APPLICATION OF A SIMPLE OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS METHOD FOR CORRECTION OF A FRAMESHIFT MUTATION

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ABSTRACT: Application of a rapid and simple method for generating site-directed mutations in double-stranded plasmid DNA is described. Insertion of four nucleotides correcting a frameshift mutation was accomplished utilizing only a single synthetic oligonucleotide. Insertion was confirmed by restriction endonuclease digestion and dideoxy nucleotide sequencing. Successful application of this rapid oligonucleotide-directed site-specific mutagenesis requires no subcloning, special bacteria or multiple primers. *(JUMMEC 1997 2(1): 27-30)*

KEYWORDS: Frameshift mutation, ScFv, site-directed mutagenesis

Introduction

Site-directed mutagenesis is commonly used for the study of structure-function relationships (1,2) and gene regulation (3). In these studies, mutations are created at specific regions or sites through insertion, deletion or base substitution in the target DNA. These mutations are accomplished by a number of mutagenesis methods. The PCR-based mutagenesis for example, has been very popular (2,3). This site-directed mutagenesis method, however, requires multiple primers, thus, is not cost effective. Furthermore, repeat amplifications of the full length plasmid DNA may introduce other mutations because of the inherent low fidelity of the Taq DNA polymerase (3). In cases where the DNA template is large, DNA polymerase with proof reading activities has to be used and this indirectly affects the cost-effectiveness of this procedure. Recently Lai *et al.* (4) reported a simple and efficient method for site-directed mutagenesis with double-stranded plasmid DNA. They reported that using their protocols, successful single oligonucleotide-directed mutagenesis could be accomplished within two weeks. Numerical results of their investigation were reported. However, they did not provide actual electrophoresis results which demonstrate the various possible heteroduplexes that could arise following annealing of the DNA strands. Thus, it is possible that due to this reason among others, their protocols did not enjoy a wide spread usage in comparison to the more popular yet tedious PCR-based protocols. In the present report, we detailed a successful application of the procedure to correct a frameshift mutation affecting the expression of the single chain variable fragment (ScFv) gene cloned into the pCANTAB 5E expression vector.

Materials and Methods

Preparation of DNA fragments

The schematic for the oligonucleotide-directed mutagenesis used in this study is as outlined in Figure 1. Initially, fragment A was obtained by digesting 6 µg of the recombinant pCANTAB 5E plasmid (Pharmacia Biotech, Sweden) consisting the 3H5-1 single-chain variable fragment (ScFv) with 60 U *Not* I and then with 60 U *Sfi* I in 1.5X universal buffer (Stratagene, USA). Fragment B on the other hand, was obtained after digestion of the recombinant plasmid with 60 U *Hind* III and 60 U *Sfi* I, sequentially in 1X NEB buffer 2 (NEB, USA). The restriction endonuclease after the first digestion was removed using the StrataClean resin (Stratagene, USA). Following the second digestion, the digestion mix was added with 2 volumes of sterile MilliQ water (sMQ H_2O; Millipore, USA) and 0.5 volume of phenol:chloroform:isoamylalcohol solution (USB, USA) and then centrifuged at 20,000 X g for 4 minutes. The aqueous phase of the mixture consisting the digested DNA was removed and placed into a new microcentrifuge tube. The DNA was then precipitated using 1/10 volume of 3 M potassium acetate, pH 5.2 and 2.5 volumes of absolute ethanol at -70° C for 2 hours. The DNA was sedimented at 12,000 X g for 15 minutes at 4° C, air dried and electrophoresed in 1% agarose gel. DNA bands corresponding to fragments A and B at the expected molecular weights were removed from the agarose gel and placed into separate

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1.5 ml microcentrifuge tubes. Three volumes of 6 M NaI were added to the agarose gel slice and incubated at 58° C until the agarose melted. Then 10 µl of pretreated silica particles solution was added to the molten agarose and the mixture was incubated at room temperature for 15 minutes with occasional mixing. Following the incubation, the tubes were centrifuged at 13,000 X g for 7-8 seconds and the supernatants were discarded. The pellets were washed 3 times by resuspending with washing buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 50% ethanol) followed by sedimentation as above. After the washings, the pellets were dried at 37° C for 4 minutes and the bound DNA fragments were eluted with 30 µl sMQ H_2O at 55° C for 10 minutes with occasional tappings. The purified DNA fragments in the supernatant were recovered by sedimentation at 20,000 X g for 4 minutes and then quantitated using the GeneQuant RNA/DNA calculator (Pharmacia Biotech, Sweden).

