A simple and efficient method for site-directed mutagenesis with double-stranded plasmid DNA

Derhsing Lai, Xueli Zhu and Sidney Pestka*

Department of Molecular Genetics and Microbiology, UMDNJ – Robert Wood Johnson Medical School, Piscataway, NJ 08854-5635, USA

Received June 10, 1993; Revised and Accepted July 23, 1993

ABSTRACT

A general, simple and efficient method for preparing site-specific mutations in double-stranded plasmid DNA without the need for special plasmids, bacterial strains or reagents is described. Only one synthetic oligonucleotide for each mutation is required, subcloning is unnecessary and a high efficiency of mutation (58–97%) was obtained. If two synthetic oligonucleotide primers are used, two separate mutations can be simultaneously created in a single reaction tube.

INTRODUCTION

Oligonucleotide-directed site-specific mutagenesis is commonly used to create mutations. Three basic methods for this purpose use single-stranded (M13),^{1,2} the polymerase chain reaction (PCR),^{3–6} and double-stranded plasmids.^{7–9} The single-stranded method developed by Zoller and Smith¹⁰ has been modified many times to achieve a higher yield of mutants. More recently, the PCR method has become a popular technique for site-directed mutagenesis. The advantages and shortcomings of these methods have been discussed.^{11,12} Limitations include the availability of restriction sites for subcloning and the instability of large insertions in M13 vectors,¹³ the low fidelity of the Taq polymerase and the expense for multiple primers in the PCR methods, and the low mutant yields with the double-stranded plasmid method.

Here we introduce another strategy using double-stranded DNA with only one new synthetic primer required for each mutant. Subcloning steps are unnecessary and the theoretical mutant yield is 100%. In addition, no special *Escherichia coli* mutant strains, unusual nucleotides or specific modification enzymes are required. The method may be used to generate base changes, insertions, and deletions in the target DNA.

EXPERIMENTAL PROCEDURES

Vector, cells and primer

The Bluescript k/s II vector (Stratagene) was modified by cutting the *Cla*I site within the polylinker, blunting the ends with the Klenow fragment of DNA polymerase I, and religating to yield

plasmid pDL1. This eliminated the *Cla*I site and introduced a frameshift (two additional bases: CG) into the coding sequence for the *lac* α -peptide, resulting in a white colony phenotype on indicator plates. Competent cells were prepared by the procedure of Nishimura *et al.*¹⁴ *Escherichia coli* DH5 α and JM109 were used to prepare competent cells. Primer I (5'-CAAGCTTATCGATACGTCG-3') was used to reconstruct the *Cla*I site in the *lac* Z gene by deletion of the two extra bases (CG) and produces the wild type *lac* α -peptide sequence which in turn restores the blue colony phenotype. Primer I was incubated with T4 DNA kinase to phosphorylate the 5'-end.

The mutagenesis method

The experimental procedure, a modification of that reported by Inouye *et al.*,¹² is outlined in Fig. 1. The experimental vector pDL1 was double-digested with restriction endonucleases *Pst*I (A), *Xba*I (B) to produce fragment I and with *Pst*I (A), *Xho*I (C) to produce fragment II. After digestion with enzymes A and B to produce fragment I, this reaction mixture only was further treated with calf-intestinal alkaline phosphatase to remove 5'-phosphate termini where noted. Gel electrophoresis (1% agarose; TAE buffer, 0.04 M Tris·acetate, 0.001 M EDTA, pH 7.6; 94 volts; 45 min.) was used to isolate the fragments and GeneClean (Bio101) used to recover fragments I and II.

Equimolar amounts (0.3–0.4 μ g) of DNA fragments I and II were mixed with a 500-fold or 2000-fold molar excess of synthetic phosphorylated oligonucleotide primer as noted. The control groups contained no primer. The reaction mixture contained 172 mM NaCl, 12 mM Tris·HCl (pH 7.5), 14 mM MgCl₂, 1.7 mM 2-mercaptoethanol in a final volume of 35 μ l. The mixture was incubated in boiling water for 3 minutes to denature the DNA fragments. After denaturation, the mixture was transferred to 30°C for 30 minutes, then to 4°C for 30 minutes, and then for at least 10 minutes on ice to allow the denatured DNA fragments to reanneal. The formation of new heteroduplex DNAs was confirmed by 1% agarose gel electrophoresis in TAE buffer before proceeding to the next step. To extend the 3'-ends and ligate the resulting heteroduplex plasmid (III, Fig. 1), 11.6 μ l of the above reaction mixture was incubated with 5 units of Sequenase or the Klenow fragment of DNA polymerase I (0.4 μ l), 2 μ l of T4 DNA ligase (2 units),

* To whom correspondence should be addressed

4 μ l 2.5 mM dNTPs, and 2 μ l 10 mM ATP in a final volume of 20 μ l at 15°C overnight. After the overnight incubation, 5 μ l of the mixture was then used to transform competent bacteria and the mixture plated on a LB agar plate containing 100 μ g/ml ampicillin. Prior to plating the bacteria; the surface of the plate was coated with 44 μ l of a solution of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (40 μ l X-gal, 20 mg/ml) and isopropyl- β -D-thiogalactopyranoside (4 μ l IPTG, 200 mg/ml) as reported (Sambrook *et al.*¹⁵). The ratio of blue to white colonies was used to calculate the mutation efficiency. Because transformation was performed with double-stranded DNA, each blue colony represents a 50% mixture of the wild type and mutant plasmids.

