A simple *in vitro* site directed mutagenesis of concatamerized cDNA by inverse polymerase chain reaction

Ghanshyam D.Heda⁺, Timothy R.Henion and Uri Galili*

Department of Microbiology and Immunology, The Medical College of Pennsylvania, 3300 Henry Avenue, Philadelphia, PA 19129, USA

Submitted May 14, 1992

Site directed mutagenesis can be performed in plasmids by inverse polymerase chain reaction (PCR) with 'back to back' primers one of which contains the mutation. The PCR product is then ligated in order to recircularize the plasmid for further transformation (1). This method is limited by the size of the plasmid, since plasmids used as expression vectors in nucleated cells are often too large for PCR. A modification of this method is presented here, where the inverse PCR is performed with concatamerized DNA. Thus the entire site directed mutagenesis procedure is performed *in vitro*; independent of plasmids, and the mutagenized cDNA is devoid of the wild type molecules. The mutations can be inserted at any site in the cDNA (Figure 1). This simple approach is potentially useful for the identification of catalytic domains in enzymes, and functional domains in various biologically reactive proteins.

The full length cDNA molecule is synthesized with upstream and downstream primers A and Z each containing a restriction site (Figure 1). The A–Z PCR product is cleaved by the restriction enzyme and subjected to overnight ligation. The ligated product is run on a 1% agarose gel and the concatamerized DNA molecules are collected onto a DEAE nitrocellulose membrane. This DNA preparation, which is devoid of the monomeric cDNA, serves as a source for all subsequent mutagenesis reactions. One nanogram of the concatamerized DNA is subjected to inverse PCR with the back to back primers 1 and 2, either one or both of which, containing the desired mutation(s). These back to back primers on opposite strands of DNA can correspond to any region of the cDNA. The 1–2 PCR product is isolated by gel electrophoresis in order to remove any concatamerized wild type cDNA sequences. This PCR product is subjected to end flushing by Klenow DNA polymerase, so that the exonuclease activity of the enzyme will remove any nontemplate added basepairs (1). Subsequently, the 1–2 PCR product is phosphorylated at the 5' end and the molecules undergo blunt end ligation in order to form concatamers. The resulting concatamers are subjected to a third PCR using primers A and Z. This A–Z PCR product has the same sequence as the original cDNA except for the mutation(s) introduced by primers 1 and/or 2. The effect of the introduced mutations on the protein encoded by the cDNA can be assessed after insertion of the mutagenized cDNA into an expression vector, using the restriction site in primers A and Z.

We introduced simultaneously two mutations into the cDNA of the glycosylation enzyme $\alpha 1,3$ galactosyltransferase ($\alpha 1,3$ GT) (2). The cDNA was prepared from the marmoset cell line B95.8. The primers used were numbered according to their location in the mouse $\alpha 1,3$ GT cDNA (2), and had the following sequences: Primer A: 5'GCCGAATTCGAGATGGCAGAAGGACTGGT (bases 207–225) and Primer Z: 5'GCCGAATTCTTGAAGT-CAGACATTATTTCT (complementary sequence of bases 1191–1171). Both primers contained an EcoRI restriction site at their 5' end. This and subsequent PCR reactions were performed with Vent™ DNA polymerase (kit purchased from Promega Corp., Madison, WI). The A–Z PCR product was cleaved with EcoRI. Five micrograms of the EcoRI cleaved A–Z PCR product were self ligated in a total volume of 20 μ l containing 3 units of T4 DNA ligase (GIBCO-BRL, Gaithersburg, MD), 15% (w/v) PEG-8000, 25 mM Tris–HCl (pH 7.6), 5 mM MgCl₂, 0.5 mM DTT and 0.5 mM ATP at 18–C for 16 hours. The concatamers resulting from this ligation were electrotitrated on NA45 DEAE nitrocellulose membrane (Schleicher and Schuell) (3). These concatamers were used as the template for PCR with primers 1 and 2. The back to back mutagenizing primers 1 and 2 contained a deletion and a substitution mutation respectively. Primer 1: 5'AAAATCCCC-TGGCCAAATGG; (sequence of complementary bases 910–889) and primer 2: 5'TATTACCATGCAGCCATTTC (bases 911–930). Primer 1 contained a deletion of C to form CCCC instead of CCCCC and Primer 2 a C substitution for T to form TTTC instead of TTTT.

The 1–2 PCR product was isolated by gel electrophoresis and subjected to end flushing treatment in a 100 μ l volume with 20 units Klenow enzyme (*E. coli* DNA polymerase I, GIBCO-BRL) and 250 μ M dNTP mix according to standard conditions (3). Phosphorylation of 5' end of the 1–2 PCR was then carried out in 25 μ l containing 20 units T₄ polynucleotide kinase (GIBCO-BRL) and 1 mM ATP (3). Subsequently the DNA was blunt end ligated (1) as described above, using 12 units of T4 DNA ligase and subjected to PCR with primers A and Z as described in Figure 1. This DNA product and the wild type $\alpha 1,3$ GT cDNA were inserted into the M13 vector and sequenced with a dideoxynucleotide kit purchased from U.S. Biochemical and α -[³⁵S]thio dATP (New England Nuclear). As seen in Figure

⁺Current address: Skin Biology Research, Schering-Plough Health Care Products, Inc., 3030 Jackson Avenue, Memphis, TN 38151, USA

* To whom correspondence should be addressed

Figure 1. Schematic representation of the procedure used for site directed mutagenesis by inverse PCR of concatamerized cDNA.

Figure 2. Sequences of the wild type (A) and mutagenized (B) region of marmoset $\alpha 1,3GT$ cDNA. Note the deletion of a G and substitution of a T with a C. Mutations are indicated by arrows.

2, the mutagenized cDNA contained a deletion of one G and a substitution of a T with a C. In ten clones sequenced, no nucleotide misincorporation by the VentTM DNA polymerase, and no addition of nontemplate nucleotides at the junction between primers 1 and 2, were observed.

ACKNOWLEDGEMENT

Supported by NIH grants AG06299 and GM40205.

REFERENCES

1. Helmseley, A., Arnheim, N., Toney, M.D., Cortopassi, G. and Galas, D.J. (1989) *Nucleic Acids Res.* **17**, 6545–6551.
2. Larsen, R.D., Rajan, V.P., Ruff, M., Kukowska-Latallo, J., Cummings, R.D. and Lowe, J.B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8227–8231.
3. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.