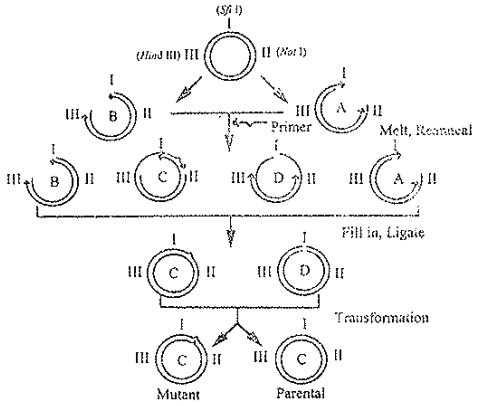

Figure 1. Oligonucleotide-directed mutagenesis scheme used in this study. Plasmids were digested with *Not* I and *Sfi* I, or *Hind* III and *Sfi* I to yield fragments A and B, respectively. After denaturation and renaturation, the parental duplexes (A and B) and heteroduplexes (C and D) were formed. Only heteroduplex C could be extended and ligated. [Diagram was adapted with modification from Lai *et al.* (4)].

Site-directed mutagenesis

Approximately 0.4 µg of each DNA fragment (A and B) were mixed with 1000-fold or 4000-fold molar excess of the phosphorylated oligonucleotide primer (5' CATTAGGGAGC**CGGC**TTACACGTTCG 3'), ligase buffer (Promega, USA) and sMQ H_2O were added to a final volume of 70 µl. The samples (two with the primer and one without the primer to serve as control) were boiled for 3 minutes and then incubated at 30°C for 30 minutes. The incubation was continued for an additional 30 minutes at 4°C and then on ice for not less than 10 minutes to allow formation of new heteroduplex DNAs. The samples were then electrophoresed in a 0.8% agarose gel to confirm the formation of the heteroduplex DNAs.

5 U of Klenow fragment of DNA polymerase I, 2 U of T4 DNA ligase and dNTP at 10 mM were then added to 17.8 µl of the above reaction mix. The newly prepared mix was incubated at 15°C overnight to allow extension and ligation to take place. After overnight incubation, 4 µl of the mixture was used to transform competent *Escherichia coli* HB 2151 cells and the transformants were then plated on 100 µg/ml ampicillin agar plate. Plasmids of the transformants were isolated using alkaline lysis protocol (5) and then digested with *Ngo*M I restriction endonuclease. The additional *Ngo*M I site was conferred by the inserted nucleotides (**CGGC**). Positive clones were then subjected to nucleotide sequencing for further confirmation.

Results and Discussion

Oligonucleotide-directed site-specific mutagenesis performed in this study was to correct a frameshift mutation in one of the recombinant clones of the hybridoma 3H5-1 single-chain variable fragment (ScFv) (6). The ScFv comprising of 732 bp DNA including the Flag detection sequences at the 3' end was cloned into the pCANTAB 5E expression vector. No expression of the ScFv could be detected by immunoblotting following transformation of the recombinant plasmid into the *Escherichia coli* HB 2151. Upon sequencing, it was determined that a single nucleotide deletion had occurred at position 663 of the ScFv (Figure 5a), thus, resulting in a translational frameshifting involving about 9% of the carboxy end of the recombinant protein. Applying the above detailed oligonucleotide-directed mutagenesis, four nucleotides were successfully inserted at position 663 of the ScFv (Figure 5b), restoring the translational reading frame, thus, allowing for expression of the Flag detection sequences (data not shown). Insertion of the four nucleotides also introduced an additional *Ngo*M I restriction endonuclease site which was used for screening of successfully mutated clones and an amino acid, proline, to the ScFv.

The mutation rate obtained in this investigation, however, was low in comparison to the 58-97% success rate reported by Lai *et al.* (4). This could probably due to ligation of the heteroduplex D which would take place when excess ligase was used or the formation of the parental plasmid after transformation (4). It was also possible that the low frequency was because the resulting DNA fragments were not dephosphorylated prior to ligation. Fragment A (~ 4.5 kb; Figure 2, lane 2) and B (~ 5.2 kb; Figure 2, lane 3) were successfully obtained after the restriction endonuclease digestions. The formation of heteroduplex DNAs following denaturation and renaturation was confirmed by the appearance of an additional ethidium bromide-stained DNA band at a higher molecular weight (~ 9.7 kb) alongside the initial two bands for the DNA fragments A and B (Figure 3, lane 1, 2 and 3).