RESULTS AND DISCUSSION

In this report, we describe a simple method to generate site-directed mutants on a double-stranded plasmid DNA with high efficiency. As shown in Table 1, the mutation rate was 58 to 97%. In this strategy (Fig. 1), restriction endonuclease A must have overhanging ends which are shared by the two double-digested fragments I and II. There are no constraints for the other restriction endonucleases B and C except that they need to produce fragments I and II. One or more B sites may be present in the gap produced by the A and B double digestion (Fig. 1); and similarly one or more C sites may be present in the gap produced by digestion with enzymes A and C (Fig. 1). After reannealing and 3'-extension, duplexes I and II cannot religate since they retain a 3'-overhang and a blunt end. When restriction endonuclease A produces a 3' overhang (e.g., *Pst*I), Duplex IV produces a blunt-ended DNA that will not be religated significantly at the low concentrations of the ligase used. This was confirmed by demonstrating that blunt-ended heteroduplex DNA produced no colonies after transformation (data not shown). Duplex III, therefore, is the only one that can religate efficiently after 3'-extension. This design provides a theoretical mutation rate of 100%. Although it was not necessary to dephosphorylate fragment I, its dephosphorylation increased mutation efficiency significantly (Table 1).

It should be noted that if restriction endonuclease A is chosen with a 5' overhang, then duplex III cannot be extended and filled in (Fig. 2). The oligonucleotide must be complementary to the

opposite strand as that shown in Fig. 1 (heteroduplex III) so that heteroduplex IV is the only one that religates the outer strand (Fig. 2).

In practice, the mutant yield may be somewhat lower than the theoretical ratios although they approximated the theoretical values as shown in Table 1. This is assumed to be due to such factors as incomplete *in vitro* DNA polymerization, primer displacement by the DNA polymerase used to fill in the strands and primer phosphorylation efficiency. We did not use a repair minus *E. coli* strain (e.g., BMH 71-18 mutS)^{16,17} in these experiments as our actual mutation rates were almost equivalent to the theoretical rates. Thus, special strains are not critical for this method.

This mutation method has some advantages over others. Its efficiency is as high as that of the single-stranded method. The procedure is more consistent and rapid compared with the single-stranded method^{1,2,10} because subcloning into a single-stranded vector or a vector with an M13 origin can be omitted. In addition,

Figure 1. Diagram of the strategy of the double-stranded DNA site-specific method with restriction endonuclease A producing a 3' overhang. The plasmids are digested with restriction endonucleases A and B, and A and C to yield fragments I and II, respectively. In this example, the A restriction endonuclease site produces a 3'-protruding end. The B and C sites can be any sites flanking the target sequence. After denaturation and renaturation, the two homoduplexes (I, II) and two heteroduplexes (III, IV) are formed. The parental duplexes (I and II) and blunt-ended heteroduplex (IV) after filling in and ligation will yield no transformants. Only heteroduplex III can be extended and efficiently ligated. The A in parentheses means the site may no longer be intact. The theoretical mutation percentage is 50%. In the example shown in the text and in Table 1, the restriction endonucleases are represented as follows: A, *Pst*I; B, *Xba*I; and C, *Xho*I. Arrowheads in the top half of the figure represent the 3' ends of the DNA strands.

Table 1. Mutation Rate

<i>E. coli</i>	DH5 α	DH5 α	JM109
Alkaline Phosphatase	No	Yes	Yes
Blue/total	138/237*	16/18	126/130*
%	58	89	97

*Represents the combinations of three separate experiments.

The experiments with *E. coli* DH5 α were performed as described in 'Experimental Procedures' except as noted. An equal amount of fragment I (plasmid pDL1 digested with *Xba*I, *Pst*I) and fragment II (*Xho*I, *Pst*I digested) were incubated with a 500-fold excess of phosphorylated primer I. Fragment I was dephosphorylated where noted in the table (alkaline phosphatase). Site-specific mutagenesis was otherwise carried out as described under 'Experimental Procedures.' The ratio of blue to white colonies was used to calculate the mutation efficiency. DNA from the blue colonies was checked by restriction endonuclease *Cla*I digestion. The experiments with *E. coli* JM109 were performed as the experiments with *E. coli* DH5 α with minor modifications: the *Xba*I, *Pst*I fragment (A-B fragment I, Fig. 1) was treated with alkaline phosphatase after the restriction enzyme digestion; *E. coli* JM109 competent cells and 2000-fold excess of phosphorylated primer I were used. White colonies represent wild type and blue colonies, mutants.

