Random insertion and deletion mutagenesis for construction of protein library containing nonnatural amino acids

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ABSTRACT
A new method was developed for the generation of a library of mutant proteins that contained nonnatural amino acids. The method, "random insertion and deletion (RID) mutagenesis", is based on the deletion of an arbitrary number of bases at random positions and, at the same time, the insertion of an arbitrary sequence into the same position. By using this method, randomly selected three consecutive bases in the gene of green fluorescence protein (GFP) were replaced by a CGGT 4-base codon. When this DNA library was expressed in E.coli, about 80% of colonies lost the fluorescence. The non-fluorescent colonies were picked up and the genes were sequenced. Replacement of three consecutive bases by CGGT 4-base codon was found in two of the four mutated genes.

INTRODUCTION
The alteration of protein structure and function by the incorporation of nonnatural amino acids has a wide potential to create useful and functional proteins (1-4). However, incorporation of a nonnatural amino acid with large side group, like a fluorescent group, often causes a suppression or a complete loss of the protein activity (5). This problem will be avoided by producing of a library of proteins in which each protein contains a single nonnatural amino acid at various positions, and the mutants that possess the desired function are screened and produced. In this study, a new method "random insertion and deletion (RID) mutagenesis", is proposed that allows to replace randomly selected three consecutive bases by a CGGT 4-base codon. Subsequently, the CGGT codon will be translated to a nonnatural amino acids. Using this DNA library, a protein library in which each protein contains a nonnatural amino acid at a random position will be constructed.

Figure 1 A general scheme of the random insertion and deletion mutagenesis for generation of a protein library in which each protein contains a single nonnatural amino acid at random positions.
RESULTS AND DISCUSSION

The key steps for the RID mutagenesis, that replaces randomly selected 3-consecutive bases to a CGGG 4-base codon, are illustrated in Figure 1. The procedure consists of eight major steps. **Step 1:** The GFP gene was amplified by PCR with a primer pair that is phosphorylated at one of the 5'-end. **Step 2:** The amplified DNA was cyclized with T4 DNA ligase. **Step 3:** The circular dsDNA was treated with T4 DNA polymerase to give a circular ssDNA. **Step 4:** The circular ssDNA was randomly cleaved at a single position by treating with Ce(IV)/EDTA complex (6). **Step 5:** The linear ssDNA was ligated with the 5'-linker and the 3'-linker. **Step 6:** The DNA attached with the two linkers at both ends was amplified by PCR. **Step 7:** The products were cleaved with *BclI* at both ends. The cleavage at the 5' end will add a CGGT 4-base codon and the cleavage at the 3' end will delete 3-consecutive bases. **Step 8:** The product was treated with T4 DNA polymerase, and cyclized with T4 DNA ligase. The PCR product was cleaved with EcoRI and *Hind*III site. The ligated DNA was electroplated into *E. coli* DH10B. Under a standard UV light (365nm) about 80% of colonies lost fluorescence, suggesting that the GFP gene was mutated (Figure 2). The non-fluorescent colonies were picked up and the mutated GFP genes were sequenced. Two of the four genes had a CGGT codon in place of 3-consecutive bases (Figure 3,a,b). The other two genes also had a CGGT codon, however, in place of 4 or 5-consecutive bases (Figure 3,c,d).

In conclusion, a new method, "RID mutagenesis", was developed for generating a library of mutant proteins that contained a nonnatural amino acid. The RID mutagenesis will be able to regulate insert and delete any number of bases on the gene at a random position, and will be a powerful tool for biological research.

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