Forty five transformants obtained (23 transformants

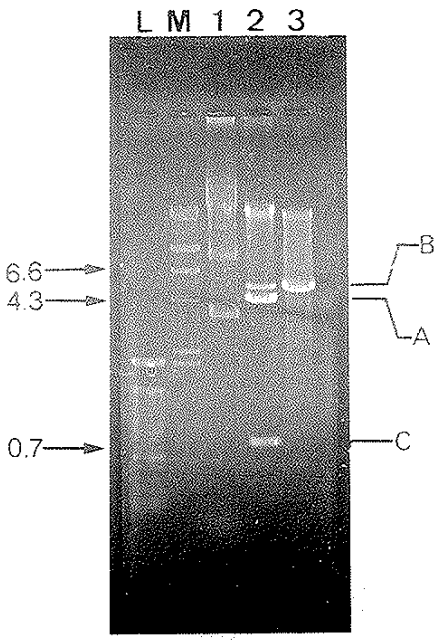

Figure 2. Restriction endonuclease digestion of the recombinant pCANTAB 5E plasmid. 3 µl of the undigested plasmid (lane 1) and 5 µl of *Not* I and *Sfi* I (lane 2) or *Hind* III and *Sfi* I (lane 3) digested plasmids were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidium bromide. 100 bp DNA ladder (lane L) and λ *Hind* III molecular weight markers (lane M) were used for DNA size indication and are shown in kilobases. A, B and C indicate the resulting DNA fragments.

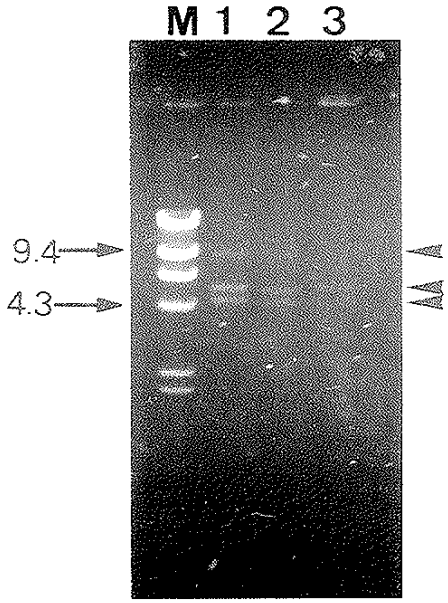

Figure 3. Formation of heteroduplex DNA fragments. 5 µl of denaturation and renaturation mixture of 1000-fold molar excess primer (lane 1), 4000-fold molar excess primer (lane 2) or without primer as negative control (lane 3) were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidium bromide. λ *Hind* III molecular weight markers (lane M) were used for DNA size indication and are shown in kilobases. Arrow heads indicate the resulting DNA fragments.

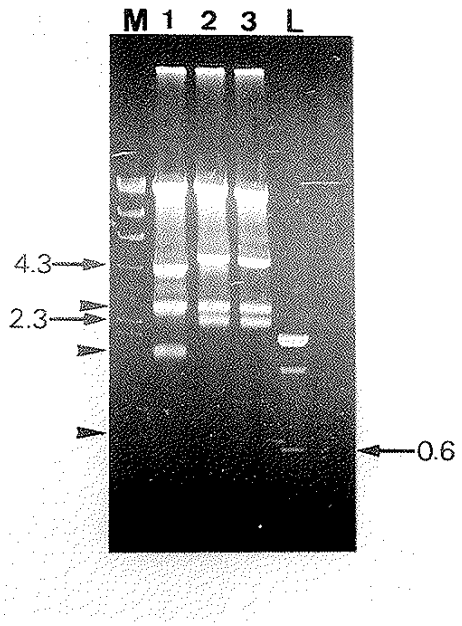

Figure 4: Screening for successful oligonucleotides insertion using restriction endonuclease digestion. Following the site-directed mutagenesis, 10 µl of the *Ngo*M I digested samples were electrophoresed in a 0.8% agarose gel in 0.5X TAE and stained with ethidiun bromide. λ *Hind* III molecular weight markers (lane M) and 100 bp ladder (lane L) were used for DNA size indication and are shown in kilobases. Arrow heads indicate the resulting DNA fragments. See text for discussion.