Figure 2. Diagram of the strategy of the double-stranded DNA site-specific method with restriction endonuclease A producing a 5' overhang. If restriction endonuclease A is chosen to be one that produces a 5' overhang, then the mutation oligonucleotide must be complementary to the opposite strand as that shown in heteroduplex III of Fig. 1 and only heteroduplex IV produces a functional closed single-stranded circle after ligation.

Figure 3. Diagram of the strategy with two synthetic primers. This represents a variation of the procedure shown in Fig. 1 with the use of two oligonucleotide primers to produce two single and one double mutation simultaneously. Dephosphorylation of Fragment I (Fig. 1) is unnecessary.

the cost for primer synthesis is less than the costs with PCR methods since only one primer is required. Furthermore, the procedure can be modified by using two primers to generate three mutants (two single mutants, one double mutant) in the same experiment (Fig. 3). We have routinely used this method to create mutants of the extracellular domain of the murine interferon gamma receptor.

This method can be used with plasmids containing three suitable restriction enzyme sites to generate base changes, deletions and insertions in the target DNA. The method is a general one that is convenient and rapid. Insertion and deletion mutants can be easily identified without the need for sequencing. In fact, single base changes can be identified rapidly by PCR by preparing an oligonucleotide with the mutant base at the 3'-end.^{18,19} As no specific plasmids or *E. coli* strains are required, the simplicity and high efficiency of this method should make it a useful general procedure for preparing site-specific mutations in double-stranded DNA.

ACKNOWLEDGEMENTS

We thank Eleanor Kells for her expert assistance in the preparation of this manuscript. This study was supported in part by United States Public Health Service Grants AI25914, CA46465 and CA52363 from the National Institutes of Health awarded to SP.

REFERENCES

- Derbyshire, K.M., Salvo, J.J., Grindley, N.D.F. (1986) A simple and efficient procedure for saturation mutagenesis using mixed oligodeoxynucleotides. *Gene* **46**, 145–152.
- Li, B., Langer, J. A., Schwartz, B., and Pestka, S. (1989) Creation of phosphorylation sites in proteins: Construction of a phosphorylatable human interferon α . *Proc. Natl. Acad. Sci. USA* **86**, 558–562.
- Hignichi, R., Krummel, B., and Saiki, R. K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351–7367.
- Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1989) Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Res.* **17**, 723–733.
- Kadowaki, H., Kadowaki, T., Wondisford, F. E., and Taylor, S. I. (1989) Use of polymerase chain reaction catalyzed by Taq DNA polymerase for site-specific mutagenesis. *Gene* **76**, 161–166.
- Ho, S., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Vlasuk, G. P., and Inouye, S. (1983) 'Site-Specific Mutagenesis Using Synthetic Oligodeoxyribonucleotides as Mutagens,' In: *Experimental Manipulation of gene expression*. (M. Inouye, ed.), Appendix 2, pp. 291–303. Academic Press, New York.
- Morinaga, Y., Franceschini, T., Inouye, S., and Inouye, M. (1984). Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA. *Bio/Technology* **2**, 636–639.
- DeChiara, T. M., Erlitz, F., and Tarnowski, S. J., (1986) Procedures for in vitro DNA mutagenesis of human leukocyte interferon sequences. *Methods in Enzymology* **119**, 403–415.
- Zoller, M. J., and Smith, M. (1982) Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment. *Nucleic Acids Res.* **10**, 6487–6500.
- Jones, D. H., and Howard, B. H. (1990) A rapid method for site-specific mutagenesis and directional subcloning by using the polymerase chain reaction to generate recombinant circles. *BioTechniques* **8**, 178–183.
- Inouye, S. and Inouye M. (1987) 'Oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA,' In: *Synthesis and Applications of DNA and RNA*. (Saran A. Narang, ed.), pp. 181–206. Academic Press, New York.

13. Cordell, B., Bell, G., Tisher, E., DeNoto, F. M., Ullrich, A., Pictet, R., Rutter, W. J., and Goodman, N. M. (1979) Isolation and characterization of a cloned rat insulin gene. *Cell* **18**, 533–543.
14. Nishimura, A., Morita, M., Nishimura, Y., and Sugino, Y. (1990) A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. *Nucleic Acids Res.* **18**, 6169.
15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning, a laboratory manual, second edition, Cold Spring Harbor Laboratory Press, 3 volumes.
16. Kramer, B., Kramer, W., and Fritz, H. J. (1984) Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E.coli*. *Cell* **38**, 879–887.
17. Zell, R., and Fritz, H. J. (1987) DNA mismatch repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. *EMBO. J.* **6**, 1809–1815.
18. Newton, C. R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C., and Markham, A. F. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17**, 2503–2515.
19. Sommer, S. S., Cassady, J. D., Sobell, J. L., and Bottema, C. D. K. (1989) A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of phenylketonuria. *Mayo Clin. Proc.* **64**, 1361–1372.