper was supported by National Institutes of Health Research grant GM23913 and by the Director of the Office of Energy Research, Office of Health and Environmental Research, contract DE-FC03-ER06015.

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Received October 11, 1994; accepted in revised form January 6, 1995.
An efficient and optimized PCR method with high fidelity for site-directed mutagenesis.

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Genome Res. 1995 4: 269-274