from 1000-fold and 22 transformants from 4000-fold molar excess of primer) were screened with the *Ngo*M I restriction endonuclease. The parental recombinant pCANTAB 5E plasmid consisted two *Ngo*M I restriction endonuclease sites which would give approximately 2600 and 2700 bp DNA fragments following the *Ngo*M I restriction endonuclease digestion. Therefore, plasmids without any nucleotides insertion would have only two DNA fragments (Figure 4, lane 3). In contrast, the mutated plasmid with an additional *Ngo*M I restriction site would show 3 DNA fragments of approximately 800, 1800 and 2700 bp respectively (Figure 4, lane 1). This is because the additional *Ngo*M I restriction site was introduced into the region of the 2600 bp DNA fragment which when digested would result in two DNA fragments of 800 and 1800 bp. Besides the three DNA fragments, there was an additional DNA fragment of about 4500 bp. This DNA fragment was probably the incompletely digested plasmid. This was because the total molecular weight of two out of the three DNA fragments (1800 and 2700 bp) was approximately equivalent to the above presumed incompletely digested DNA fragment. Whereas, the largest DNA fragments (> 4500 bp) in the other two samples (Figure 4, lane 2 and 3) were probably the linearized plasmid since the molecular weights were approximately 5300 bp which is the reported molecular weight of the plasmid. Samples shown in lane 2 (Figure 4), however, had an additional band of about 1800 bp in addition to the three DNA fragments (2600, 2700 and 5300 bp) similarly present in samples in lane 3. This could be due to insufficient restriction endonuclease digestion of the plasmid or there was a mixture of mutated and unmutated plasmids in the sample. Nevertheless, successful insertion of the four nucleotides was confirmed by nucleotide sequencing (Figure 5).

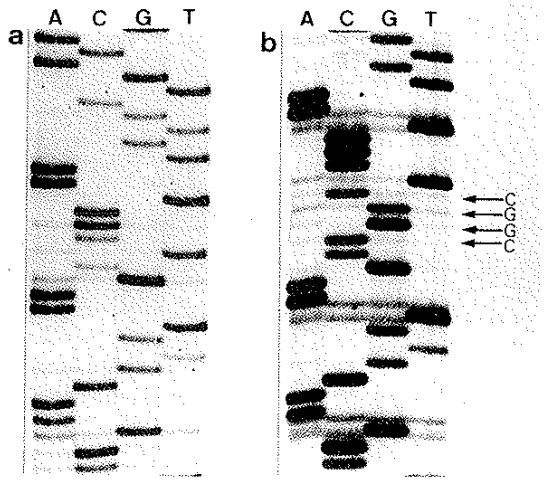

Figure 5. The nucleotide sequences before (a) and after (b) site-directed mutagenesis. Arrows indicate the inserted 4 nucleotides.

In summary, we detailed here a successful application of a simple site-directed mutagenesis of double stranded DNA using a single oligonucleotide. The entire procedure was performed within two weeks without any need to subclone or use of any additional new enzymes.

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References

1. Lake DF, Lam KS, Peng L and Hersh EM. Molecular cloning, expression and mutagenesis of an anti-insulin single chain Fv (scFv). Mol Immunol 1994; 31: 845-56.
2. Martin A, Toselli E, Rosier MF, Auffray C and Devignes M-D. Rapid and high efficiency site-directed mutagenesis by improvement of the homologous recombination technique. Nucl Acids Res 1995; 23: 1642-3.
3. Picard V, Ersdal-Badju E, Lu A and Bock SC. A rapid and efficient one-tube PCR-based mutagenesis technique using *Pfu* DNA polymerase. Nucl Acids Res 1994; 22: 2587-91.
4. Lai D, Zhu X and Pestka S. A simple and efficient method for site-directed mutagenesis with double-stranded plasmid DNA. Nucl Acids Res 1993; 21: 3977-80.
5. Sambrook J, Fritsch EF and Maniatis T. Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory, 1989.
6. Chee HY. Construction of a single-chain Fv fragment of an antibody recognizing dengue 2 envelope protein [dissertation]. Universiti Malaya, Kuala Lumpur, Malaysia, 1997.